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Influence of maturation on hypoxic ventilatory responses

in P0-P30 neonatal rats *in vivo*

A Dissertation Presented

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Inefta M Reid

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Abstract of the Dissertation

Influence of maturation on hypoxic ventilator responses in P0-P30 neonatal rats *in vivo*

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Hypoxia is a common feature of many pathologies including but not limited to asthma, spinal cord injury, chronic obstructive sleep pulmonary disease, and sudden infant death syndrome (SIDS). Even though neuromodulatory effects mediated by maturation have been widely studied in other disciplines, such efforts have been lacking in the area of respiratory control. This dissertation is motivated by questions such as how does a known change in respiratory neurotransmitters manifest differently in the modulation of known respiratory output, especially in response to moderate, mild, and sever hypoxia. Aimed at understanding the characteristics and underlying mechanisms involved maturation of the hypoxic ventilator response in neonatal rats in vivo. Although a limited number of studies have been done on newborn rodents, the mechanisms responsible for long-term and short-term variability and complexity of these dynamics within the neural network during postnatal development is not yet well-studied. Understanding the characteristics and underlying mechanisms of neural plasticity

is important to the study of respiratory control during normal physiology as well as pathological disorders. In our first aim we discovered that neonatal rats exhibit a higher likelihood of gasping and the duration of gasping and number of gasp also had a maturational trend as well. Most importantly the network complexity (organization) was reduced during gasping. In the second part of our study data demonstrated that acute intermittent hypoxia (AIH) elicited respiratory long-term facilitation (LTF). However unlike the traditional response to AIH in the developing rat respiratory LTF primarily took the form of an enhancement in burst frequency, although an increase in burst amplitude was seen in some rats. Insight gained from this work will highlight that a distinct group of neurotransmitters are responsible for the varying responses to hypoxia and that they also manifest as a developmental response to exhibiting a similar behavior from the neonatal rat.

DEDICATION

To God: without the strength, wisdom and grace bestowed upon me, this dissertation would not be possible. Though His continual guidance the future holds nothing but open doors and promises for me.

To my husband, who has patiently allowed me to pursue my dreams. Thank you for all your help, encouragement but mostly for your tolerance. Love you forever and always. It's your time to shine now!

To my children, Naya and Allison. With the completion of this degree it is my way of telling you that 'All Things Are Possible'. Dare to be greater than I could ever be and don't ever stop short of reaching the stars.

To my mother, my father, my aunts, my uncles, my brothers, my sisters, my husband, nieces, nephews, cousins, in-laws, my church, and everyone else that makes up who I am my support system thank you always for your words of encouragement throughout this process.

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Acknowledgments

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CHAPTER 1

INTRODUCTION

Influence of maturation on hypoxic ventilatory response in neonatal rats *in vivo*

SPECIFIC AIMS

The main hypothesis in this proposal is that developmentally-regulated changes observed in neurotransmitter systems in the respiratory brainstem produce corresponding alterations in the complexity (organization) of the inspiratory neural network that will be manifested as developmental differences in inspiratory motor output, including the ventilator responses to hypoxia.

The focus of this thesis work is to identify the characteristics and underlying mechanisms involved in maturation of hypoxic ventilatory responses (HVR) in neonatal rats *in vivo*. Although a number of studies have been performed on newborn rodents (Liu *et al*., 2006, Waters and Gozal, 2003, Wang *et al*., 1996), the mechanisms responsible for long-time-scale and short-time-scale dynamics of the inspiratory neural network during postnatal development have not been identified.

This body of work characterizes the association between developmental changes in the respiratory nuclei and the Hypoxic Ventilatory Responses in neonatal rats *in vivo*. The two sets of experiments described investigate the hypothesis that maturational changes observed in the neonatal rat respiratory related nuclei will manifest as functional differences in responses of hypoxia. Insight gained from this work will help to broaden our understanding of postnatal changes in neurotransmitter systems and its impact on varying responses to hypoxia in the neonatal rat.

The specific aims are to: (1) characterize and quantify the inspiratory motor output response to acute severe hypoxia at different postnatal maturational stages on inspiratory motor output in neonatal rats, (2) characterize and quantify respiratory long-term facilitation (LTF) induced by acute intermittent hypoxia (AIH) at different postnatal maturation stages in P9-P15 neonatal rats.

BACKGROUND AND SIGNIFICANCE

Neural Control of Breathing

Breathing is a dynamic behavior from the first to the last breath of life. It is a complex behavior that is accomplished through the coordinated activation of respiratory-related skeletal muscles. The most important respiratory muscle is the diaphragm, which contracts to enlarge the thoracic cavity and create a pressure difference between the lungs and the external environment, thus drawing air into the lungs (i.e., inspiration). In anesthetized animals, the phrenic nerve, which innervates the diaphragm, is often recorded as a measure of ventilatory output. The other respiratory muscles include the inspiratory and expiratory intercostal muscles, expiratory abdominal muscles, and upper airway muscles (e.g., genioglossus).

The primary function of the respiratory system is to maintain proper concentrations of oxygen (O_2), carbon dioxide (CO_2), and hydrogen ions (H^+) in the tissues of the body. In order to achieve this, O_2 is transferred into the blood while, at the same time, CO_2 is removed from the blood. This process of gas exchange takes place in the alveoli of the lungs where, due to pressure gradients and factors related to chemical affinity, O_2 diffuses from the alveoli into the blood and CO_2 diffuses from the blood into the alveoli. Breathing is therefore the chemical homeostasis of O_2, CO_2, and H^+ in the blood and tissues, which is under tight regulation mediated by input from central and peripheral chemoreceptors. The chemoreceptors are highly sensitive to changes in gas exchange, and provide immediate feedback to the brainstem respiratory control system, such that breath-to-breath adjustments can be made. Thus, the respiratory system acts to control these variables by altering ventilation and regulating the amount of air (O_2) inspired and metabolic waste product (CO_2) expired. Due to the reaction combining CO_2 and water to form carbonic acid, $PaCO_2$ levels directly affect arterial pH, which must be strictly regulated as it can affect normal protein folding.

While the most powerful stimuli evoking adjustments in ventilation are believed to be related to alterations in CO_2 and H^+ concentrations (Feldman et al., 2003), breathing is also modified by changes in O_2 tension (PO_2). While the precise stimulus evoking activation of central chemoreceptors is still debated, these chemoreceptors appear to monitor H^+ changes in cerebral spinal fluid (CSF), which are in part the result of changes in blood CO_2 levels; the blood-brain-barrier is almost impermeable to H^+ whereas the lipid-soluble CO_2 rapidly passes into the central nervous system (CNS) and subsequently reacts with water to form carbonic acid that in turn dissociates into H^+ and bicarbonate ions. Due to this reaction, CO_2 levels directly affect pH, which must be strictly regulated, as it can affect normal protein folding. On the other hand, the peripheral chemoreceptors, which include the carotid bodies located at the bifurcations of the common carotid arteries and the aortic bodies, are sensitive mainly to changes in O_2 although they respond to changes in CO_2 and H^+ as well ((Feldman et al., 2003). Sensory signals from the peripheral chemoreceptors are transmitted via the glossopharyngeal and vagal nerves, respectively, into a primary relay station located in the nucleus tractus solitarus (NTS).

The Respiratory Brainstem

The main areas involved in ventilatory control are located in the brainstem – the dorsal respiratory group (DRG) located in the NTS, the ventral respiratory group (VRG) located in the ventrolateral part of the medulla, and the pontine respiratory group located in the dorsal lateral pons (Feldman and McCrimmon, 1999) – and previous studies have revealed that the primary centers responsible for the generation of respiratory rhythm are located in the medulla, as sectioning the brainstem below the medulla abolishes all respiratory activity while sectioning above this region does not. Complex interactions between neurons located in these brainstem regions as well as influences from other parts of the brain upon these neurons, will under normal conditions, lead to an adequate control of breathing.

Respiratory Rhythm Generation

Respiratory efforts are generated and regulated via a complex integrative system consisting of multiple feedback mechanisms. Within the medulla, there are "inspiratory neurons" that are active during inspiration and inactive during expiration. In contrast, "expiratory neurons" are active during expiration. These two subtypes of respiratory neurons are proposed to automatically maintain a rhythmic cycling pattern of inspiration and expiration, and they are aggregated in the pons, DRG, and VRG (Duffin, 2004). The DRG contains neurons that fire during inspiration, and these neurons project contralaterally to the phrenic and intercostal motoneurons in the spinal cord to provide excitatory inspiratory drive. The VRG consists of a long column of respiratory neurons that exhibit inspiratory (in the rostral VRG) and expiratory (in the caudal VRG) firing patterns, and includes the nucleus ambiguus inspiratory neurons that project to the larynx, pharynx, and tongue to dilate the upper airways to minimize airway resistance during inspiration. Also within the VRG are the Bötzinger complex (BötC) and pre-Botzinger (PBC) complex, which created a oscillator system, which are hypothesized to be critical for respiratory rhythmogenesis (Duffin, 2004; Feldman *et al.*, 2003; Smith *et al.*, 1991). The BötC is the primary source of inhibition in the respiratory network, and it uses a complex network of inhibitory, mainly GABAergic, neurons to inhibit both premotor and motor neurons (Feldman and McCrimmon, 1999), thus being responsible for the silent periods of the respiratory cycle. The PBC has been hypothesized to be the primary locus of inspiratory rhythm generation (Smith *et al.*, 1991; Ramirez and Viemari, 2005; Ramirez *et al*., 1998; Gray *et al*., 2001; Solomon, 2002, 2003; Wenninger *et al*., 2004). Even after being isolated from all afferent inputs, the pre-BötC in a medullary slice preparation is still capable of generating various forms of inspiratory activity, including fictive eupnea, sighs, and gasps (Ramirez and Lieske, 2003). Previous studies have demonstrated the necessity of the pre-BötC in respiratory rhythm generation. Activation of pre-BötC neurons *in vivo* increases the frequency of phasic inspiratory discharges and in some cases, produces tonic excitation of inspiratory motor activity (Chitravanshi and Sapru, 1999;

McCrimmon *et al*., 2000; Monnier *et al*., 2003; Solomon, 2003; Solomon *et al*., 1999), while ablation of neurons within this region abolishes respiratory rhythm (Gray *et al*., 2001; Ramirez *et al*., 1998; Wenninger *et al*., 2004) and electrophysiological recordings, pharmacological manipulations, and focal lesions in this area, have confirmed that the PBC is critically involved in the generation of the respiratory rhythm both *in vitro* and *in vivo* (Gray *et al.,* 2001, Smith *et al.,* 1991, Solomon *et al.,* 1999). Respiratory rhythm in the pre-BötC is thought to be the result of the combination of synaptic and intrinsic membrane properties that includes neurons with bursting pacemaker properties (Ramirez *et al*., 2004; Del Negro *et al*., 2002). Three main respiratory rhythms are generated: eupnea (*e.g.,* normal resting respiration); sighing (*e.g.,* large inspiratory efforts overlying and interspersed within eupnea) l and gasping (*e.g.,* short inspiratory efforts of high amplitude preceding a long expiratory pauses). Ultimately, the aim of this complex respiratory system is to supply the body with oxygen, remove CO_2, and balance acid-base status.

Eupnea and Gasping

Eupnea, or normal breathing, is characterized by an augmenting or ramp-like discharge pattern in phrenic motor output, which peaks later in the inspiratory burst. In both in vitro and in vivo preparations (St.-John, 1990, 1996, and 1998; St-John and Patton, 2003) eupnea is characterized by an augmenting population burst in where neuronal activities during this firing pattern include cells with pre-inspiratory, post-inspiratory or expiratory activities, as well as inspiratory cells with an augmenting depolarization pattern (Lieske *et al*., 2000) such patterns can be produced under a normoxic or hyperoxic. This pattern of activity is believed to be a result of an orderly recruitment of phrenic premotor and motor units throughout the period of inspiration (Akay and Sekine, 2004).

In contrast to normal breathing efforts, severe hypoxia results in another form of respiratory excitation, which is referred to as gasping. Gasping is defined by an abrupt onset, short duration, decrementing pattern majority of the inspiratory activity is found at the begging of the burst (St.-John, 1990, 1996, and 1998). Phrenic nerve discharge is of greater amplitude and shorter duration than that observed in eupnea and the frequency of phrenic discharge (or breathing) is decreased due to an increase in the period between bursts. In mammals, exposure to hypoxic or severe hypoxic conditions results in a characteristic pattern of responses that may include gasping. The initial phase of the hypoxic response is characterized by hyperpnea (in chemo-intact mammals), or an increased breathing frequency with increased amplitude, after which a primary apnea occurs. Within a variable period of time following apnea (gasp latency), gasping respiratory activity will emerge and continue until terminal apnea or death. Gasping can be elicited in animal models with intact peripheral chemoreceptors as well as in animals that have been deafferented, and has been demonstrated to result from centrally-mediated hypoxic effects (St. John, 1996; Lieske *et al*., 2000, Solomon, 2000), although afferent inputs can modify the timing (onset) of hypoxia-induced gasps (Melton *et al*., 1993). During gasping, inspiratory (i.e., phrenic, hypoglossal) nerve discharge is characterized by a rapid rise to peak activity, high amplitude (although this is not seen in some mammalian models studied), short duration burst of activity (St. John and Knuth, 1981; St. John, 1996); thus, the inspiratory burst pattern differs from that seen in eupnea. Hypoxic gasping is also associated with a prolongation of expiration, yielding a reduced burst frequency. Developmental influences on the gasping response will be discussed below (see Development and the HVR).

In the in vivo cat model, Richardson (1986) distinguished between eupneic and gasp patterns based not only on the burst shape, duration, and amplitude of the phrenic burst, but also on the underlying spectral components within the inspiratory effort (Richardson, 1986).

Akay and Sekine (2004) evaluated the complexity (or orderliness) of the phrenic neurogram during the development of hypoxic respiratory depression and during reoxygenation following severe hypoxia (gasping) using approximate entropy (ApEn) analyses. ApEn is a statistical index that measures and quantifies the regularity in a time series. They found that complexity values observed during eupnea had a maturational dependence, with complexity decreasing as piglet age increased. In addition, the complexity values of the phrenic neurogram were significantly reduced during gasping efforts, but gradually became higher during reoxygenation, suggesting that severe hypoxia reduces the complexity of the phrenic neurogram and reoxygenation reverses this reconfiguration of the respiratory neural network (Akay and Sekine, 2004; Akay *et al*., 2006).

OXYGEN SENSING AND THE PERIPHERAL NERVOUS SYSTEM

Peripheral Chemoreceptors

The peripheral chemoreceptors, which include the carotid body and aortic chemoreceptors, sense changes in arterial O_2, CO_2, and pH, and alter breathing to maintain constant levels of these important blood gas parameters (see schematic). The arterial chemoreceptors were first discovered by Heymans and Bouckaert in 1930, in which they demonstrated that even the slightest of variations in O_2 lead to changes in respiration. Since this initial finding, our understanding of the ability of different organs to detect and respond to changes in O_2 levels has evolved dramatically. The carotid body is located bilaterally at the bifurcation of the common carotid artery. The carotid bodies are highly vascularized and have been estimated to receive the highest blood flow relative to size compared with other organs in the body. This allows for efficient sensing in systematic changes in PO_2, $PaCO_2$, and pH. While the arterial chemoreceptors are the primary hypoxia sensors involved in ventilatory control, there are a variety of cells and organs that are capable of varying their activity in response to changes in O_2 tension (PO_2).

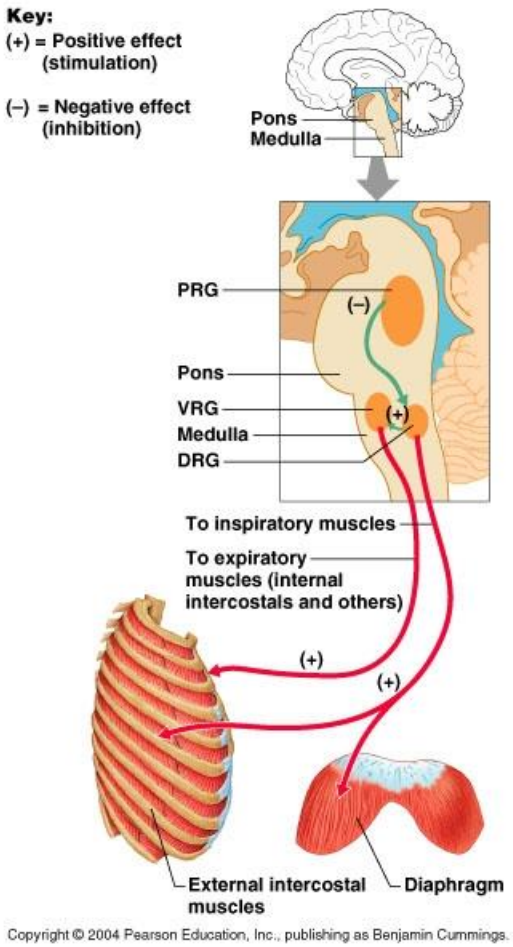


Figure 1: Respiratory Center. Control of breahting neural pathways are integrated in the brainstem and relayed to the respiratory muscles via nerve impluses. These impluses are paired and functionally related autonomic nuclei responsible for rhythm generation. Image taken from: Neurology June 12, 2007 vol. 68 no. 24 2140-2143

The carotid bodies maintain a tonic output under normoxic conditions (~80-100 mm Hg PaO_2) but are activated when reduced levels of hypoxia (~50-60 mm Hg PaO_2) are sensed (Lopez-Barneo *et al*., 2001). Type I carotid body glomus cells are electrically excitable and when stimulated they depolarize and send signals up carotid sinus afferent nerve fibers to the NTS. Through inhibition of membrane potassium channels, hypoxia is sensed (Weir *et al.,* 2005; Ortega-Saenz 2006).

The primary role for O_2 sensing mechanisms from a survival perspective, is to maintain appropriate function of the cardiovascular and respiratory systems to ensure appropriate O_2 delivery to the tissues. Oxygen sensing and the subsequent response vary according to the degree and duration of the hypoxic stimulus, with decreases in blood oxygenation being sensed primarily by the carotid body, and to lesser extent the aortic chemoreceptors. Decreases in PaO_2 or pH and increases in $PaCO_2$ all stimulate ventilation, and these changes initiate an increase in action potential firing frequency in the carotid sinus nerve, which is relayed to the brainstem where it is integrated with other afferent information to increase breathing. This augmentation of ventilation serves to increase O_2 availability in an attempt to restore chemical homeostasis. While hypoxia is not as potent of a respiratory stimulus as hypercapnia, the degree of hypoxia and factors that modulate oxygen-hemoglobin affinity, such as disease state and changes in arterial pH and PCO_2, are important determinants of the effect of hypoxia-induced ventilatory drive. Vasoconstriction of the small resistance pulmonary arteries can also occur in response to acute hypoxia in order to optimize O_2 transfer in the lung (Weir *et al.,* 2005).

The brain has limited O_2 reserves and is therefore crucially dependent on O_2. In times of limited O_2 supply, there are populations of neurons within the CNS that can act as hypoxia chemoreceptors that work to modulate critical functions necessary for the overall survival of the whole organism (French 1972; Lopez-Barneo *et al.,* 2001; Neubauer and Sunderram, 2004). The thalamus, hypothalamus, pons, and medulla have each been implicated in modulating respiratory and sympathetic activity (which can be either inhibitory or excitatory) (Dawes *et al.,* 1984; Horn and Waldrop, 1997; Solomon *et al.,* 2000; Neubauer and Sunderram, 2004), and likely form a complex chemosensitive network (Neubauer and Sunderram, 2004). The CNS displays both stimulatory and depressant responses to hypoxia, with one of the most widely researched examples of this being the biphasic ventilatory response that occurs during mild to moderate hypoxia. There are also several other well documented physiological changes that occur within the cardiovascular system, including the direct vasodilatory effect of hypoxia on peripheral arterioles, which is overridden by chemoreceptor-mediated reflexes to induce peripheral vasoconstriction

that redistributes cardiac output toward the myocardial and cerebral circulations with clear survival advantages (Scholz, 2002). It is important to note that the characteristics of these responses may differ widely according to the duration and degree of hypoxia (Neubauer, 2001) which will be further disscuessed later in this document.

OXYGEN SENSING AND THE PERIPHERAL NERVOUS SYSTEM

Central Chemoreceptors

The brain has limited oxygen reserves and is therefore crucially dependent on oxygen. While, low oxygen levels (hypoxia) is sensed peripherally via the carotid body and transmitted through the carotid nerve. There are certain population of neurons within the brain that act as chemoreceptors and in times of hypoxia they modulate critical functions necessary for the overall survival of the organism. Central ventilatory efforts are stimulated by the acidification of the central nervous system (Guyenet *et al*., 2008) and must be maintained within a very narrow range of oscillations by balancing respiratory efforts and metabolism. Furthermore, feedback from chemoreceptors, which are CO_2 mediated, provides a tonic, chemical drive to breathe (Cohen, 1968, Pillipson, *et al*., 1981, Sullivan, *et al*., 1978).

The first suggestion that the brainstem was CO_2 sensitive came from studies where acidic fluids were perfused into the cerebral ventricles of anesthetized dogs (Leusen, 1954). The influx of acidity into the subarachnoid region of the ventrolateral medulla (VLM) caused large increases in respiratory efforts, making claim to the vitality of the ventral medullary surface for brainstem chemosensation.

Many cells in the body respond to CO_2 or H^+. Whether the chemosensitive activating signal is CO_2 or intra/extracellular H^+ ions is unknown as both have been speculated to play a role in chemosensation. Putnam and colleagues (2001) suggested multiple factors responsible for neuronal chemosensitive signaling in response to CO_2 or H^+ involves multiple cellular signals and multiple ion channel targets. It has been speculated that intracellular and extracellular CO_2/H^+ play a role in

chemosensation and there is some evidence that hypercapnia inhibits pH sensitive K^+ channels, although the specific channel subtype(s) relevant to this behavior have yet to be identified.

Despite their wide distribution and unclear mechanism of action, Putnam and colleagues plus other labs have suggested a few conditions that must be met in order for a cell to be classified as chemoreceptors. To be considered a central chemoreceptor, CO_2/H^+ sensitive cell, these neurons must increase c-fos expression in response to elevated CO_2, increase firing rates in response to CO_2 *in vitro*, project to a respiratory site and alter respiration when stimulated.

In brain slices, presumed chemoreceptor cells have been identified in the RTN (Wellner-Kienitz & Shams, 1998; Mulkey *et al.,* 2004), medullary raphe (Richerson, 1995), and NTS (Dean *et al*., 1989). While synaptic blockade was attempted in all of these experiments, intrinsic chemosensitivity cannot be proven conclusively because of the possibility that residual neurotransmission or coupling by gap junctions might have contributed to the CO_2/H^+ response in some neurons.

Further evidence suggests potential chemoreceptor sites in vivo by using focal acidification. Utilizing microinjection of carbonic anhydrase inhibitor acetazolamide or microdialysis with a 25% CO_2 equilibrated solution, investigators have identified potential chemoreceptor sites. In anesthetized cats (Cotes et al., 1993; Bernard et al., 1996; Xu et al., 2001) respiratory activity was elevated following acidification on the ventral medulla. In wake animals stimulating the RTN, medullary raphe, and NTS with microdialyzed CO_2 caused an increase in respiratory output (Nattie & Li, 2001; Nattie & Li, 2002a). There is also evidence suggesting that preBötC, the site of respiratory rhythm generation, contains CO_2 chemoreceptors as well (Solomon *et al*., 2000).

HYPOXIA – HYPOXIC VENTILATORY RESPONSE (HVR)

Overview

Hypoxia is a low oxygen level and is a common feature in many respiratory disorders of which many are also associated with significant morbidity. Hypoxia in clinical disease can devlop rapidly (e.g. acute bronchospasm in response to an allergen, or more gradually, progressive lung disfunction) in response to illness. During sleep hypoxia can be prolonged, hypoventilation syndrome, or short and repetitive, such as in chronic obstructive sleep apnea. Whatever the etiology, hypoxic conditoins results from a failure of the respiratory system to properly maintain homeostasis in gas exchange as a result of the pathology. In many cases hypoxia occurs alongside an increase in respiratory load. This is why several compensatory and protective mechanisms have been put in place to combat these impediments to normal respiratory gas exchange.

Thruoghout the literature it has been demonstrated that the physiological responsse to hypxoa is diverse. This response varies between species, maturational day, wake versus sleep, duration and degree of hypoxic exposure. Even when experimental conditions are replicated discrepancy still exist among studies. Numerous studies have demonstrated that the ventilatory (phrenic) responses to and following hypoxia exhibit complex, time-dependent modulation (see review by Powell *et al.*, 1998). The precise response to hypoxia depends upon the pattern (i.e., sustained or intermittent), severity (i.e., mild, moderate, or severe), and duration of the hypoxic exposure(s), and therefore may involve several underlying physiological mechanisms.

Ventilation increases in response to hypoxia when small changes in Pa_{O2} and most importantly Pa_{CO2} are detected. The ventialtory resoinse to hypoxia is dependent upoing the timing and pattern fo the hypoxic stimulus shown in Figure 2. Sustained hypoxia ranging from 5 to 20 minutes results in the subsequent depression of ventilation most commonly known as Hypoxic Ventilatory Decline (HVD) or "ventilatory roll –off". During HVD ventilation is decreased relative to the acute response and this decrease in ventilation can persist for upto 60 minutes after normoxia is restored. On a longer time sclare,

in the years follwing hypoxia, hypoxic densensitization (HD) will occur. During HD ventilation is decreased in comparrison to sbjuects exposed to hypoxia for a short period of time and the acute HVR becomes blunted. The experiments outlined in this dissertation will be focused on characterizing the HVR following seconds to minutes of hypoxic exposure during maturation on the neonatal rat to determine the potential delerterious effects of hypoxia and maturational. For the work described in this dissertaiton, I will focus on (1) the acute hypoxic ventilatory response, which is elicited by a single brief (seconds to minutes) hypoxic exposure, (2) the gasping response, which is elicited in response to severe brain hypoxia or ischemia, and (3) LTF of inspiratory motor output, which is elicited by acute intermittent (episodic), but not sustained, hypoxia.

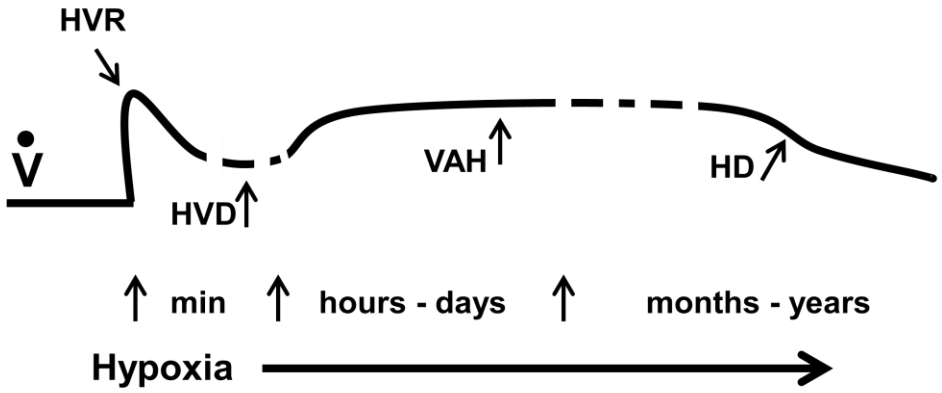


Figure 2: Hypoxic Ventilatory Response. The ventilatory response to hypoxia is dependent on the duration and severity of hypoxic stimulus. Upon initial exposure to hypoxiam ventilation increases: acute Hypoxic Ventilatory Reponse (HVR). Following minutes of hypoxia, ventilation "rolls off" (Hypoxic Ventilatory Decline, HVD). A resulting increase in ventilation following hours to days occurs Hypoxic Ventilatory Acclimitization to Hypoxia(VAH). Months to years of hypoxia leas to Hypoxic Decline (HD). Modified image from Powell *et al*., 1998

PHYSIOLOGICAL RESPONSE TO HYPOXIA

The Acute Hypoxic Ventilatory Response – PCR

The acute sustain iscocapnic hypoxia ventilatory (phrenic) response produces a biphasic ventilatory response characterized by a rapid augmentation, with a peak response occuring in 3-5 minutes, followed within minutes by depression of ventialtion that although is above baseline levels is reduced in comparrison to the acute response. This is characterized by an increase in minute ventilation (or minute neural activity), which is mediated by an increase in both tidal volume (phrenic amplitude) and breathing frequency. The response, which is initiated by hypoxic stimulation of the carotid body chemoreceptors (Dutton *et al.*, 1973), has been observed in all animal models studied thus far, although the magnitudes of the increases in tidal volume (phrenic amplitude) versus breathing frequency are highly variable, and influenced by a variety of factors, including the state of the preparation (i.e., anesthetized vs. unanesthetized) and species (see review by Powell *et al.*, 1998). HVD is characterized by a decrease in ventilation and is proposed to occur centrally as evidence by continuous elevation of carotid sinus nerve activity during acute isocapnic hypoxia (Vizek *et al*., 1987). Furthermore it has been demonstrated that blocking pheripheral chemoreceptors using somaostatin abolishes the initial hyperpnea and unmaks a pure depression of ventilation (Easton and Anthonisen, 1985). Researchers have come up with several mechanisms for hypoxia-induced central depression of ventilation. It has long been understood that a shift in the balance of excitation and inhibition through neurotransmitters within the central nervous system is believed to be important. Regional or global pharmalogical techniques have provided insights into the role of neuromodulatory effects on respiration. In additon, mild to moderate hypoxia reduces the synthesis or release of excitatory neurotransmitters (i.e. acetylcholine, monoamines, glutamate, etc.) and increases the synthesis or release of inhibitory neurotransmitters (i.e. GABA, adennosine, endogenous opiods, etc.) (Neubauer, 1990). If the hypoxic exposure is allowed to progress (minutes), short-term potentiation of tidal volume (phrenic amplitude) and short-term depression of breathing frequency will be observed (Powell *et al.*, 1998). Re-oxygenation reverses these ventilatory effects although return to pre-hypoxic

levels is not immediate. Although the HVR can vary between species and experimental preparations (Erecinska and Silver, 2001), discrepancies in the observed respiratory behaviors have fueled many investigations in this area although only a limited number of studies have focused on the influence of postnatal maturation on the acute HVR (e.g., Liu *et al.*, 2009; also see Wong-Riley *et al.*, 2013). Developmental influences on the acute HVR will be discussed below (see *Development and the HVR*).

Intermittent Hypoxia and LTF

LTF a form of respiratory plasticity elicited by repeated acute bouts of hypoxia (*i.e.*, acute intermittent hypoxia, AIH; see review by Mitchell *et al.*, 2001). It is characterized by a long-lasting progressive increase in ventilation (respiratory motor output) that lasts for minutes to hours after the hypoxic stimulus is removed (e.g., Fregosi and Mitchell, 1994; Bach *et al.* 1996; Turner and Mitchell, 1997; Mateika and Fregosi, 1997; also see review by Mitchell *et al.*, 2001). LTF has been observed in most animal models studied thus far (e.g., cat, Milhorn *et al.*, 1980; Gallman and Millhorn, 1988; dog, Cao *et al.*, 1992; rat, Hayashi *et al.*, 1993; Bach *et al.*, 1996; Baker and Mitchell, 2000; Olson *et al.*, 2001; Bavis and Mitchell, 2003; goat, Dwinnell *et al.*, 1997; Turner and Mitchell, 1997; duck, Mitchell *et al.*, 2001; mouse, Kline *et al.*, 2002; also see review by Mitchell *et al.*, 2001) as well as in humans (Babcock and Badr, 1998). Aalthough the magnitude and time course associated with LTF appear to be influenced by a variety of factors, including species studied, state of the preparation (i.e., anesthetized vs. unanesthetized), level of arterial PCO_2, and AIH protocol used (Turner and Mitchell, 1997; Powell *et al.*, 1998; Olson *et al.*, 2001; Nichols *et al.* 2012; also see review by Mitchell *et al.*, 2001). It should be noted that LTF appears to be more robust in anesthetized preparations (see review by Mitchell *et al.*, 2001) in compasrrison with wake studies.

Development and the HVR

While the effects of hypoxia on respiration have been studied extensively, far less is known regarding the effects of maturation on hypoxia-induced respiratory activities. In the developing fetus *in utero*, hypoxia constitutes a stressor of critical importance, and severe acute hypoxia or more moderate prolonged hypoxia can result in major neurological sequelae with life-long consequences. As O_2 availability is reduced *in utero*, fetal O_2 sensors respond to lower absolute PaO_2 values (~20-40 mmHg). The fetus also has specialized O_2 sensors, including neuroepithelial bodies in the lungs, chromaffin cells of the fetal adrenal medulla, smooth muscle cells of the resistance pulmonary arteries, fetoplacental arteries, systemic arteries, and the ductus arteriosus (Weir, *et al.*, 2005). The response of the fetus to hypoxia is characterized by an absence of respiratory facilitation. Instead, breathing movements are reduced, often to complete silence, and accompanied by bradycardia (Boddy *et al.*, 1974; Bissonnette, 2000). This is believed to reflect central inhibition rather than an immature chemoreceptor response (Bissonnette, 2000; Gozal *et al.*, 2001), and this trait may have an important survival advantage *in utero* when, during an asphyxial insult, respiratory movements are suppressed and energy is conserved.

In the neonate, there is a biphasic ventilatory response, consisting of an initial increase in ventilation followed within minutes by depression of ventilation that is sometimes below baseline levels (Blanco et al., 1984, Eden and Hanson, 1987, Neubauer et al., 1990, Rehan et al., 1996). This biphasic hypoxic response has also been observed in the neonatal rat transverse medullary slice preparation, suggesting that there may be a centrally-mediated mechanism involved in both the hypoxic excitation and depression of ventilation in neonates (Ramirez et al., 1996). With maturation advancing age, the initial augmentation increases and the subsequent depression declines (Bissonnette, 2000); however, hypoxia-induced depression of ventilation is still clearly evident in the adult. While hypoxia-induced depression of central autonomic function may be life saving in the fetus, its function in adults is far from clear, and it is possible that this is a residual function carried forward from fetal life, which in the adult can prove deleterious to cardiorespiratory function.

Newborn mammals have also been shown to be more tolerant than older mammals to anoxic exposures (Fazekas *et al.*, 1941), and the hypoxic gasping response appears to be affected by postnatal developmental. In this case, in response to anoxia, both the duration of gasping and the number of gasps have been shown to decrease with increasing postnatal age in rats (Fewell *et al.*, 2005; Gozal, 1996; Gozal *et al.*, 1998). Recovery from anoxia-induced gasping upon re-oxygenation has also been shown to be developmentally influenced in various strains of mice (Jacobi *et al.*, 1991).

While much is known about the AIH-induced respiratory LTF response in adult mammals, little is known about the response to AIH in neonatal animals. To date, only one study has reported respiratory LTF in the neonatal rat, and in this study, an enhancement of genioglossus muscle activity was observed in response to episodic hypoxia in P3-P7 neonatal rats (McKay *et al*., 2004).

CLINICAL IMPLICATIONS AND THE HVR

Numerous cardiovascular and respiratory pathologies result in hypoxia (hypoxemia). Moreover, the critical period of respiratory development appears to be important, as it might be related to the proposed critical period noted in the pathogenesis of Sudden Infant Death Syndrome (SIDS). SIDS is associated with a very specific time in an infant's life, with ~90% of SIDS deaths occurring in the first six months of life. Although, the precise pathogenesis of SIDS is unknown, over the years, several etiologies have been proposed to explain SIDS. These include infections (Blackwell *et al*., 1999; Howatson, 1992), metabolic disorders (Bonham and Downing, 1992; Burchell *et al*., 1992), environmental perturbations (Kemp and Thach, 1993; Lewis and Bosque, 1995), and both cardiac (Schwartz *et al*., 1998; Ackerman *et al*., 2001) and neurologic (Kinney *et al*., 1991; O'Kusky and Norman, 1995; Carpentier *et al.*, 1998) abnormalities. These etiologies have often been intertwined and difficult to categorize; thus, Filiano and Kinney proposed the "Triple Risk Model", in which SIDS cases are thought to occur when a vulnerable infant (congenital defect, metabolic disorder, etc.), at a critical developmental period, and an exogenous stressor (infection, heat stress, CO_2 rebreathing, etc.) converge (Filiano and Kinney, 1994). This model was adopted as the central hypothesis for the National Institute of Child Health and Human

Development's (NICHD) strategic plan for SIDS research (Kinney *et al.*, 2001). Failure to gasp, which represents autoresusitation or the spontaneous recovery from O_2 deprivation, is believed to be the downfall of all SIDS cases. Although other underlying respiratory deficiencies, known and unknown, can lead to autoresusitation failure, the failure to recover is the actual cause of death.

As stated earlier, the peak incidence for SIDS is between two and four months of age (Brooke *et al*., 1997; Carroll-Pankhurst and Mortimer, 2001). The first six months of human life are reported to be a critical integration period for arousal and cardiorespiratory control. Stable configuration of the system is established by approximately six months of age (Kinney et al., 1992; Kralios and Kralios, 1996; Kahn et al., 2002; Patzak, 1999). Periodic breathing, characterized by periods of deep breaths followed by short shallow breaths or cessation, are reported to start during the first few days of human life and to decrease rapidly after the fourth month of life (Patzak, 1999). Responses to CO_2 challenge in infants are reported to vary with age. Specifically, respiratory responses to challenge with CO_2 are less robust in children three months of age than for children one or six months of age (Campbell *et al*., 1998). SIDS cases are reported to occur more often at night, with a peak in the early morning hours suggesting a glitch in the arousal state (Kelmanson, 1991). Circulating histamine levels are elevated while epinephrine is reported to be depressed in the early morning hours (Kraft and Martin, 1995). Asthma attacks are reported more commonly at night than during the day (Kraft and Martin, 1995). Furthermore, blood pressure and heart rate are reported to be at their lowest point in humans during the peak hours of the morning (Kraft and Martin, 1995). These developmental changes and/or circadian rhythms, emphasize the vulnerability of infants during the first few months of life, yet their role in SIDS remains to be determined.

DEVELOPMENTAL RESPIRATORY NEUROBIOLOGY

Neonatal Respiration

Development of the intrinsic properties and functional organization of the central respiratory network continues after birth in order to maintain homeostasis relative to size. Not only are there known changes in motor pattern and neurotransmitter systems within the respiratory brain stem with advancing postnatal age (Paton, 1994; Ramirez 1996), but there are also maturational changes in dendritic morphology and increases in synaptic connections and myelination after birth (Purpura, 1974). Together, these changes work to maintain homeostasis and help stabilize respiratory activity during the postnatal period.

In the adult mammal, the HVR occurs in two phases. The initial phase involves an increase in ventilation, which is the result of peripheral carotid body receptor stimulation. The second phase is a depression of ventilation, which is a result of pH changes due to a dip in O_2 either in the blood or in the brain's interstitial fluid as a result of the hyperventilatiory response during the first phase of the HVR. It has been reported by Gozal and colleagues that immediately after birth in the rodent, the initial phase of the HVR is virtually absent (Gozal, 2003). However, the initial phase increases with increasing postnatal age, reaching a peak response on or about the second post natal week of life while the HVD response decreases. Unfortunately these studies are not completed in that most research previously done 1) group animal ages from the first and second week second post natal week when there are known changes in the brainstem neurochemistry that are known to play a key role in the responses to hypoxia and 2) do not address the day to day changes in the HVR with maturation. Since respiratory patterning can change dramatically in one to two day postnatal day up to the time of weaning (Liu, 2006; Liu 2009), it is important to look at day to day changes. This factor must be heavily considered when commenting on maturational changes of physiological importance.

Development of peripheral chemoreceptors

In the neonatal rat, both the carotid body and central chemoreceptors are undeveloped. The developmental changes associated with the HVR parallel the two distinct phases of carotid body development (Jansen *et al*., 1991). The first stage occurs immediately after birth and is referred to as the "silencing phase." During this stage, the peripheral chemoreceptors are practically unresponsive to hypoxia. It is thought this is due in large to part to a resetting of the hypoxic threshold required in the transition from fetus to infant (Gauda *et al.*, 2009). The next phase is a coexisting gradual increase in the responsiveness with increasing age. The afferent carotid bodies demonstrated increase in sensory discharge with similar levels of oxygen tension (Carroll *et al.*, 1993. Marchal *et al.*, 1992). Within the first week of normal development, tyrosine activity decrease and remains at a steady state until at least P21, decrease of tyrosine hydroxylase activity indicates a reduction in dopamine content and an increase in the responsiveness to hypoxia throughout the postnatal period, since dopamine within the carotid body attenuate the hypoxic chemosensitivity (Hertzberg *et al.*, 1990).

Development of central chemoreceptors

Peripheral chemoreceptors are not the only site responsible for hypoxia detection. Central chemosensitivity also plays an important role in modulating neonatal respiration. At birth, the central respiratory network is not fully mature, and numerous studies have demonstrated postnatal developmental changes in intrinsic membrane properties and synaptic transmission within the central respiratory network (Cummings, 2011; Cummings, 2005; Liu, 2011; Lui, 2006; Liu, 2002; Gozal, 1998; Katz, 2005). The ventilator responses to CO_2 in healthy term neonates is similar to that seen in adults (Avery, 1963), indicating that he central chemoreceptors are indeed fully functional after birth. This is supported by evidence that primary central chemoreceptor area the ventral medullary surface exhibits *c-fos* mRNA expression directly after birth and this expression is further enhanced by hypercapnia at one day after birth (Wickstrom, 1999).

While arterial chemoreceptors are functional in utero, they become inactive immediately after birth. However, within the first few days of postnatal life, peripheral chemoreceptors increase their responsiveness towards adult levels (Hertzberg *et al.,* 1990; Blanco, 1984) This resetting process most likely results from a rise in pO2 concentrations at birth (Blanco,1988). Peripheral chemo sensitivity continues to develop during the postnatal period and plays an important role in respiratory regulation (Bur Genetics also play a role in the development of the respiratory system. Mutations in c-ret proto-oncogene, a tyrosine kinase receptor necessary for proper neural crest development, results in an increase apnea frequency, longer apnea duration, as well as periodic breathing compared wild-type animals (Aizenfisz *et al.*, 2002).

Most investigations, however, focus on a subset of developmental stages, with many studies using neonatal animals ranging from P0-P5 to study electrophysiological and biophysical membrane properties of respiratory-related neurons and motoneurons and from P0-P21 in 1-week increments (i.e., P0, P7, P14, and P21) to study various aspects of central and peripheral mechanisms in respiratory control as well as gene/protein expression in respiratory-related CNS structures. Recently, however, a number of investigations have focused on the day-to-day maturational changes in various neurochemical systems in the respiratory brainstem. These studies have identified a critical period in respiratory development, in which alterations in multiple neurochemical systems, including receptor subunit composition, receptor expression levels, neurotransmitter abundance, and neurotrophic factors involved in central respiratory control (Liu and Wong-Riley, 2009; Liu and Wong-Riley, 2010; Liu and Wong-Riley, 2012) occur simultaneously. This critical period of respiratory development corresponds to the end of the second postnatal week, occurring at approximately postnatal day 12 (P12). Amongst the changes occurring at this time point, the expression levels of (1) the excitatory neurotransmitter glutamate and its NMDA receptor are dramatically decreased, (2) the inhibitory neurotransmitters GABA and glycine and the $GABA_B$ receptor are substantially increased, (3) the serotonin (5-HT) receptors 5-HT_{1A}, 5-HT_{1B}, and 5-HT_{2A} as well as the 5-HT transporter (SERT) are significantly reduced, and (4) the neurotrophic factor

BDNF and its TrkB receptor are markedly decreased. These neurochemical changes, at least in part, lead to a neurochemical imbalance that results in neurological instability of respiration at P12 (Liu and Wong-Riley, 2002) and an attenuation of the ventilatory response to acute hypoxic (Liu *et al*., 2006). While both 5-HT and BDNF/TrkB signaling have been implicated in the AIH-induced LTF response in adult rat, it remains to be determined whether these developmentally-regulated neurochemical changes affect the AIH-induced LTF response in the developing neonatal rat.

ANIMAL MODELS OF SIDS

Traditionally animal models of human disease have been divided into two broad categories; spontaneous and induced models. Because the cause of SIDS is unknown, no induced and/or spontaneous animal model of SIDS has been reported. Swine were specifically investigated as a model for spontaneous SIDS, but without success (Lavoue *et al*., 1994). Further complicating the production of an SIDS animal model is the developmental and environmental risk factors that are reported for SIDS would need to be addressed in the animal model while simultaneously investigating a given etiology (Blackwell *et al*., 1999). In spite of these limitations, a few species have been proposed as either spontaneous produce sudden death of unknown causes, while other species have been utilized to investigate specific risk factors of SIDS. Specifically, it has been reported that a line of German shepherd dogs exhibit sudden death around five months of age. Death is thought to be due to ventricular arrhythmia similar to that seem in long QT interval syndrome, though long QT was not reported in these dogs (Merot *et al*., 2000). Specific claims for use of these dogs as a spontaneous model for SIDS have not been reported. However, because sudden unexpected death from acute cardiac failure has been proposed for SIDS, further investigation of this canine line appears warranted (Schwartz *et al*., 1998; Merot *et al*., 2000). Another possible SIDS model is the magnesium deficient rat reported by Caddell in 2001 (Caddell, 2001). In this model, juvenile rats (~30g) were fed a magnesium deficient diet for six days. This diet induced seizures, often followed by death. Furthermore, the author reported lesions consistent with SIDS, cyanosis and more specific to my thesis work, rats of this age were in a critical developmental period, although supporting evidence for

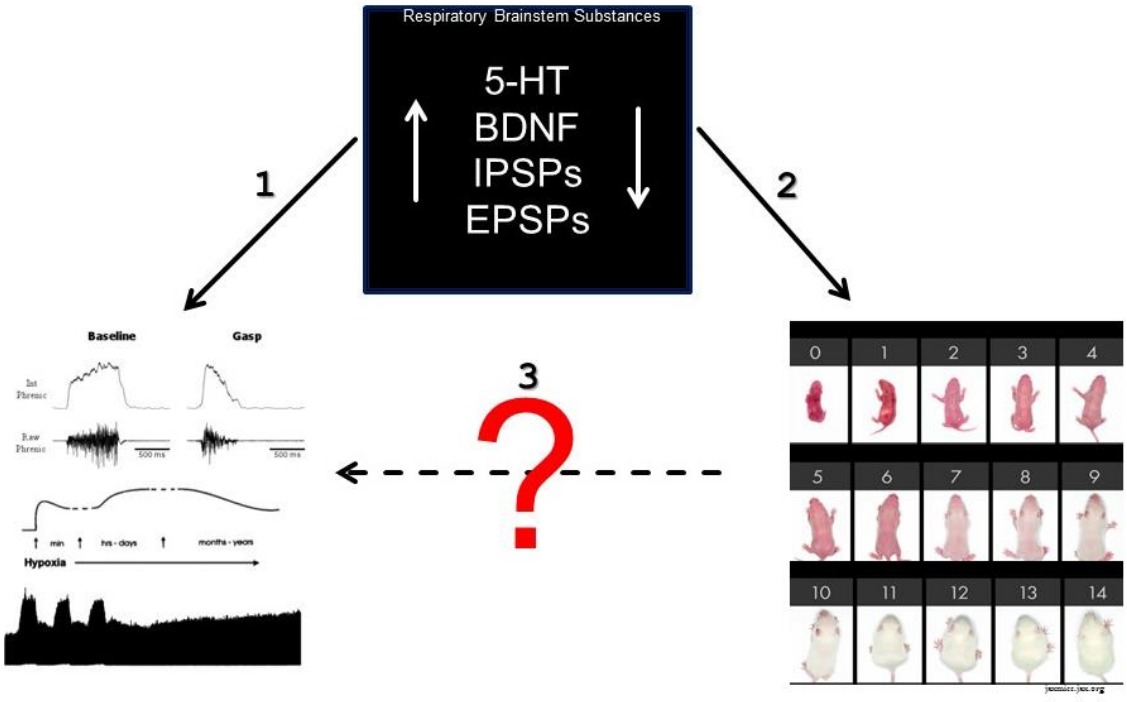
that claim was not provided. Further investigation with tissues from SIDS cases should provide evidence critical for future consideration of this model and hypothesis. In addition to the previous model, rats have been used to investigate other hypotheses regarding the pathogenesis of SIDS. More commonly, investigations of prenatal nicotine exposure in rats has revealed that nicotine decreased the respiratory response to hypoxia, possibly through depression of adrenal catacholamine release (Slotkin *et al*., 1995; St.-John and Leiter, 1999; Fewell *et al*., 2001). Prenatal nicotine exposure also lowered dopamine content within the carotid bodies, and in combination with hypoxia resulted in damage to cardiac myocytes (Holgert *et al*., 1995; Tolson *et al*., 1995). Guinea pigs have been extensively studied in respiratory physiology and pathology (Friberg *et al*., 2001; Li *et al*., 2001b). Environmental tobacco smoke exposure excited afferent lung C fibers, as well as nucleus tractus solitarius neurons, which prolonged expiratory apnea in the young guinea pigs. These findings have led to further understanding of the relationship between smoking and SIDS (Bonham *et al*., 2001). Adult and fetal rabbits have also been used to understand potential etiologies of SIDS. Studies have focused on cocaine exposure, toxigenic bacteria, and rebreathing of CO_2 (Gingras and Weese-Mayer, 1990; Kemp and Thach, 1993; Siarakas *et al*., 1997). Finally, because SIDS may be associated with thermal stress, Elder and coworkers (1996) (Elder *et al*., 1996) utilized 5 - 6 day old swine to investigate the effects of hyperthermia. They reported lung edema and hemorrhage consistent with that reported in SIDS. As with the previous swine model of SIDS, further reports of investigations with these models have not be of much use. Sheep have also been used to investigate SIDS, where intravenous nicotine reduced arousal and respiratory responses to hypoxic challenge (Hafstrom *et al*., 2000). It remains to be seen whether this model will provide useful information in understanding the pathogenesis of SIDS.

Animal models for human disease are often developed when there is a complete understanding the mechanisms involved as well as the disease etiology. Because this is not the case for SIDS, future animal models should mimic normal cardiorespiratory and neurologic development reported for infants two to four months of age. The model would be further enhanced if risk factors outlined in the “Triple

Risk Model" are incorporated into the model. Discovery of the etiology of SIDS, will facilitate the development of multiple animal models suitable for investigation of pathophysiologic mechanisms

SUMMARY

The overall goal of the present body of work was to characterize and quantify the hypoxic ventilatory responses in the developing neonatal rat. It has been previously shown that neurotransmitters are responsible for the differences in the hypoxic ventilatory responses (1) and it is also been shown that there are developmental changes in neurotransmitter system in the neonatal rat (2). What is unknown is the reverse (3) if these developmentally-regulated changes in observed in neurotransmitter systems will also manifest as maturational alterations in the inspiratory motor output responses to hypoxia. (See Schematic below)



CHAPTER 2

GENERAL METHODS

Human and Use of Laboratory Animals In this chapter are the general methods used to acquire and process data across both Specific Aims of this dissertation. Specific protocols for each aim are addressed in their respective chapters.

Animal model/Justification

Neonatal Sprague-Dawley (SD) P0-P30 rats of both sexes were used. All experiments were conducted under protocols approved by the Institutional Animal Care and Use Committee at the State University of New York at Stony Brook in compliance with Animal Welfare and in accordance with Public Health Service Policy on Human and Use of Laboratory Animals.

For the presented studies, *in vivo* urethane-anesthetized spontaneously breathing neonatal rat preparation were used. We selected this preparation based on the following: (1) the anesthetized rat is the primary animal model used for studying hypoxia-related ventilatory control (and LTF appears to be more robust in anesthetized as opposed to unanesthetized preparations), (2) developmental changes in neurotransmitter systems in brainstem respiratory-related regions are well established, and (3) spontaneously breathing, afferent intact animals exhibit HVR behaviors similar to those seen in humans/infants.

General Methods

Neonatal rats were anesthetized by an intraperitoneal (i.p.) injection of urethane (1.8 g/kg); and the level of anesthesia were assessed, and deemed appropriate, by absence of withdrawal reflex to a noxious paw pinch; if responsive, supplemental doses of anesthesia (*ca.* 10% of initial dose, i.p.) were given as needed. Body temperature was monitored using a rectal probe and maintained at ~37 °C throughout the experiment, using a heating pad and a heat lamp as necessary. All rats were allowed to breathe spontaneously while supplied a gas mixture of 40% O_2 in a balance of N_2 from a nose cone (basal conditions), and a thin platinum-iridium twisted wire bipolar electrode was inserted into the right side of the diaphragm (just right of the midline) for recording of diaphragm electromyogram (EMG) activity. The specific recording protocols, including the fractional concentrations used for the hypoxic exposures are identified in the *Experimental Protocol* sections associated with the individual Specific Aims.

Data Acquisition and Analysis

Diaphragm EMG (EMG_{DIA}) activity was amplified (x1k), notch filtered at 60 Hz, and filtered to pass frequencies between 10 Hz and 1 kHz. Filtered signals was full-wave rectified, and a moving average was obtained using 50-ms time constant. The raw and moving-averaged signals was recorded on digital tape at a sampling rate of 2.5 kHz (DAT, Cygnus Technologies) and on a computer at a sampling rate of 2 kHz (Chart 5.0, PowerLab, ADInstruments) for off-line analyses (MatLab 7.0.1). EMG_{DIA} data were then be segmented to obtain to data lengths corresponding to the inspiratory burst.

Temporal Analysis

EMG_{DIA} signals were analyzed in both the time domain (temporal characteristics) and information domain (inspiratory network complexity). Time domain data included inspiratory burst duration (T_I), time between bursts (T_E), burst frequency (burst/min), burst amplitude, and in some cases, minute activity (MA). MA was calculated as the product of burst amplitude and frequency, and both burst amplitude and MA data were normalized to baseline levels of discharge, which was set to 100% in each preparation. These variables were measured for eupnea, gasping, and post hypoxia respiratory rhythm.

Approximate Entropy Analysis

Information domain data consisted of the *approximate entropy* (ApEn) measure. The complexity of a signal can be quantified using ApEn analysis, which provides a statistical index that measures and quantifies the regularity (orderliness) in a time series. Higher values of ApEn are associated with irregularity and greater randomness, and thus reflect less system order or higher system complexity. Conversely, lower values of ApEn are associated with a higher degree of regularity and predictability, and thus reflect an ordered system or lower system complexity. ApEn was calculated as a function of the parameters m, the embedding dimension, and r, the tolerance (threshold) level. For the spontaneously breathing neonatal rat, m and r were set to the following values: 2 and 0.25 xSD, respectively. ApEn was calculated according to the methods described by Pincus (1991).

Approximate Entropy Analysis

All data are reported as mean±SE. The specific comparisons and statistical evaluations are described below in the *Statistical Analysis* sections associated with the individual Specific Aims.

CHAPTER 3

CHARACTERIZE AND QUANTIFY THE GASPING RESPONSE TO ANOXIA IN P0-P30 NEONATAL RATS

INTRODUCTION

Oxygen deprivation activates four respective stages of what is classically referred to as the hypoxic ventilatory response (HVR): hyperpnea, primary apnea, hypoxic gasping, and terminal or secondary apnea. Hyperpnea is characterized by deep and rapid breathing resulting from stimulation of the peripheral (carotid body) chemoreceptors. If the hypoxic stimulus is severe, this excitation is followed by respiratory depression that leads to apnea, which is the complete secession of breathing. If hypoxia persists beyond this point, a new form of respiratory excitation, hypoxic gasping, emerges (Thatch et al., 1991; St. John, 1996; St. John and Paton, 2003 Gozal, *et al*. 1996). Although the precise mechanisms generating gasping are not known, it is believed that the pre-Botzinger complex (PBC) located in the ventrolateral medulla senses severe hypoxia and elicits the gasping response following apnea (Solomon 1999; Solomon 2000; Solomon 2006). Hypoxic gasping is the last mechanism for survival; thus, gasping continues until either autoresusitation succeeds in restoring enough oxygen to re-establish normal breathing, or if that fails, terminal gasping is succeeded by secondary, terminal apnea. Terminal apnea is accompanied by a decrease in cardiac activity until death.

The sudden and unexplained death of an infant (SIDS) has been known since ancient times. Currently, SIDS is the third leading cause of death among infants from the day of birth up to 12 months of age. Epidemiologic studies established an association between prone sleeping and SIDS (Mitchetll *et al*., 1991), which led to the "Back to Sleep Campaign" that was endorsed by the American Academy of Pediatrics in order to reduce the incidence of SIDS.

With advances in our medical understanding, we can now offer a definitive diagnosis for the cause of death – autoresuscitation failure. Many propositions have been made to answer the question: What causes autoresusitation failure? Over the years, several etiologies have been proposed to explain SIDS. These include infections, (Blackwell *et al*., 1992; Howatson, 1992), metabolic disorders (Bonham and Dowing, 1992; Burchell *et al*., 1992), environmental perturbations (Kemp and Tach, 1993; Lewis and Bosque, 1995), as well as cardiac (Schawrats *et al*., 1998; Ackerman *et al*., 2001) and neurological abnormalities (Kinney *et al.,* 1991). These etiologies are often merged and difficult to categorize. For this reason, Filiano and Kinney proposed the "Triple Risk Model", in which SIDS cases are thought to occur when a vulnerable infant (congenital defect, metabolic disorder, etc.), a critical developmental period, and an exogenous stressor (infection, heat, CO_2, etc.) converge (Filiano and Kinney, 1994).

To date, it is common knowledge that SIDS occurs in children under one year of age although deaths after this time frame may also occur. Although the precise age in which a child succumbs to SIDS varies, most cases occur between one month and six months of age. Thus, there has been an increase in research focused on developmental respiratory neurobiologyin an attempt to identify potential causes for SIDS in the context of the "Triple Risk Model". Additionally, since failure to autoresuscitate is an important component of SIDS deaths, research focusing on identifying potential mechanisms of hypoxic gasping, which may or may not consider developmental aspects, has re-emerged.

Starting with work from Fazekas in 1941, it was recognized that the developing mammal is more tolerant to exposureto anoxic conditions (Fazekas and Alexander, 1941). Since this initial finding, a number of laboratories have characterized the autoresuscitation response during development. Gasping is an essential part of the autoresuscitation response, and it corresponds to the breathing pattern observed when mammals are exposed to prolonged anoxia or asphyxia, representing the final effort of the respiratory system to recover from anoxic episodes, such as those occurring in SIDS cases (Selle and Witten, 1941; Thach *et al*., 1991). Gasps are very characteristic in their pattern, which includes a short inspiratory duration, very high air flow, and enhanced inspiratory volume in comparison to normal spontaneous basal breaths (Thatch *et al*., 1991; St. John, 1996; St. John and Paton, 2003), and this type of breathing occurs as a result of a very high respiratory drive. Thus, if air is available, gasping provides the needed oxygen for survival, and restoration of breathing and cardiovascular function. In addition to newborn mammals being more tolerant than older mammals to anoxic exposure (Fazekas *et al*., 1941), the gasping response also appears to be developmentally regulated, with the duration of gasping, the effects of intermittent or repetitive hypoxia on gasping, and the number of gasps being shown to decrease with increasing postnatal age in different species, including mice and rats (Stafford and Weatherall, 1960; Chen *et al*., 2013; Fewell, 2000; Fewell *et al*., 2005; French *et al*., 1972; Gershen *et al*., 1990; Gozal *et al*., 1996; Gozal *et al*., 1998; Gozal *et al*., 2002; Jacobi *et al*., 1991; Jacobi and Thach 1989; Yuan *et al*., 1997). Although the above studies have identified developmental differences, they examined only a limited number of postnatal ages.

As previously mentioned, breathing encompasses the alternating phases of inspiration and expiration, behaviors that are generated by nuclei located in the brainstem. The coordination of these phases occurs under involuntary control, and is functional immediately following birth although changes in the neural network controlling breathing continue to occur as the mammal matures. In fact, ongoing research has demonstrated that there is a period of modification that occurs during early postnatal neurodevelopment of the respiratory system, and that may play a critical role in understanding SIDS.

From this research, it has been shown that the developing rat brain undergoes neurochemical changes during the first two weeks of postnatal life. These neurochemical changes are suggested to represent a critical age of respiratory network development, and it has been proposed that this critical period might be related to SIDS since SIDS occurs at a very specific time point of an infant's life, with 90% of the cases occurring in the first six months where neural development is critical (Fewell, 2000).

To begin to understand respiratory brainstem neurodevelopment, Wong-Riley and colleagues examined the day-to-day expression levels associated with various neurotransmitter systems and their components (e.g., receptors, transporters, etc.) in respiratory-related brainstem nuclei. They found in the neonatal rat that the first two postnatal weeks appear to be the critical time period for neural changes, with postnatal day 12 (P12) being identified as the primary critical age due to multiple developmental changes occurring simultaneously (Wong-Riley, 2008, Wong-Riley, 2005). They further proposed that at this age, changes in neurotransmitters and their receptors are potential causes for instability of breathing (Wong-Riley, 2005, Wong-Riley, 2008). Among the changes noted, both excitatory and inhibitory neurotransmitter systems known to be involved in the neural control of breathing were shown to be affected, with the expression levels of the main excitatory amino acid neurotransmitter glutamate and its N-methyl-D-aspartate (NMDA) receptor being dramatically decreased and the expression levels of the main inhibitory neurotransmitter gamma-aminobutyric Acid (GABA) and the inhibitory glycine receptor being markedly increased (Wong-Riley, 2008, Wong-Riley, 2005). In addition they proposed that, changes in receptor subunit dominance of key receptors responsible for exciting or inhibiting breathing contributed to this imbalance (Wong-Riley, 2008, Wong-Riley, 2005). Together, these neurochemical changes and the subsequent imbalance would lead to more inhibition and less excitation, resulting in a higher risk of autoresusitation failure (Wong-Riley, 2005).

In addition to the above observations, significant changes in the expression levels of serotonin (5HT)-related neurotransmitters, receptors, and receptor sub-types during the first two weeks of life has been demonstrated, including a significant reduction of ~25% in 5-HT_{2A} receptors at P12 in the pre-Botzinger, which is the primary area for respiratory rhythm generation, nucleus ambiguus, and the hypoglossal motor nucleus (Lui and Wong-Riley, 2010). This is of great significance to this body of work since brainstem abnormalities, which potentially could accelerate a failure of normal protective homeostatic responses to physiological stressors, involves 5-HT, and corresponds to a common abnormality found within the medulla in human SIDS brainstems (Kinney *et al*., 2003; Ozawa and Okado, 2002; Panigrahy *et al*., 2000; Paterson *et al*., 2006).

While the interaction between excitation and inhibition may be essential for respiratory rhythm generation, modulatory neurotransmission appears to confer the high degree of adaptability necessary for function of such a highly dynamic system. Neuromodulation has been defined as "the ability of neurons to alter their electrical properties in response to intracellular biochemical changes resulting from synaptic or hormonal stimulation" (Kaczmarek and Levitan, 1987). As previously mentioned, respiratory neuronal networks are modulated by several neurotransmitters, including those that are considered classically excitatory (e.g., glutamate) or inhibitory (e.g., glycine), as well as, more specific to this body of work, through monoamine transmitters (*e.g., 5-HT).* 5-HT acts as both a signaling molecule and a neurotransmitter, and in mammals, it is synthesized by enterochromaffin cells in the gastrointestinal tract by a select population of neurons located in the midline of the brainstem region known as the serotonergic raphe nuclei (Jacobs and Azmitia, 1992).

Neurons that govern respiratory control in the pons and medulla are innervated by 5-HT neurons (Connelly *et al*., 1989, Holtman *et al*., 1990), and receptors for 5-HT are located both pre- and post-synaptically as well as localized extra-synaptically on the cell membranes of neurons and other cell types. These receptors hail from a broad range of the 5-HT receptor families: 5-HT_1–5-HT_7 receptors consist of multiple subtypes that differentially mediate either excitatory or inhibitory effects. Due to the wide

number of receptor subtypes and a variety of systemic effects amongst experimental models, the exact effects of 5-HT have remained unclear. 5-HT has been shown to produce a variety of responses, including facilitatory effects on the respiratory rhythm generator via medullary 5-HT_{1A} receptors, the recruitment of phrenic motoneurons via 5-HT_{2A} receptors, and inhibitory effects on the transmission of the central drive to phrenic motoneurons via presynaptic 5-HT_{1B} receptors (Lindsay et al., 1993). Therefore the role of 5-HT in respiratory control has been the source of much speculation over many years of research, having been at once deemed net inhibitory, stabilizing or regulatory, and excitatory - the latter of the three appearing to have gained the most favor in recent years (Richerson, 2004).

Serotonergic defects in the CNS have been discovered postmortem by neurohistological studies; thus, numerous research efforts have been dedicated to understanding such deficiency (Duncan *et al.* 2010). 5-HT appears to play an important role in the maturation and regulation of neurons involved in respiratory control, even as early as the embryonic stage of development. Evidence supporting a critical role for endogenous 5-HT comes from experiments in which blocking medullary 5-HT_{1A} receptors in embryonic day 18 (E18) rats induced respiratory arrest while activating them increased breathing frequency (Di Pasquale et al., 1994). Additional insight into the role of 5HT comes from studies in transgenic mice The transgenic mouse models developed include a knockout mouse model lacking the 5-HT transcription factor Pet-1 and a conditional knockout mouse model of the factor Lmx1b, which lack 70% and 99.0% of 5-HT neurons, respectively (Ding *et al*., 2003; Hendricks *et al*., 2003). Neonatal Pet-1 KO mice display a slow and unstable respiratory rhythm while in adult male mice, the respiratory phenotype manifests as a blunted response to CO_2 (Erickson *et al*., 2009, Hendricks *et al*., 2003; Hodges *et al*., 2008). This latter phenotype is also a feature present in Lmx1b conditional knockout mice (Hodges *et al*., 2008). These findings suggest that 5-HT neurons play an important role in establishing eupnea as well as the hypercapnic ventilatory response. Application of exogenous 5-HT rescues the response to CO_2 in Lmx1b deficient mice, suggesting that 5-HT may act in an additional capacity to enhance hypercapnic chemosensitivity of non-5-HT neurons (Hodges *et al*., 2008). Similarly, in neonatal rats with 5-HT

lesions, there is a high mortality rate due to their inability to autoresuscitate following repeated challenges of anoxia (Cummings *et al*., 2011)

While great scientific advances have been made in understanding many aspects of respiratory brainstem neurochemistry and its developmental significance, more work needs to be done. Of relevance to this dissertation, additional information is needed to understand the potential influence of these day-to-day changes in the respiratory brainstem neurochemistry on physiological processes that participate in the pathogenesis of SIDS. Thus, one goal of our study is to investigate the influence of postnatal age on the gasping response in the neonatal rat.

Since SIDS infants do not usually show any symptoms of respiratory abnormalities before death, it is important to identify a way to diagnose SIDS before it occurs in order to try to prevent it. Observations from human infants that died of SIDS and non-SIDS conditions revealed that gasping is characterized by the presence of multiple gasping patterns, including doublet and triplet gasps (Sridhar, 2003). In general, gasps occur as singlet events that are separated by long expiratory pauses (T_E); however, doublet and triplet gasps are characterized by two and three consecutive (or premature) gasps, respectively, that are separated by a very short T_E. Thus, doublet and triplet gasps represent unusual patterns of gasping. In the above study, the incidence of multiple gasp patterns was significantly higher in SIDS cases than non-SIDS cases, with four out of five SIDS cases containing both doublet and triplet gasps. In addition, the non-SIDS cases were initially suspected to be SIDS, but following re- examination, were found to be caused by other known influences (Sridhar, 2003). Based on these observations, it was suggested that the presence of multiple gasping patterns may be a way of predicting when SIDS might occur, and that instead of predicting when in the first year of life SIDS will occur, which is already known by statistics, this may be a preferable way to predict if an infant is vulnerable to SIDS or when an infant would be at an extremely high risk of a SIDS-related respiratory event.

Although multiple gasping patterns have been previously reported in dying preterm infants (Peiper, 1963, Jacobi *et al*., 1991), very few studies, especially studies using animal models, have reported doublet and triplet gasp patterns. In fact, doublet and/or triplet respiratory-related burst patterns in animal models have only been included in a few scientific papers as a side note to the main topic under investigation. For example, doublet bursts were reported in respiratory-related cervical and hypoglossal discharges in neonatal rat brainstem-spinal cord preparations during cyanide (CN)-induced anoxia (Taccola, 2007).In addition, ectopic and doublet neuronal bursts were observed in adult rats during hypercapnic hypoxia-induced gasping or the early phase of recovery from hypercapnic hypoxia (Fortuna *et al*., 2008). Finally, doublet phrenic bursts were observed in response to chemostimulation of the adult cat PBC (Solomon, 2000). While the above studies demonstrate that doublet respiratory-related burst patterns may be observed in animal models, it remains to be determined whether doublet and triplet gasps are seen in the in vivo neonatal rat, and if so, how postnatal maturation affects this behavior.

Thus, the experiments proposed in this aim were designed to (1) characterize the time course and temporal characteristics of the severe hypoxic response, including the PCR and gasping response in spontaneously breathing P0-P30 neonatal rats, (2) determine whether gasping is evoked in response to severe hypoxia in neonatal rats at each postnatal developmental stage being studied, and (3) characterize "unusual" gasping patterns observed in young neonatal rats.

METHODS

General Methods

All experiments were performed under protocols approved by the Institutional Animal Care and Use Committee at the State University of New York at Stony Brook in accordance with Public Health Service Policy on Human Care and Use of Laboratory Animals. Experiments were conducted using an in vivo urethane-anesthetized spontaneously breathing P0-P30 neonatal Sprague-Dawley rat (Taconic Farms, Germantown, NY) preparation (n=258).

Rats where anesthetized with an intraperitoneal injection of urethane (~1.8 g/kg). The adequacy of anesthesia was regularly verified by absence of withdrawal reflex to a noxious interdigitary pinch and was supplemented (5-10% of initial dose) as necessary. Diaphragm electromyogram (EMG_{Dia}) activity was recorded using a thin twisted platinum wire bipolar electrode inserted into the right side of the rat's diaphragm. Body temperature was measured using a rectal probe and maintained at ~37 °C throughout the experiment, using a heating pad and a heat lamp as needed.

Experimental Protocol

EMG_{Dia} activity was recorded under baseline conditions (40% O_2 in a balance of N_2) and during anoxia (100% N_2) to evaluate the HVR and gasping. Stable baseline activity was recorded for at least 10 minutes, after which the anoxic challenge was initiated. The anoxic challenge was variable in duration, but was maintained until terminal apnea ensued. The recording protocol is shown below:

Data Acquisition

EMG_{Dia} discharge signals were amplified (1k), notch filtered at 60 Hz, and filtered to pass frequencies between 10 Hz and 1 kHz. Filtered signals were rectified, and a moving average was obtained using a 50-ms time constant. The raw and moving-averaged inspiratory motor discharges were recorded on digital tape at a sampling rate of 2.5 kHz (DAT, CygnusTechnologies) and on a computer at a sampling rate of 2 kHz (Chart 5.0, PowerLab, ADInstruments) for off-line analyses (MatLab 7.0.1). The data were then segmented to obtain to data lengths corresponding to the inspiratory burst. These segmented data were digitally band-pass filtered (10-50 Hz) using a 6-pole Butterworth filter.

Data Analyses

Part I – Temporal Analyses:

Data from individual experiments were output for temporal analyses. Inspiratory burst duration (T_I), the duration between bursts (T_E), and burst amplitude (normalized to percent baseline) were determined for an average of 20 bursts during baseline, and for all primary gasps recorded. For the anoxia, the duration of the PCR, apnea, and gasping were determined, and the onset latency to gasping and the total number of gasps elicited were also evaluated.

Approximate Entropy Analyses: Approximate entropy (ApEn), which serves as an index of inspiratory neural network complexity, was calculated as a function of the parameters *m*, the embedding dimension, and *r*, the tolerance (threshold) level. For these analyses, m=2 and r=0.25 xSD. ApEn was calculated according to Pincus (1991) as follows:

$$\mathrm{ApEn}(m, r, N) = \Phi^m(r) - \Phi^{m+1}(r)$$

$$\text{where } \Phi^m(r) = (N-m+1)^{-1} \sum_{i=1}^{N-m+1} \ln C_i^m(r)$$

$$\text{and } C_i^m(r) = N^m(i)/(N-m+1)$$

$$N^m(i) = \text{no. of } d[X(i),\ X(j)] \leq r$$

where $X(i)$, $X(j)$ are vectors defined by $X(i) = [u(i), u(i + 1), ..., u(i + m – 1)]$ and $X(j) = [u(j), u(j + 1), ..., u(j + m – 1)]$ from the original time series $u(1), u(2), ..., u(N)$.

ApEn values were calculated for 20 consecutive EMG_{Dia} bursts during the last minute of baseline conditions and for all primary gasps. The average ApEn value was then determined from these data.

Statistical Analyses: All data are reported as mean±SE. Statistical significance was determined using a paired *t-test* for comparisons of variables between basal bursts and gasps, and *one-way ANOVA*, followed by Holm-Sidak *post hoc analyses* for multiple comparisons was used for comparisons across the age groups. The criterion level for determination of statistical significance was $P<0.05$.

RESULTS

Gasping Response in the Neonatal Rat

The gasping response to severe hypoxia was observed in 145 of the 258 experiments conducted. In these experiments, the neonatal rats exhibited the classic PCR, apnea, and gasping; however, differences in the patterning and timing the severe hypoxic response were observed in rats at different postnatal ages. Example traces showing typical recordings of basal EMG_{Dia} activity and the response to severe hypoxia from experiments conducted in two different postnatal aged rats are provided in Figure 1. A more detailed characterization of the responses seen during the severe hypoxic challenge are provided in the sub-sections below.

Incidence of Gasping

In response to severe hypoxia, gasping was elicited in subset of the neonatal rats studied. The incidence of gasping exhibited a developmental trend, with gasps being observed in nearly for all of the ≤P12 rats. In contrast, gasps were observed in 50% or fewer of the P13 and ≥P15 rats. The percentage of neonatal rats that gasped in response to severe hypoxia for each postnatal age group studied is shown in Figure 2.

Timing and Patterning of the gasping response in the neonatal rat

In experiments in which severe hypoxia elicited gasping, differences between the younger and older neonatal rats were noted for the timing and patterning of the gasping response. These differences included the onset latency to gasping, the duration of gasping, and the total number of gasps, and both the data from individual experiments and summary data illustrating these differences are provided in Figure 3. Specifically, the onset latency to gasping was longer in young neonatal rats with the mean onset latencies being 177.8±32.5 s, 158.5±11.6 s, 113.2±20.9 s, 145.0±14.1 s, and 89.2±4.0 s for P0-P5, P6-10, P11-P14, P16-P23, and P23-P30 rats, respectively. Compared to the P0-P5 age group, the onset latency to gasping was shortened by 11%, 36%, 18%, and 50% for the P6-10, P11-P14, P16-P23, and P23-P30 rats, respectively. While the onset latency to gasping was longer in the younger rats, this difference was only statistically significant for the differences between the P0-P5 and P6-P10 age groups versus P23-P30 age group (P=0.004). The duration of gasping was also longer in young neonatal rats with mean durations being 853.0±110.4 s, 259.2±33.9 s, 147.1±29.1 s, 51.3±8.3 s, and 40.3±5.2 s for P0-P5, P6-10, P11-P14, P16-P23, and P23-P30 rats, respectively, and the total number of gasps was greater in young neonatal rats with the mean number being 112.9±14.0, 94.7±12.1, 82.9±18.7, 29.4±7.0, and 16.8±1.8 for P0-P5, P6-10, P11-P14, P16-P23 and P23-P30 rats, respectively. While in younger rats the numbers of gasp were somewhat variable, for most of the neonatal rats ≤P14, the number of gasps ranged from 102 to 217 while for the neonatal rats ≥P16, the number of gasps ranged from 10 to 50. Overall, the effects of maturation on both the duration of gasping and the number of gasps were significantly different among the age groups indicated in Figure 3 (P<0.001). Regardless of the magnitudes of the responses associated with these measures, there was a clear developmental trend in all three measures, clearly demonstrating that each measure decreased as postnatal age increased.

Temporal Characteristics of Gasps in the neonatal rat

In our experiments, gasping was characterized as high amplitude, short duration EMG_{Dia} bursts that were separated by longer pauses when compared with basal breathing (see Figure 1). While these basic gasp characteristics are present for nearly all of the neonatal rats studied, our data demonstrate that these characteristics also took on a developmental trend. Moreover, the temporal characteristics of the basal EMG_{Dia} bursts also exhibited developmental differences. The effects of maturation on both basal burst and gasp burst characteristics is described below, and both the data from individual experiments and summary data illustrating these differences are provided in Figure 4.

Basal EMG_{Dia} activity exhibited developmental differences in both burst duration (T_I) and the time between bursts (T_E). In general, T_I increased and T_E decreased with increasing postnatal age. Compared to the P0-P5 age group, a significant increase in T_I of 38%, 53%, and 75% was observed in the P11-P14, P16-P22, and P23-P30 age groups , respectively ($P<0.001$), and a significant decrease in T_E of 38%, 45%, 56%, and 61% was observed in the P6-P10, P11-P14, P16-P22, and P23-P30 age groups, respectively ($P<0.001$).

The relationship between basal bursts and gasps as well as gasp burst activity (i.e., T_I, T_E) also exhibited developmental differences. When compared to basal bursts, gasps were characterized by a significant reduction in T_I ($P<0.001$) for all neonatal rat age groups except P0-P5 where the gasp T_I was unchanged due to predominantly a lengthening of the burst in the P0-P1 rats. The magnitude of the reduction in T_I from the basal burst to the gasp burst was greatest in the P11-P14 age group ($P<0.001$) although gasp T_I was shortest in the P6-P10 and P11-P14 age groups after which it increased ($P<0.001$), being longest in the P23-P30 age group. Thus, for gasp burst activity, T_I initially decreased and then increased. In contrast, compared to basal bursts, gasp T_E was significantly increased for all neonatal rat age groups ($P<0.001$), with the longest T_E being noted in the P0-P5 age group ($P<0.001$). While gasp T_E was increased over basal burst T_E for all age groups, gasp T_E decreased with increasing postnatal age,

being shortest in the P16-P22 rats. Comparing T_E under basal conditions to those observed during gasping, T_E increased from 1047.5±141.6 ms to 11579.1±4073.3 ms for P0-P5 rats, 644.2±83.7ms to 2685.7±318.9 ms for P6-P10 rats, 569.4±69.8 ms to 1874.9±328.9 ms for P11-P14 rats, 459.1±21.9 ms to 1553.4±134.5 ms for P16-P22 rats, and 403.0±14.6 ms to 2079.6±261.5 ms for P23-P30 rats, respectively (P<0.001).

While gasp burst timing characteristics exhibited a developmental trend, gasp burst amplitude did not. Compared to basal burst amplitude, gasp burst amplitude was significantly increased, ranging from an increase of 56% to 195% above baseline levels (P<0.001); however, no differences in the magnitude of the amplitude change were noted across the age groups.

Approximate Entropy and Maturation

ApEn values were reduced during gasping, as compared to basal bursts, for each of the age groups studied; however, the magnitude of the decrease exhibited a developmental difference. The data from individual experiments and summary data illustrating these differences are provided in Figure 5. Overall, there was a 5-23% reduction in ApEn values during gasping, with the P6-P10 and P11-P14 age groups exhibiting the lowest ApEN values. Compared to the P0-P5 age group, however, gasps in the P6-P10 age group had significantly lower ApEn values while gasps in the P16-P22 and P23-P30 age groups had significantly higher ApEn values (P<0.001). In addition to the developmental trend noted for gasp ApEn values, a maturational trend in ApEn values for basal bursts was also noted. In this case, ApEn values significantly increased with maturation (P≤0.001).

Multiple Gasping Patterns

While our initial analyses identified gasps in response to severe hypoxia, re-examination of our traces revealed that doublet and triplet gasps (i.e., unusual gasping patterns) were also present in a subset of our experiments. Examples of these gasping patterns are provided in Figures 6 and 7, with Figure 6 showing two episodes of multiple gasping patterns from a single experiment and Figure 7 showing all of the gasp pattern behaviors recorded in two different aged rats. It should be noted that by examining the traces in a long-duration record, such as that provided in Figure 6, the specifics on these patterns are almost unrecognizable; however, using an expanded time scale, the differences in gasp duration as well as the duration between each gasp burst can be detected. This expanded time scale is provided in both Figures 6-7 to highlight the temporal details.

These unusual gasp patterns also exhibited a developmental trend in their occurrence, timing, and patterning characteristics. Specifically, unusual gasping patterns were observed only in P0-P12 neonatal rats, with 95% (43 of 45) of the P0-P6 aged neonatal rats showing unusual gasping patterns and 35% (14 of 40) of the P7-P12 aged neonatal rats showing unusual gasping patterns (Figure 8). For both age groups, most of the gasps observed exhibited the classic singlet gasp pattern; however, for the unusual gasping patterns, a greater number of doublet and triplet gasps were observed in the P0-P6 age group compared to the P7-P12 age group. Additionally, the numbers of doublets were greater than the numbers of triplets for both age groups. While the incidence and number of unusual gasps differed between the two age groups, the total number of gasps was not significantly different. These data are quantified and provided in Figures 8-9.

Characteristics of Multiple Gasping Patterns

For these analyses, singlet gasps and the first gasp in a doublet or triplet is designated as G1, the second gasp in a doublet or triplet is designated as G2 and, the third gasp in a triplet is designated as G3. Compared to the classic singlet gasp which had a gasp T_I of 75.4±.8.6 ms, gasp T_I for a doublet was increased to 90.1±1.6 ms for doublet G1 and 83.7±1.2 ms for doublet G2, and gasp T_I for a triplet was increased to 94.5±7.0 ms for triplet G1, decreased to 73.2±2.9 ms for triplet G2, and increased to 84.0±2.6 ms for triplet G3 in the P0-P6 age group. In the P7-P12 age group, gasp T_I values were reduced from 61.9±.72 ms in singlet G1 to 37.1±1.4 ms in doublet G1 and 49.3±2.3 ms in doublet G2, and 31.0±11.9 ms, 32.7±9.0 ms, and 44.5±6.2 ms in triplet G1, G2, and G3, respectively. For gasp T_E, singlets and time between gasp events were associated with a prolongation, the T_E of doublet (G1, G2) and the triplet (G1, G2, G3) gasps were significantly shorter in both age groups. These data are presented in Figure 10. In addition, doublet gasps were further subdivided into two categories of doublet gasps: connected and separated (data not shown) based on their T_E timing characteristics. Connected doublet gasps were characterized as those in which the second gasp of the pairwas superimposed onto the first gasp (i.e., the first burst was not finished firing before the second burst began) and separated double gasps were characterized as those in which two concurrent gasps exhibited a defined pause in between them. Both connected and separated doublet gasps were observed in each of the two age groups; however, a few distinct findings are worth mentioning.

First, the duration of an experiment in which gasping included a short series of doublet and/or triplet gasps was usually shorter than the duration of an experiment that began with a string of singlet gasps. Second, during experiments where doublets and/or triplets were found interspersed between singlet gasps, T_E was highly variable, and thus, rendered the gasping response less stable. Third, if an experiment contained both connected and separated doublet gasps, the connected doublets almost always occurred in the beginning of the gasping response and the response always ended with singlet gasps. And lastly,

although doublets tended to cluster in the gasping response, the particular region and timing of this clustering was not predictable.

ApEn values were also altered in doublet and triplet gasp components in both age groups albeit the differences were not always significant. For the P0-P6 rats, ApEn values, which were 0.318±0.008 for basal bursts, decreased to 0.279±0.003 for singlet gasps, and to 0.3063±3.2 for doublet gasps, but were not altered in triplet gasps. In contrast, for the P7-P12 rats, ApEn values, which were 0.3700±3.6 for basal bursts, decreased to for singlet gasps, to 0.2538±2.9 and 0.1891±8.2 for doublet G1 and G2 gasps, and 0.2282±.01, 0.1164±.01, 0.1545±.03 for triplet G1, G2, and G3 ($P \leq 0.001$).

DISCUSSION

The initial part of this work set out to characterize the maturational response to severe hypoxia in the neonatal rat. This work further describes age-dependent changes in the incidence and characteristics of the neonatal gasping response to severe hypoxia. Importantly, we identified a high incidence of gasping during the early stage of development as compared to more mature neonatal rats. Furthermore, postnatal age significantly influenced the gasping response and the timing of this response such that many variables examined either significantly increased or decreased with maturation. In general, both inspiratory duration and network complexity were reduced during gasping (except in the younger neonatal rats). Novel findings to this body of work include the evidence that neonatal rats are capable of producing unusual gasping patterns similar to those seen in human infant SIDS patients, in response to severe hypoxia, and these patterns like other parameters examined where developmentally regulated.

Overall the response to severe hypoxia was not identical across all ages examined. In addition, during gasping, the duration of inspiration and timing between bursts was altered significantly in the older neonatal rats examined, demonstrating a maturational trend that centers on a specific developmental window. These results are in general agreement with the respiratory brainstem neurochemical studies reported by Wong-Riley and colleagues (Wong-Riley *et al*. 2013), in which they report that in respiratory brainstem regions, there seems to be a critical age in which the changes in neurochemistry shift.

Of the central mechanisms that could be responsible for altered respiratory responses to hypoxia, the role of neurtotransmitters/neuromodulatory systems have received much attention. Numerous studies have demonstrated developmental changes in intrinsic membrane properties and synaptic transmission within the central respiratory network. Researchers have examined maturational changes in expression of various neurotransmitters, neuromodulatory and neurotrophic factors involved in central respiratory control systems during development (Wong-Riley, 2008, Wong-Riley, 2005).

As far as we know, the neurochemical basis for this inverse relationship between postnatal age and gasping characteristics in the neonatal rat is unknown. It can be speculated, however, that changes in various neurotransmitter and/or neuromodulatory systems (Wong-Riley, 2008, Wong-Riley, 2005) may affect the firing pattern in neurons in the tegmentum, a general area within the brainstem that is responsible for unconscious homeostatic control and reflex pathways (Wang *et al*., 1996). Specifically, the initiation and duration of gasping during exposure to a single period of hypoxia may be determined by activation of neurotransmitter systems involved in the neural control of breathing. In 1998, Gozal and colleagues demonstrated that administration of N-nitro-$_{L}$-arginine, an inhibitor of nitric oxide and also a vasoconstrictor increases the duration of gasping, and that the effect was more pronounced in older versus younger neonatal rats (Gozal *et al*., 1998). Its plausible then that nitric oxide in the tegmentum could mediate maturational changes in the gasping response to severe hypoxia in the neonatal rat (Gozal *et al*., 1998).

In the current study during baseline breathing, ApEn values increased with postnatal maturation, suggesting maturational changes in the central respiratory neural network complexity with older neonatal rats, exhibiting higher ApEn values (less system order) compared to younger neonatal rats. In younger neonatal rats, the central respiratory neural network would be fairly immature (key players) and simple, and thus, it could be reasoned that low values of ApEn make sense. With maturation, a greater degree of connections in the network would be established, and thus, higher values of ApEn would be expected. Although, from this investigating, it is interesting to note, that despite the maturational trend seen in ApEn values during basal breathing, with the shift to gasping, there is an overall reduction in ApEn values for all ages examined, and although decreased from baseline values, the ApEn for each experimental age group was developmentally increased. Due to limited data set for the younger neonatal rats studied, however, additional experiments would be needed to support these findings.

Implementation of ApEn analyses in this study has provided an additional tool to further characterize the inspiratory neural control network during eupnea and gasping. We found, similarly to Akay and colleagues (Akay and Sekin, 2004; Akay et al. 2002) a reduction of ApEn following the shift from eupnea to gasping in each of animal ages examined, suggesting that gasping is more highly ordered than producing basal inspiratory bursts. Although the absolute magnitude of the reduction in ApEn values for the urethane-anesthetized spontaneously breathing neonatal rat *in vivo* during gasping were not as large as that seen in the anesthetized piglet (Akay and Sekine, 2004), the percent change was comparable for our data set. Although the precise reason for this is unknown, it can be related to a host of differences including difference in animal models, experimental preparations, and maturational age. In spite of the magnitude difference, both studies in response to severe hypoxia demonstrated bursts that were abrupt onset, short in duration, and decrementing in pattern. Both the work from this study and that from Akay and colleagues demonstrate that unlike initial theories of the respiratory control system being rigid, the respiratory control system in fact is capable of being highly dynamic and that the complexity of the underlying interactions although changes in response to a developing system, also exhibit changes when exposed to different conditions. Whether this is a result of removal of premotor respiratory neurons that are silenced during hypoxia or a more highly synchronized and orderly functioning system remains to be determined. Research suggests that additional mechanisms are at play to reduce ApEn (Akay *et al*. 2002; Akay and Sekine, 2006). The reduction is a result of the depressive effect of hypoxia on the respiratory neural control system during gasping. Thus, elements of the respiratory rhythm that are typically responsible for the generation of respiratory rhythm under baseline conditions are no longer in control. In essence, the reduction seen in ApEn during severe hypoxia does not rely on neurotransmitter or neuromodulatory systems which are typically in play during basal respiratory rhythm. This leads to suggestions that the complexity of gasping is a reflection of only the most minimal elements needed to coordinate a life-sustaining gasp. Identifying these key player(s) in the developing rat may prove to be crucial in understanding successful autoresuscitation efforts. Therefore, ongoing studies assessing potential mechanisms that may contribute to central respiratory neural control are of great importance.

Additional studies are needed to further clarify the precise mechanism responsible for the differences in the central respiratory network complexity among the age groups studied as well as between eupnea and gasping.

Multiple gasping patterns, typically seen in infants that have succumbed to SIDS, were also seen in the spontaneously breathing urethane-anesthetized neonatal rat.

The neural substrate responsible for generating the gasping behavior has been assigned to multiple areas in the medulla, including the lateral tegmental field (St. John, 1996; St. John *et al*. 1984) and the PBC (Solomon *et al*. 1999, 2000). This concept has been further expanded to incorporate that there are critical periods of development for respiratory development in the brain. These multiple gasping patterns were only identified in neonatal rats aged P0-P12, demonstrating that the proposed critical period of respiratory development exhibits an alternate type of gasping response.

We suggest that these observations are consistent with the idea that postnatal day 12 represent a critical day in development of respiratory neural systems. Our work revealed that although gasping could be elicited in most neonatal rats studied, multiple gasping patterns were only seen in rats at and younger than P12. This finding may be interpreted to suggest that spontaneously breathing neonatal rats P0-P12 could serve as an adequate animal model for studying unusual gasping patterns that are associated with SIDS. SIDS infants have been reported to exhibit doublet and triplet gasps (Sridhar, *et al*. 2003), and in our study, more doublet and triplet gasps were observed in the younger (P0-P6) neonatal rats compared with older (P7-P12) neonatal rats.

The analysis of doublet gasps in this study was somewhat complicated because two categories of doublets were identified, connected and separated doublet gasps. In Part 1 of this study, the T_I and T_E of the individual gasp components (G1 and G2) were characterized for doublet bursts, but these measurements did not take into consideration the different patterns of doublet bursts later identified. As a result, the values reported for both T_I and T_E may be influenced by the incidence of multiple gasping

patterns. Both age groups exhibited a difference in the number of doublet and triplet gasps. Since the values reported for T_I and T_E reflect averaged values, the inclusion of such bursts could result in higher temporal values than those associated with multiple gasping patterns. Similarly to what has been reported before, it has been suggested that younger mammals exhibit lower gasp frequency than older mammals (Fazekas, 1941). Young mammals are reported to be more tolerant to periods of anoxia because they have large stores of cardiac glycogen, which may serve a protective function during oxygen deprivation. Conversely, older mammals cannot withstand anoxia for too long because they have low amounts of cardiac glycogen (Fazekas, 1941). This work is consistent with previous observations on developmental gasping frequency.

Another interpretation of the frequency data could also be used to support the idea that high frequency gasping may deplete glycogen stores faster. In this interpretation, doublet and triplet gasps would be considered as high frequency gasps compared to singlet gasps. Thus, a string of doublet and/or triplet gasps would be expected to result in a short gasping duration if reoxygenation remains inadequate. In the current experiments, this may be responsible for the failure of the rats to sustain gasping when the gasping run exhibited an early series of doublets and/or triplets. As a high degree of variability in T_E was seen when doublet and/or triplet gasps were interspersed between singlet gasps, it appears that the presence of a series of unusual gasping patterns appears to be detrimental to the successful outcome of the gasping response. In this light, multiple gasping patterns may be unfavorable not only because they disrupt stability but also because they could potentially lead to premature (terminal) apnea. For example, doublet gasps tended to cluster in the time course of any given gasping response; however, if singlet gasps were interspersed in the doublet pattern, there was a waxing and waning of the gasping event T_E between low (singlet T_E values) and high (T_E doublet gasp values) durations. In contrast, doublet gasps which tended to cluster elsewhere in the time course of the gasping run seemed to provide stability to the gasping behavior. These patterns of behavior may be related to reconfiguration of the central respiratory network, which is constantly trying to correct itself in order to maintain homeostasis, even in the

developing rat under strenuous conditions. This possibility would be consistent with the explanation provided for network resetting associated with pre-mature or ectopic respiratory bursts (Lieske, 2000).

What is unclear from the current study is how the demonstrated changes in brainstem neurochemistry at P12 (Thach 2008, Wong-Riley 2008) fit in with the current observations. It may be that the neurochemical changes minimize the potential for manifestations of hypoxic excitation that result in gasping or in this case, multiple gasping patterns. Previous studies from our laboratory have demonstrated that application of a glutamate to the PBC produces a rapid series of high amplitude, short duration gasp-like inspiratory bursts (Solomon, 2000). Since the neurochemical changes occurring at P12 favor enhanced inhibitory neurotransmission, the respiratory network may not be able to produce these doublet and triplet excitatory events. This explanation may also represent a reasonable suggestion for why multiple gasping patterns were not observed in rats >P12, which show further enhancements of the inhibitory neurotransmitter system (Thach 2008, Wong-Riley 2008). If this explanation is valid, it could be concluded that a more excitatory network, such as that seen in young neonatal animals, may be responsible for the generation of multiple gasping patterns. This may also explain the increased incidence of multiple gasping patterns seen in SIDS infants. However, this explanation does not account for the waning seen in T_E following an unusual gasping event.

Limitations

Our studies do not provide insight into the role of specific changes in brainstem and spinal cord neurochemistry implicated in respiratory neural development. Litter sizes were monitored but the litters were not culled to a constant size (although a litters of less than 10 rats were not used) and rats from each litter were used at various ages randomly and not with any specific guidelines. Additionally, both male and female rats were used in this study, and in the older female rats, the estrus cycle was not monitored; thus, potential hormonal influences could affect our observations.

The current study utilized only the neonatal rat. The neonatal rat pup is very immature compared with other species (i.e. piglets, cat) and extrapolation of our findings from one species to another can be subjected to further discussion.

The use of anesthesia is an important limitation of this body of work. Although urethane is a commonly used anesthetic in the field of central respiratory control because of its minimal effects on respiratory output, an EEG monitor was not utilized to ensure that all animals were in similar anesthetic plane.

Conclusions

With increasing postnatal age, there was a decreased likelihood for neonatal rats to gasp in response to severe hypoxia. Similarly, the incidence, the onset, the duration, the number of gasps, and burst timing characteristics were also affected by the postnatal age. Our findings also demonstrate that for the ages studied, ApEn values were developmentally regulated.

Incidence of Unusual Gasping Patters in the Neonatal Rat

In addition, this is the first study to show that neonatal rats are capable of producing both doublet and triplet gasping patterns in response to severe hypoxia. Our findings demonstrated that the incidence of unusual gasping patterns was also developmentally regulated, with younger neonatal rats being more susceptible to unusual gasping patterns. However, there are distinct differences in the temporal characteristics and ApEn values between the P0-P6 and P7-P12 age groups as well as a difference in the likelihood of encountering a multiple gasping pattern. These data suggest that multiple gasping patterns are developmentally-dependent, and we propose that these changes rely on a reconfiguration of the central inspiratory neural network.

From our observations, we have demonstrated that maturational changes reflect a reconfiguration of the central inspiratory network; however, the precise role, if any, in the timing of these changes remains unclear. Furthermore, these findings provide insight into the potential effects of maturation and its associated changes in inspiratory network complexity.

Our findings with regards to development and the gasping response require clarification, even in light of the previous studies suggesting a critical age for neurochemical development. Our findings can neither support nor refute any of these suggestions as our data can be interpreted in many ways. Is it plausible that the loss of gasping with increasing postnatal age is a result from the removal of neuronal elements that are normally involved in the production of a gasp? For example, the changes that occur neurochemically are not manifested physiologically in the neonatal rat; thus, by removing or changing the

activity of neurons relying on 5-HT receptor activation, less neuronal activity would be synchronized allowing for an abrupt onset, high amplitude, short duration burst with prolonged duration between bursts patterns? Could the shifts in neonatal rat brain neurochemistry be responsible for the manifestation of the unusual gasping patterns seen in the neonatal rat?

With the increase of 5-HT release that is expected to occur in response to hypoxic conditions (Richter et al., 1999), the ability of 5-HT to act on 5-HTreceptor subtypes that are expressed at reduced densities or affinities within the respiratory brainstem, could lead to a reduction in the establishment or maintenance of the hypoxic ventilatory response. In addition, because 5-HT_{2A} receptors located on phrenic motor neurons are not the only excitatory neurotransmitter receptor responsible for driving the action potential down the nerve, it may be suggested that the increase of glutamate also resulting from hypoxia/ischemic exposure (Richter et al., 1999) is acting more heavily on NMDA-mediated synapses.

Generally, it has been demonstrated that gasping results in a reduction of ApEn values, as observed both *in vitro* and *in vivo* (Akay et al., 2004; Akay and Sekine, 2006). However, this is the first study to address network complexity during eupnea and gasping in the spontaneously breathing neonatal rats, and therefore, further insight into the mechanisms that generate the nonlinear dynamics reflected by ApEn values might provide an explanation as to the changes reported in this dissertation. Therefore, additional studies addressing this question are required.

FIGURE LEGENDS

Figure 1. Illustrative physiological recording of hypoxia-induced gasping response in a *in vivo* neonatal rat. Top panel: Example traces corresponding to ~20 minute severe hypoxia exposure of integrated (Int) and raw EMG_{Dia} recording in a P3 rat. Expanded time scale of a gasp is shown below trace.Bottom panel: Example traces corresponding to ~5 minute severe hypoxia exposure of integrated (Int) and raw EMG_{Dia} recording in a P22 rat. Expanded time scale of a gasp is shown below trace.These traces that gasping is observed during severe hypoxia in both rats shown however, the burst amplitude, onset latency to gasp and the duration of gasping response is markedly different.

Figure 2. Effects maturation on incidence of gasping. Percentage of successful autroresuscitation attempts in response to severe hypoxia in a spontaneously breathing urethane anesthetized neonatal rat EMG_{Dia} preparation.

Figure 3. Effects of maturation on the time course general characteristics of gasping in response to severe hypoxia. Left panel: Data acquired from each experiment conducted for each animal age that showed a gasping response to severe hypoxia. Right panel: Overall Mean (±SE) number of onset to gasping (top panel), gasping duration (middle panel), and number of gasp (bottom panel) in all animal age groups examined. *Values are mean and symbols represent a statistically significant difference ($P<0.05$) across age groups examined.*

Figure 4. Summary data showing the effects of maturation on temporal characteristics of EMG_{Dia} in response to severe hypoxia. Left panel: Data acquired from each individual experiment conducted. Right panel: Summary data for group averages (Mean±SE) for baseline (dark bars) vs gasp (gray bars), for burst duration (T_I) and the duration between burst (T_E) and burst amplitude burst duration mean animal age groups. Generally changes were seen in all parameters examined however there was only a significant decrease in (T_I, T_E, and burst amplitude in some ages). In response to severe hypoxia, gasping elicited significant decreases in T_I and increases in T_E for all experimental age groups examined (except T_I in P0-P5). *All symbols represent a statistically significant difference ($P<0.001$) from BL, BL vs. G, BL across age groups, G across age groups.*

Figure 5. Inspiratory neural network complexity during basal and gasping in the *in vivo* anesthetized spontaneously breathing neonatal rat preparation. (Top and Bottom) The effect of maturation on ApEn underlying EMG_{Dia} burst. (Top) Data acquired from each experiment conducted. Note: dark circles (BL) values are predominantly higher than gray circles (G) (Bottom) Summary data (Mean±SE) for experimental age groups during BL (dark bar) and gasping in response to severe hypoxia (gray bar) Overall, ApEn values significantly increase with maturation during baseline recording. In response to severe hypoxia all age groups demonstrated a reduction in ApEn values *albeit* the magnitude of this reduction was different among each age group. *Symbols represent a statistically significant difference ($P<0.001$) from BL, BL vs. G, BL across age groups, G across age groups.*

Figure 6. Example traces of demonstrating EMG_{Dia} long time scale gasping patterns *vs.* short time scale gasping patterns from *in vivo* neonatal rat. Example traces of basal, PCR, and gasping in response to severe hypoxia exposure of integrated (Int) and raw EMG_{Dia} recording in a P3 rat. Expanded time scales of unusual gasping patterns during EMG_{Dia} recording. Demonstrating patterns of doublet and triplet gasps

Figure 7. Example traces of unusual gasping patterns. Traces from neonatal rats (at ages indicated) in response to severe hypoxia showing characteristics of singlet, doublet, and triplet gasps. Note changes in the spacing between burst for neonatal gasping response for each age examined.

Figure 8. Incidence of multiple gasping patterns. (A) Variation in the incidence of multiple gasping patterns in neonatal rat. (B) Number of unusual gasping events in age groups P0-P6 and P7-P12. Data represented as (mean±SE) for each gasping pattern. *Asterisks represent a statistically significant difference between doublet gasping events ($P<0.05$) between doublet gasping events.*

Figure 9. Proportion of multiple gasping patterns seen in neonatal rats P0-P12. (A-B) Incidence of singlet, doublets, and triplets.

Figure 10. Temporal characteristics of EMG_{Dia} associated with unusual gasping patterns in neonatal rat ages P0-P12. (A-C) Changes in temporal characteristics during multiple gasping patterns. (A) P0-P6 T_I shows significant difference between baseline and singlet gasping burst. P7-P12 T_I is significantly different for all multiple gasping types when compared with baseline values. In addition, T_I is significant difference between age groups examined. (B) Summary data demonstrated that T_E is reduced between multiple gasping patterns compared with time between burst during singlet gasping patterns. Overall time between burst T_E is extended when compared to baseline values. Data are represented as (mean±SE) for baseline (BL), singlet (G1), doublet (G1 G2) and triplet (G1 G2 G3) gasping patterns. Note: Singlet gasping T_E is values are considerably higher in P0-P6 rats as compared with P7-P12. *Symbols represent a statistically significant difference ($P<0.001$) from BL, BL vs. Singlet (G1), BL vs. Doublet (G1, G2), BL vs. Triplet (G1, G2, G3), Singlet vs. Doublet (G1, G2), Singlet vs. Triplet (G1, G2, G3) for both age groups.*

Figure 1

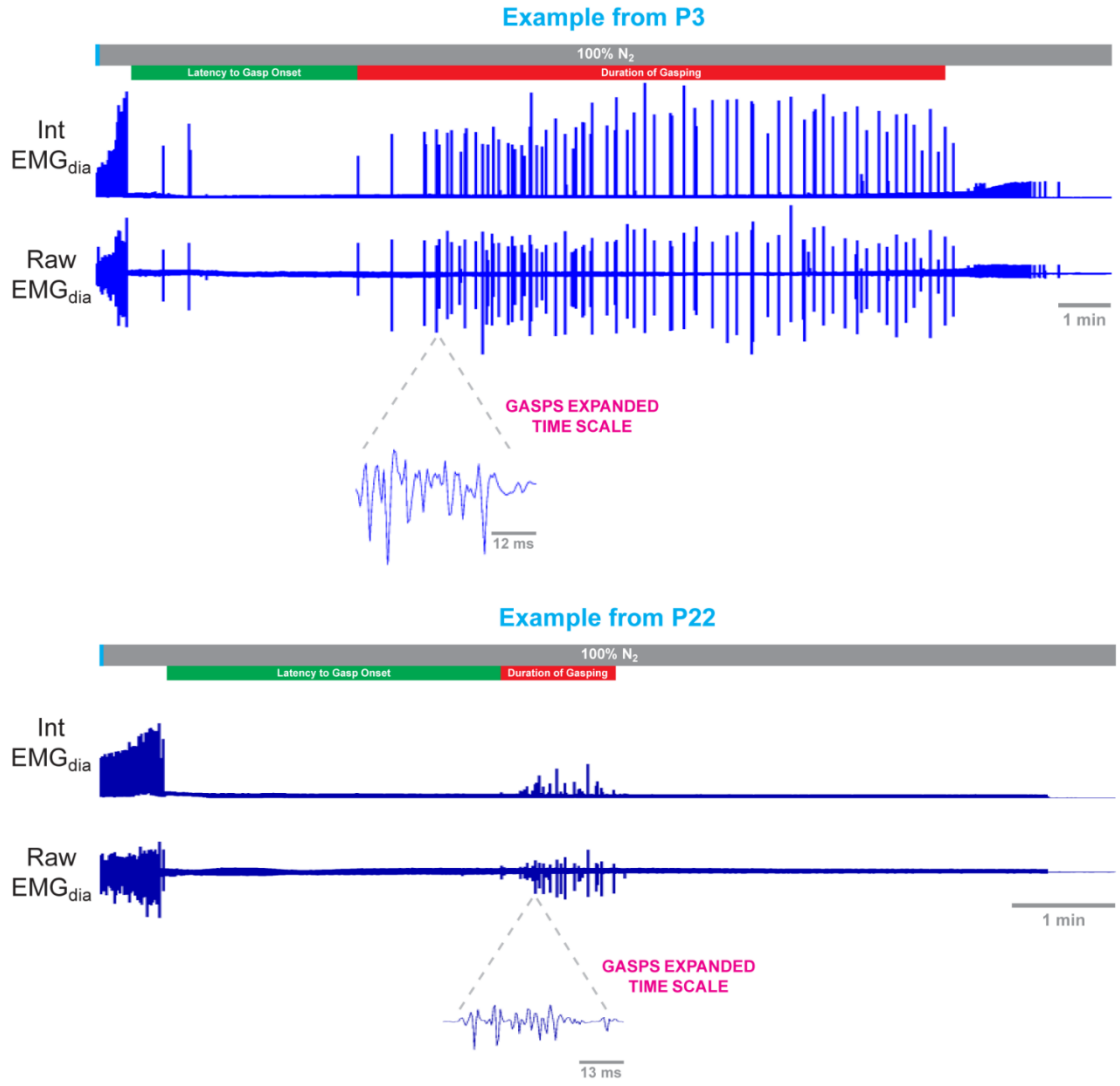

Example from P3
100% N_2
Latency to Gasp Onset
Duration of Gasping
Int EMG_{dia}
Raw EMG_{dia}
1 min
GASPS EXPANDED TIME SCALE
12 ms
Example from P22
100% N_2
Latency to Gasp Onset
Duration of Gasping
Int EMG_{dia}
Raw EMG_{dia}
1 min
GASPS EXPANDED TIME SCALE
13 ms

Figure 2

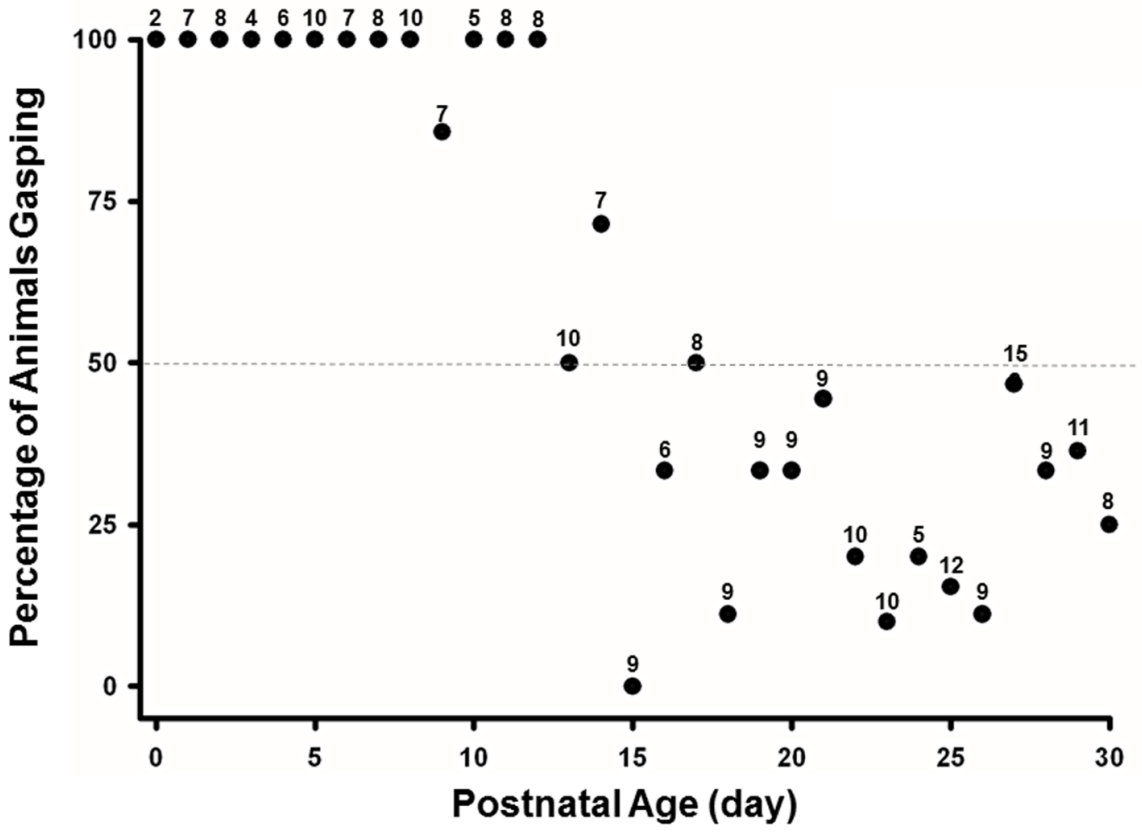

Percentage of Animals Gasping
Postnatal Age (day)
100
75
50
25
0
0
5
10
15
20
25
30
2 7 8 4 6 10 7 8 10 5 8 8
7
7
10
8
6
9 9
9
9
9
10
5
12
9
10
15
9
11
8

Figure 3

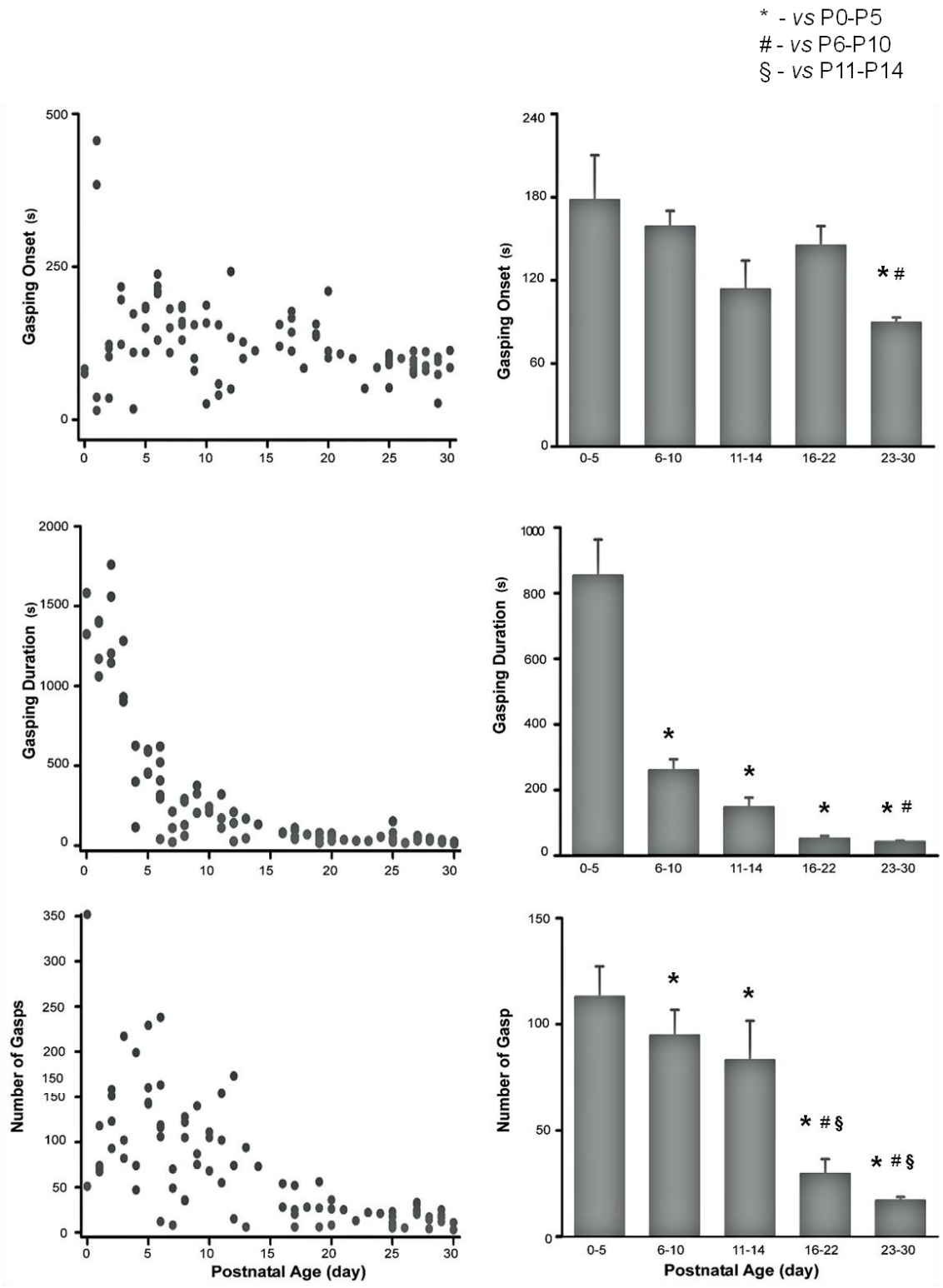

* - vs P0-P5
# - vs P6-P10
§ - vs P11-P14
Gasping Onset (s)
Gasping Duration (s)
Number of Gasps
Number of Gasp
Postnatal Age (day)
0-5
6-10
11-14
16-22
23-30

Figure 4

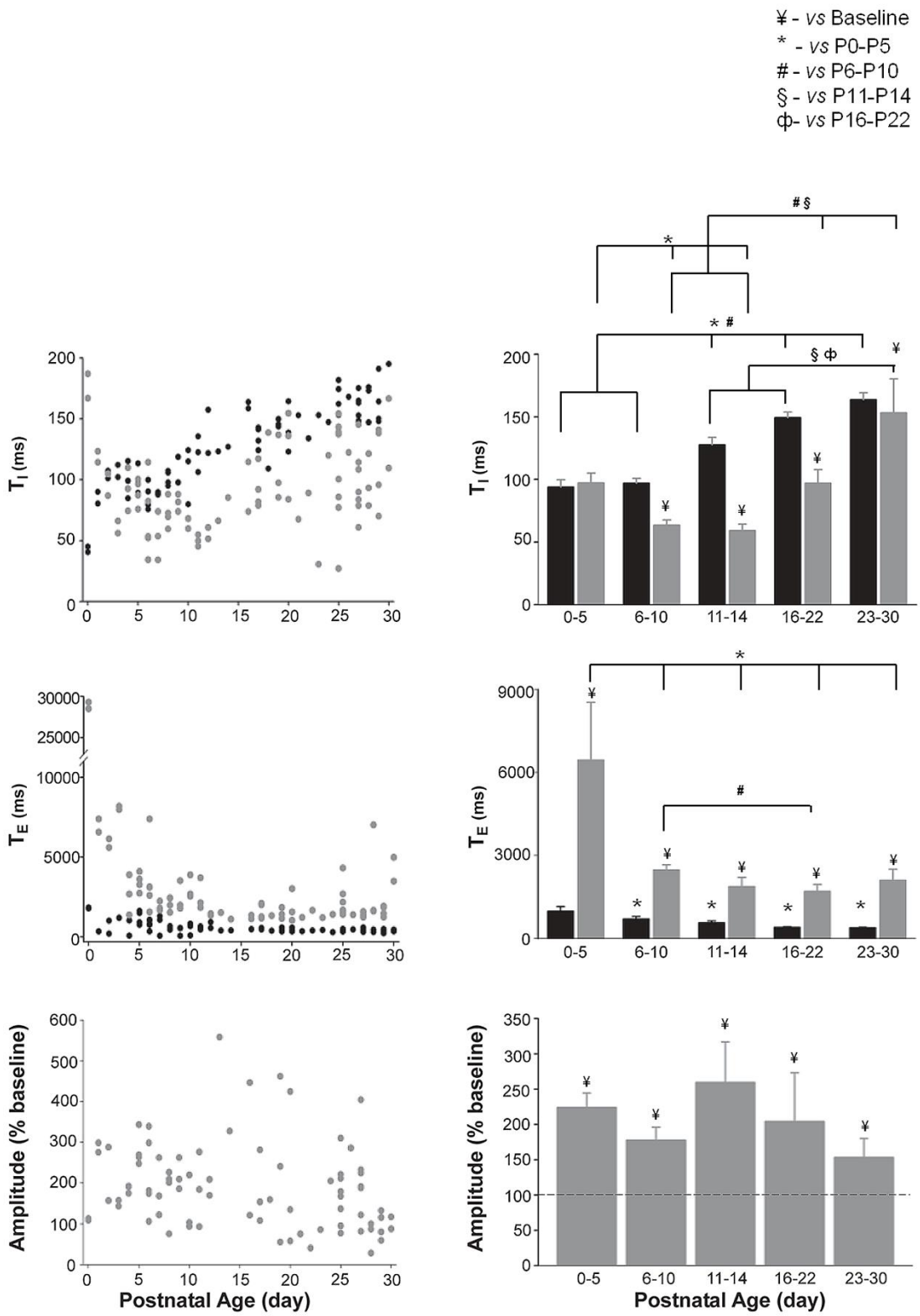

¥ - vs Baseline
* - vs P0-P5
# - vs P6-P10
§ - vs P11-P14
ф- vs P16-P22
T_I (ms)
T_E (ms)
Amplitude (% baseline)
Postnatal Age (day)
0-5
6-10
11-14
16-22
23-30

Figure 5

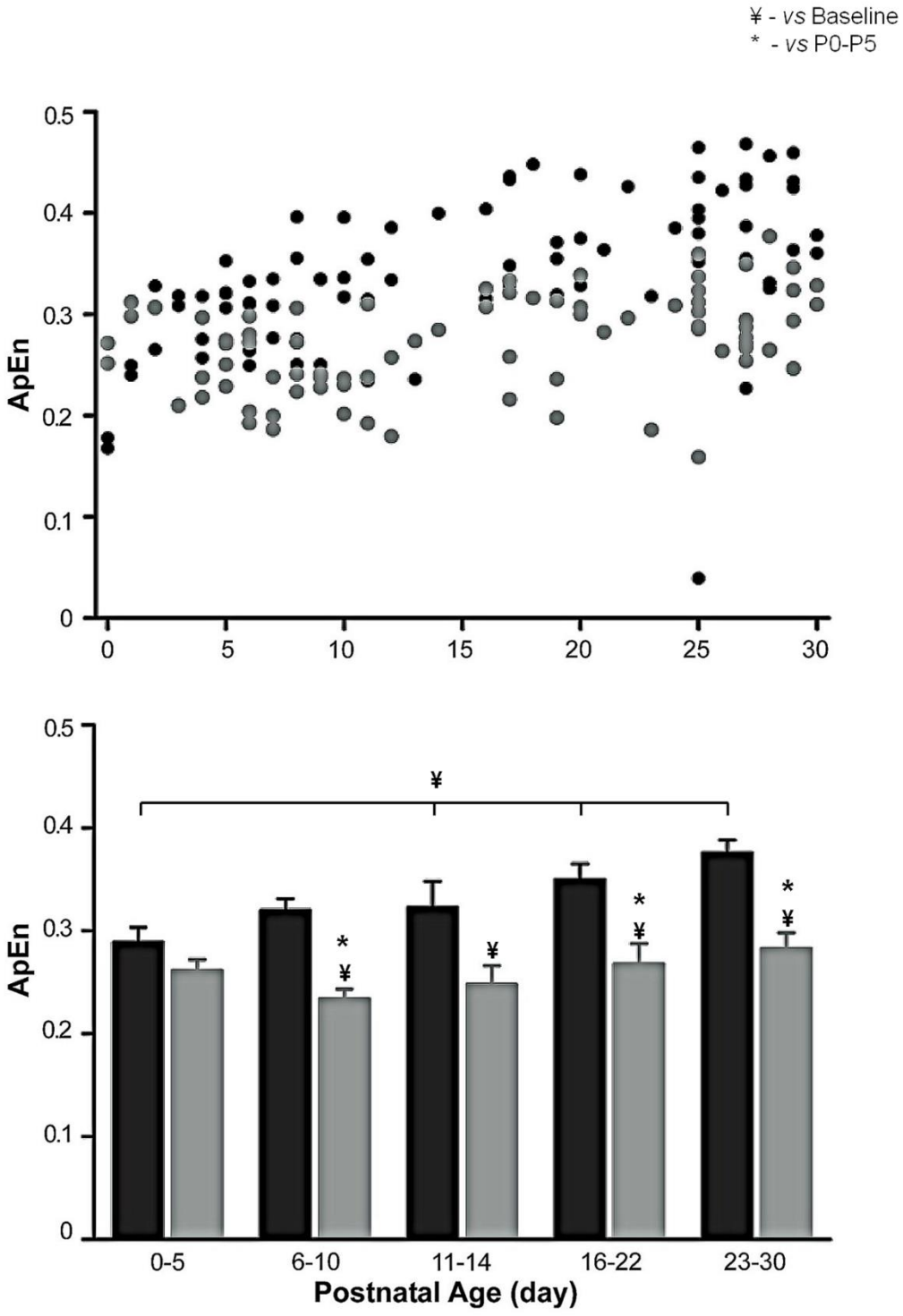

¥ - vs Baseline
* - vs P0-P5
ApEn
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15
20
25
30
ApEn
¥
*
¥
0-5
6-10
11-14
16-22
23-30
Postnatal Age (day)

Figure 6

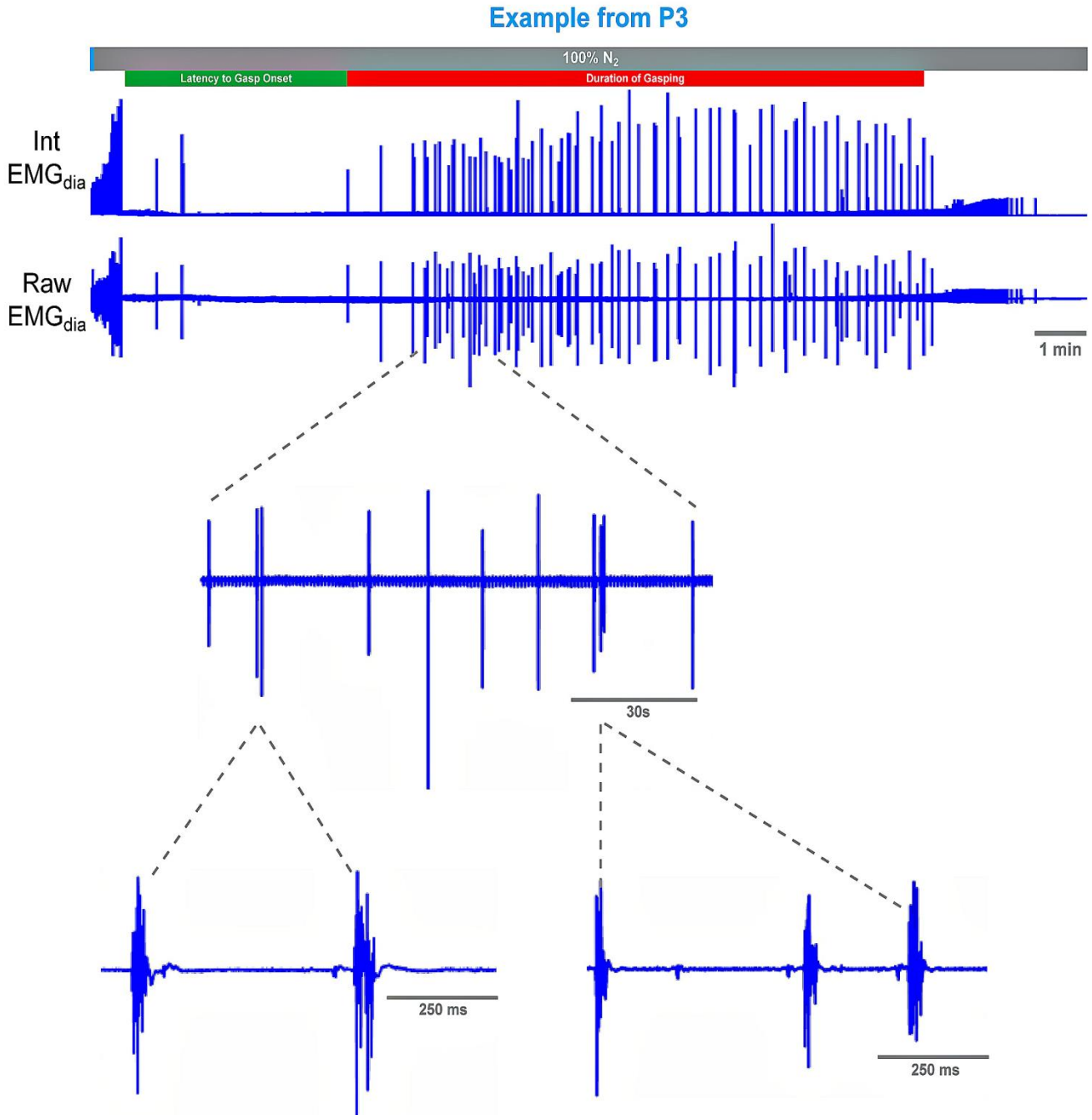

Example from P3
100% N_2
Latency to Gasp Onset
Duration of Gasping
Int EMG_{dia}
Raw EMG_{dia}
1 min
30s
250 ms
250 ms

Figure 7

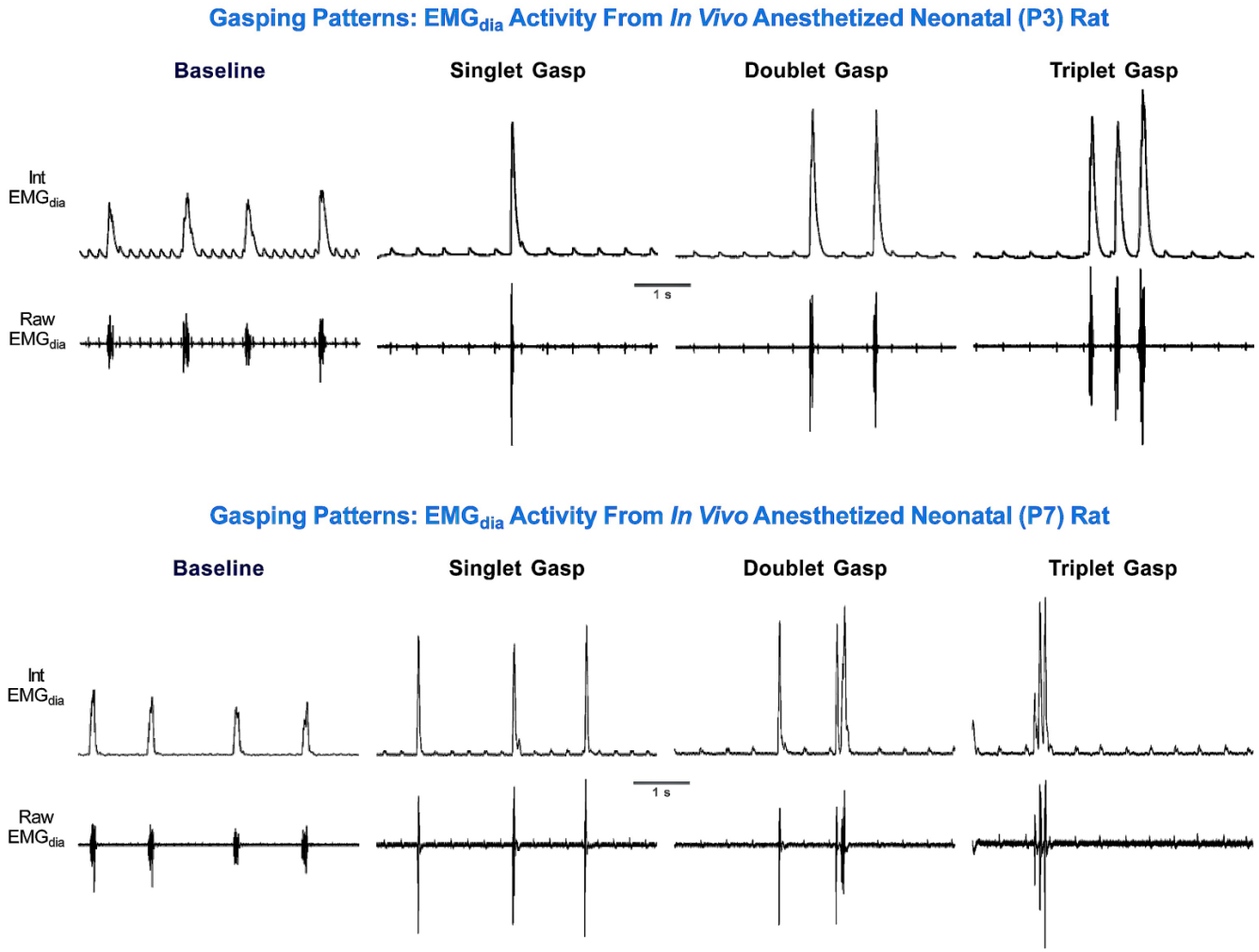
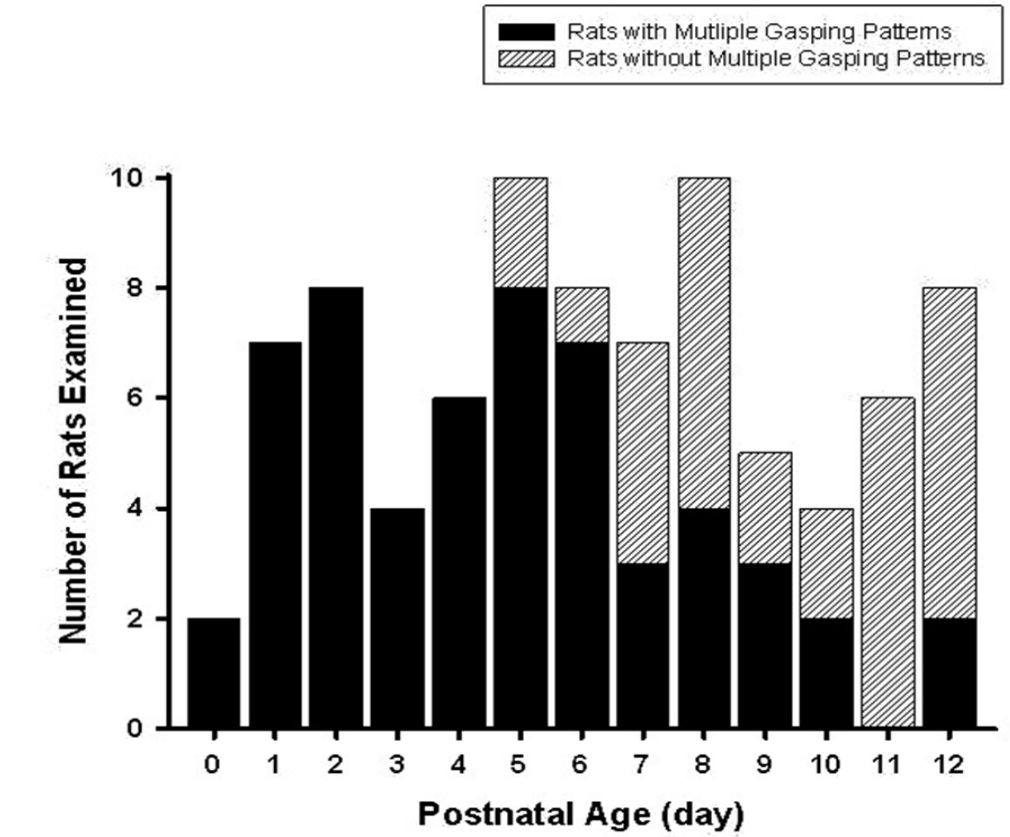

Gasping Patterns: EMG$_{dia}$ Activity From *In Vivo* Anesthetized Neonatal (P3) Rat
Baseline
Singlet Gasp
Doublet Gasp
Triplet Gasp
Int EMG$_{dia}$
Raw EMG$_{dia}$
1 s
Gasping Patterns: EMG$_{dia}$ Activity From *In Vivo* Anesthetized Neonatal (P7) Rat
Baseline
Singlet Gasp
Doublet Gasp
Triplet Gasp
Int EMG$_{dia}$
Raw EMG$_{dia}$
1 s

Figure 8


Rats with Mutliple Gasping Patterns
Rats without Multiple Gasping Patterns
Number of Rats Examined
0
2
4
6
8
10
0 1 2 3 4 5 6 7 8 9 10 11 12
Postnatal Age (day)

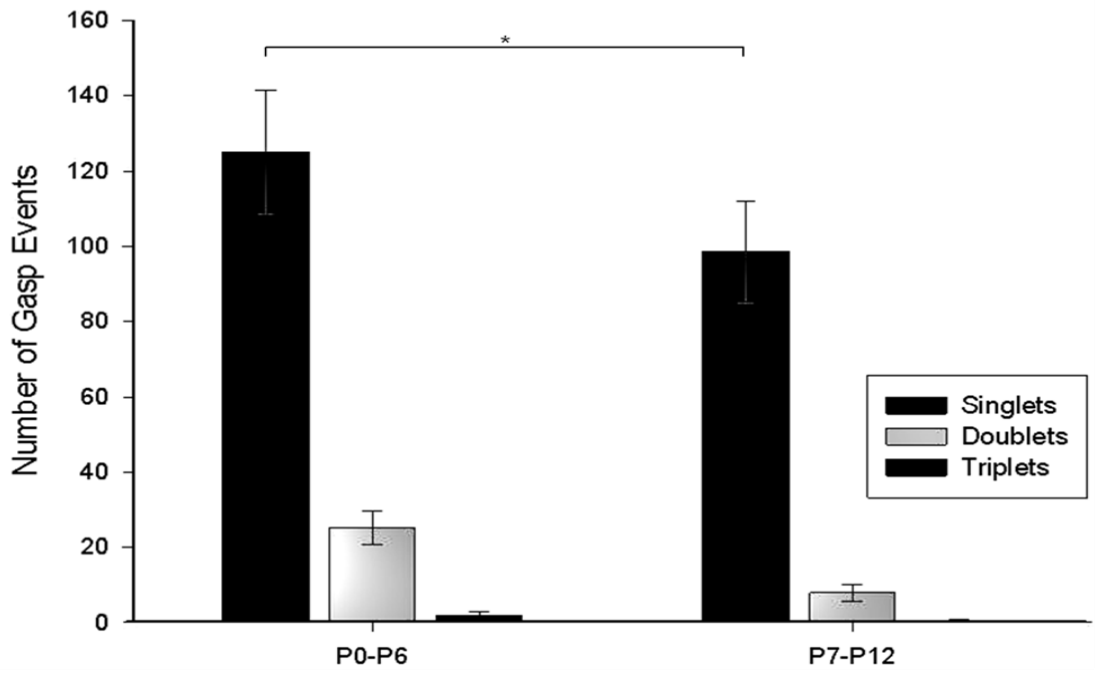

*
Number of Gasp Events
0
20
40
60
80
100
120
140
160
Singlets
Doublets
Triplets
P0-P6
P7-P12

Figure 9

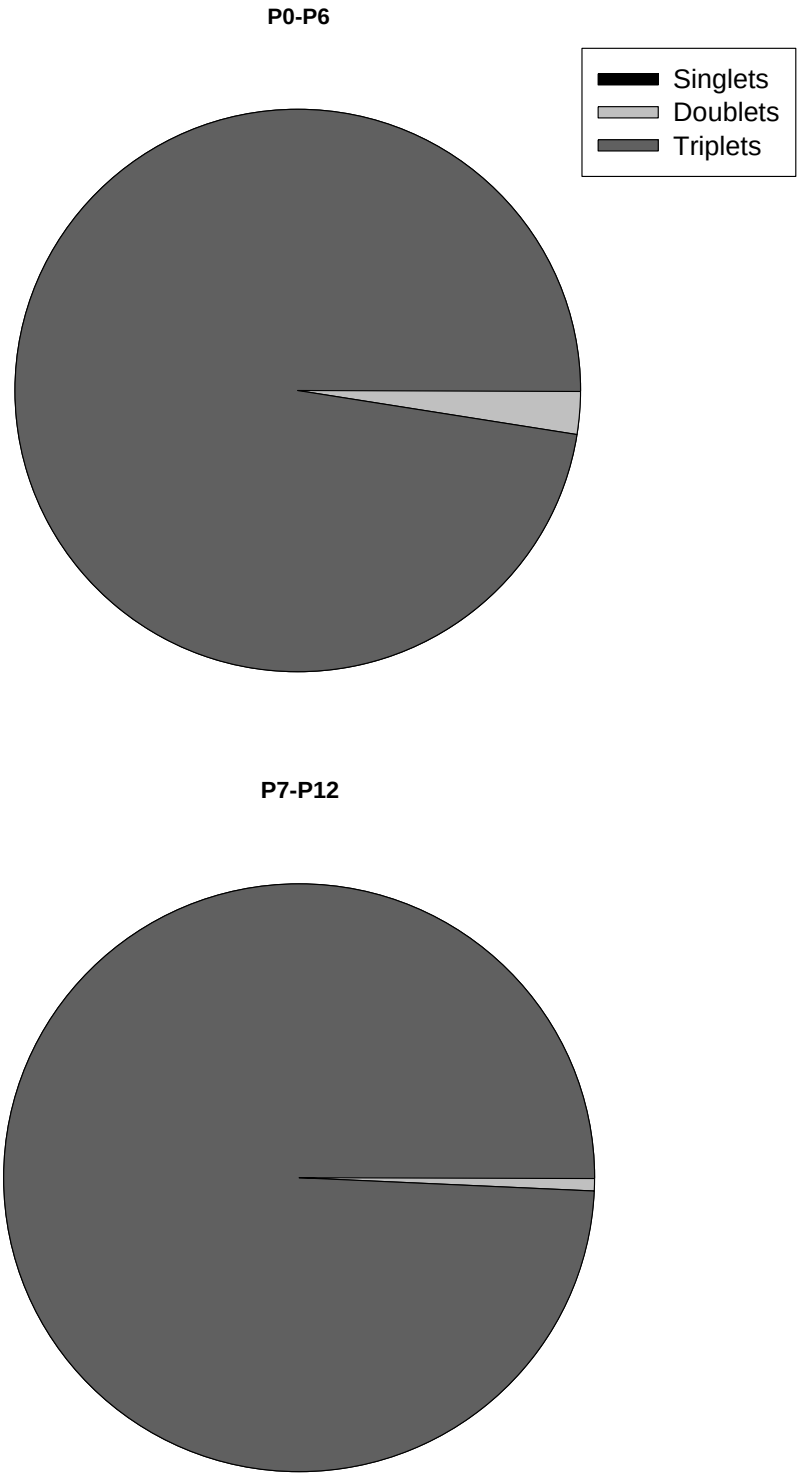
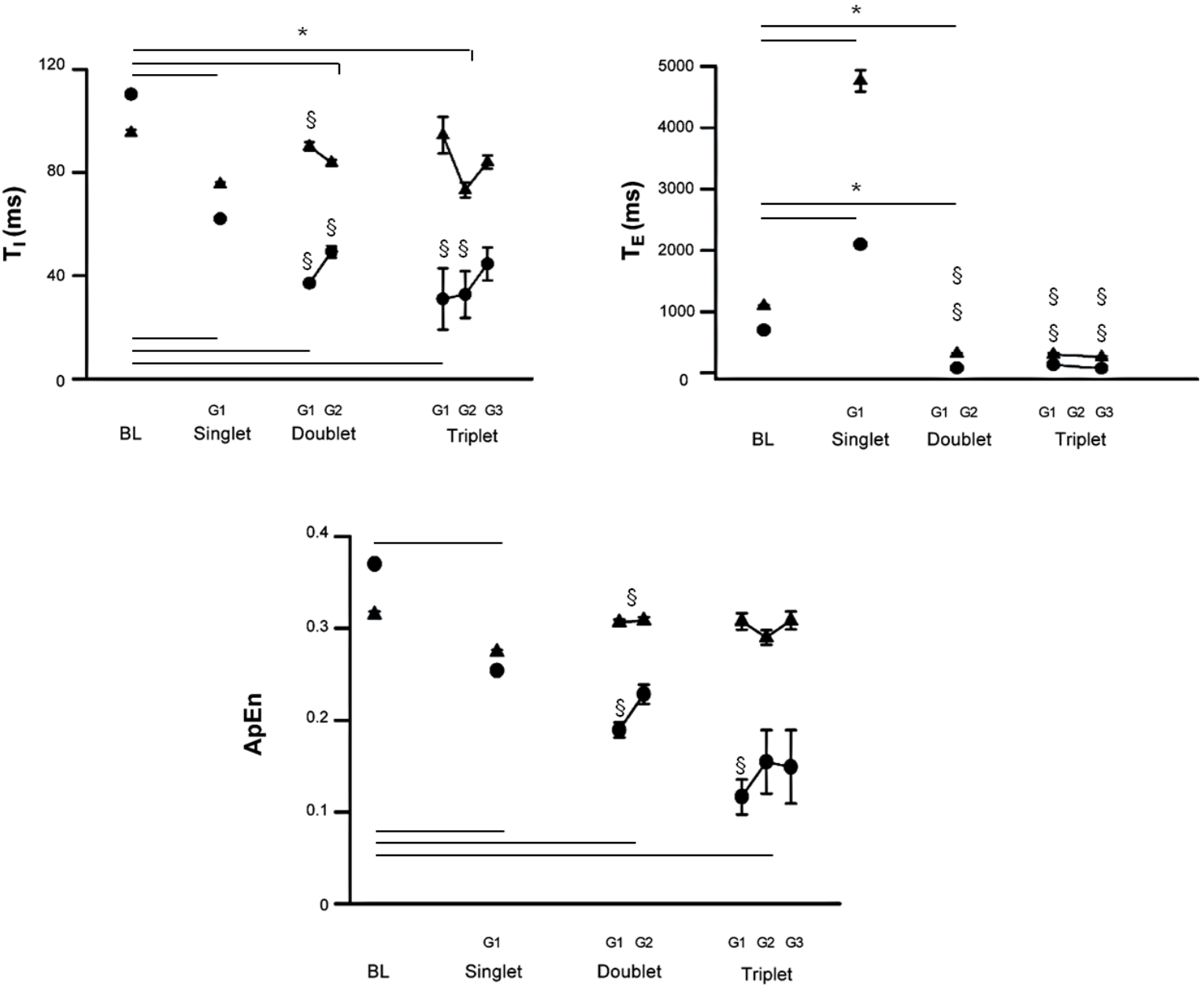


Figure 10


T_I (ms)
120
80
40
0
*
§
BL
G1
Singlet
G1 G2
Doublet
G1 G2 G3
Triplet
T_E (ms)
5000
4000
3000
2000
1000
0
ApEn
0.4
0.3
0.2
0.1

CHAPTER 4

QUANTIFICATION OF RESPIRATORY LONG-TERM FACILITATION (LTF) INDUCED BY ACUTE INTERMITTENT HYPOXIA (AIH) AT DIFFERENT MATURATIONAL STAGES IN P9-P15 NEONATAL RATS

INTRODUCTION

The respiratory system was initially viewed as rigid and non-malleable. We now know, however, that this is not the case, and the respiratory system is seen as attentive, adaptive, and capable of learning (Mitchell, 2001).

The respiratory system is capable of remembering challenges in order to fine tune its motor activity by modulating neuronal strength. Enhancement of synaptic communications functions to increase motoneuron excitability, and therefore, increase respiratory drive. This enhancement is not fixed and instead works through the effectiveness of communication of neurotransmission between neurons (Kuffler *et al*., 2001). In fact, it has been demonstrated that the synaptic efficacy between neurons can undergo changes that result from patterns of repeated activity (Kuffler *et al*., 2001). A change in synaptic strength is termed synaptic plasticity, which can be evoked and identified based on its occurrence (once or multiples) and duration (short-term or long-term). In response to repeated bouts of acute hypoxia (i.e., acute intermittent hypoxia, AIH), a form of respiratory plasticity known as long-term facilitation (LTF) is elicited (Mitchell *et al*., 2001).

The first account of these long-term changes in mammalian synapses was discovered in the hippocampus in anesthetized rabbits (Bliss and Lomo, 1973). Increases in hippocampal field potentials resulted from brief, high frequency stimulation of the inputs to the dentate gyrus. While Bliss and Lomo were not aware of the mechanism responsible for the long-term changes in hippocampal field potentials, they hypothesized that short high frequency stimuli increased neurotransmitter release, thereby increasing the amount of neurotransmitter release onto the synapse and altering the sensitivity of postsynaptic functions (Bliss and Lomo, 1973).

Following this, Millhorn and colleagues documented that repeated electrical stimulation of the cut central end of the carotid sinus nerve augmented phrenic inspiratory activity for up to 90-minutes post-stimulation in anesthetized vagotomized adult cats (Millhorn *et al*., 1980). Subsequent studies conducted in the anesthetized adult rat extended this initial observation, and further provided tremendous insight into the cellular and synaptic mechanisms for the induction and maintenance of hypoxia-induced phrenic LTF. A number of subsequent studies, provide additional evidence supporting the idea that the respiratory system displays plasticity, which can be initiated by different stimuli, in various animal models, and under several conditions (Millhorn *et al*., 1980, Fuller *et al*., 2000, Mitchell *et al*., 2001, Tadjalli *et al*., 2010). In the second part of my work, the focus is on a form of respiratory plasticity known as long term facilitation (LTF).

In breif, LTF is characterized by a progressive rise in ventilation (or a neural correlate of ventilation; e.g., inspiratory neural or motor discharge) above baseline levels after return to normoxia following repeated carotid body stimulation. Its presence may be potentially beneficial by way of stabalizing respiration and upper airway patency. This would be accomplished through preventing periods of low respiratory drive (Millhorn *et al*. 1980, Fuller *et al*., 2000, Mitchell *et al*., 2001, Tadjalli *et al*., 2010). While classically LTF is induced by repeated stimulation of the carotid sinus nerve, it can also be evoked by repeated bouts of hypoxia and repeated vagal afferent modulation (examples associate with

each method will be discussed further in the section that follows). Although it has been shown that multiple respiratory muscles can exhibit LTF, the majority of LTF studies look at respiratory output using either the phrenic or hypoglossal motor pools or their associated muscles (diaphragm and genioglossus, respectively) (Fuller *et al*. 2001, Baker-Herman *et al*., 2004; Tadjalli *et al*., 2010).

Carotid Sinus Nerve Stimulation LTF

The initial experiments that demonstrated LTF recorded phrenic nerve activity in response to repeated electrical stimulation of the carotid sinus nerves in cats (Millhorn *et al*. 1980). The protocol for stimulation used five episodes of two minute duration stimulation, and the results consisted of a long-term enhancement of phrenic nerve activity following termination of the repeated bouts of stimulation. The observations were obtained in urethane-anesthetized, paralyzed, mechanically ventilated, and bilaterally vagtomized adult cats. In this study, paralysis was used to reduce myoeletctric activity, mechanical ventilation was used for controlling arterial blood gases at normal physiological levels, and vagotomy was used to eliminate lung and airway vagal afferent input and entrainment to the ventilator. Thus, with all parameters tightly controlled, other inputs that could drive phrenic nerve activity were excluded, indicating that repeated carotid sinus nerve stimulation could elicit long-term changes in respiratory output, suggesting that the respiratory system was indeed plastic and could adapt/adjust its output. In subsequent studies, Millhorn and colleagues identified specific regions responsible for the manifestation of this enhanced respiratory output. Spinal cord transections at C7-T1 did not prevent LTF from occurring; however, using a serotonin antagonist did. This discovery opened the door for researchers to study LTF using the respiratory system, which responded within the physiological range to hypoxia – an effective yet still natural stimulus of the respiratory system.

Hypoxia-induced Respiratory LTF

Although some laboratories are currently discovering new ways to elicit LTF, the most popular method to initiate LTF is AIH. AIH-induced LTF is long lasting and responses in the phrenic (spinal) and/or hypoglossal (brainstem) nerves are usually studied (Mitchell *et al*., 2001; Baker-Herman and Mitchell, 2002; Feldman *et al*., 2003). AIH-induced LTF typically implements the classic protocol, where experiments are carried out using anesthetized, paralyzed, vagotomized, and mechanically ventilated animals exposed to three five-minute episodes of hypoxia (Baker-Herman and Mitchell, 2008). AIH-induced LTF is pattern sensitive in that intermittent bouts of hypoxia are needed to induce respiratory LTF, as a single continuous bout of hypoxia with the same duration does not induce respiratory LTF (Baker and Mitchell, 2000). Furthermore, each hypoxic episode is accompanied by a recovery period in which the animal is supplied with baseline levels of oxygen for five minutes before the next hypoxic challenge. While the optimal spacing interval between hypoxic bouts has not been clearly defined (ranging from 1 to 30 minutes, Hoffman *et al*., 2010), alternating hypoxic episodes and recovery are critical in initiating respiratory LTF (Bach *et al*., 1999). It should be noted, however, that although numerous hypoxic exposure protocols are currently used to elicit respiratory LTF, the most commonly used is the three five-minute hypoxic episodes that are each separated by five minutes of hyperoxia, see Figure 1.

Furthermore, although it was initially thought that the intensity of the hypoxic challenge and the duration of the exposure did not significantly affect the magnitude of LTF (Fuller *et al*., 2000; Mahammed and Mitchell, 2008), a recent study has shown that severe hypoxia elicits enhanced respiratory LTF when compared to that elicited by moderate hypoxia (Nichols *et al*., 2012). Additionally, the mechanism of LTF seems to be pattern sensitive in that the level of oxygen used could determine the outcome of the post-IH response (Ling *et al*., 2001).

Experimental preparations that do not involve the use of anesthetics or mechanical ventilation, but rather use an awake animal model or the arterially perfused (a.k.a., working heart-brainstem) rodent preparation, have demonstrated different results in the variables that are commonly associated with respiratory (phrenic) LTF (McGuire *et al*. 2002, Tadjalli *et al*., 2007). In awake rats, for example, both the intensity and frequency of hypoxia have been shown to determine the specific behaviors associated with respiratory LTF (McGuire *et al*., 2002), with the optimal hypoxic level producing ventilatory LTF being ~10% oxygen and the number of hypoxic episodes increasing the duration, but not the magnitude, of ventilatory LTF (McGuire *et al*., 2002). Additionally, in the arterially perfused neonatal rat preparation, respiratory plasticity was seen as an elevation of respiratory frequency, and not as an elevation in phrenic nerve amplitude, which is traditionally reported. (Tadjalli *et al*., 2007).

Respiratory plasticity has been observed in several animal species. As stated earlier, cats were the original animal model for respiratory LTF studies (Millhorn *et al*., 1980); however, this phenomenon has also been reported in ducks, mice, goats, dogs, and rats (Cao *et al*., 1992, Hayashi *et al*., 1993, Bach and Mitchell, 1996, Turner and Mitchell, 1997, Mitchell *et al*., 2001, Peng and Prabhakar, 2003, Sokolowska and Pokorski, 2006).Respiratory LTF has also been documented in awake humans; however, to induce respiratory LTF, sustained elevated levels of carbon dioxide in addition to the episodes of hypoxia were required (Harris *et al*., 2006). While the adult rat animal model is the most commonly used to examine the mechanisms for respiratory plasticity, genetic variations do exist between strains of rats and it has been shown that these genetic variations play a significant factor in the expression of respiratory LTF. For example, Sprague-Dawley rats from Harlan colony 236 did not express LTF in hypoglossal nerve activity whereas those from Charles River colony K62 did (Fuller *et al*., 2001). Thus, a variety of factors need to be considered - animal models, age of animals, and parameters of the hypoxic stimulus used, as they can all influence mechanisms mediating respiratory plasticity.

Cellular Mechanisms involved in Hypoxia-induced Respiratory Plasticity

Mechanisms involved in respiratory LTF have been extensively studies *in vivo* and *in vitro* (Zabka *et al*., 2001; Feldman *et al*., 2003; Mitchell and Johnson, 2003; Baker-Herman *et al*., 2004; Bocchiaro and Feldman, 2004; McKay *et al*. 2004; Mahamed and Mitchell, 2007). Respiratory LTF has largely been attributed to centrally-mediated mechanisms that vary in duration from several minutes to hours depending on the species and protocol (Turner and Mitchell, 1997; Powell *et al*., 1998; Olson *et al*., 2001).

Respiratory LTF and 5-HT

There is evidence to suggest that respiratory LTF is a serotonin (5-HT) dependent process (Bach and Mitchell, 1996). 5HT receptor activation is necessary for phrenic LTF in adult rat preparations since repeated bouts of hypoxia could not elicit LTF if the animals were pretreated with methysergide or ketanserin (Bach and Mitchell, 1996). Thus, 5-HT, and more specifically 5HT receptor activation, is crucial for the manifestation of phrenic LTF although this study did not specifically determine whether 5-HT receptor activation was necessary for induction, maintenance, or both. To address this issue, Fuller and colleagues demonstrated that if 5-HT receptor activation was blocked using ketanserin after the hypoxic episodes, phrenic LTF was not seen, indicating that activation of spinal 5-HT receptors initiates, but does not maintain, phrenic LTF (Fuller *et al*., 2001, Baker-Herman and Mitchell, 2002). Ongoing research is focused on investigating the role of downstream intracellular cascades, triggered by 5-HT in AIH-induced respiratory LTF.

However, in addition to central mechanisms, recent evidence suggests that peripheral modulation may be involved (Cummings and Wilson, 2005). Several molecules have been implicated in the synaptic mechanism of respiratory LTF. 5-HT receptors are Gq protein-coupled metabotropic receptors that activate downstream kinases like protein kinases C (PKC) (Baker-Herman and Mitchell, 2002, Feldman *et al*., 2003, Baker-Herman *et al*., 2004). The current hypothesis throughout the literature suggests that

activation of 5-HT receptors on phrenic motoneurons activates PKC, which further initiates protein synthesis and neurotrophic cascades that include synthesis of new brain-derived neurotophic factor (BDNF), which has been demonstrated to be necessary for the expression of respiratory LTF (Baker-Herman and Mitchell, 2002, Baker-Herman *et al*., 2004). BDNF is proposed to induce respiratory LTF via TrkB receptor activation because inhibition of receptor tyrosine kinase prevents respiratory LTF (Baker-Herman *et al*., 2004). Furthermore, activation of TrkB receptors by BDNF is hypothesized to trigger LTF via the enhancement of presynaptic glutamate release, increase in glutamatergic excitation of phrenic motoneurons, and reduction in the amount of presynaptic GABA transmission, all of which impact membrane potentials and excitability of phrenic motoneurons (Levine *et al*., 1998, Lui *et al*., 2003, Kafitz *et al*., 1999, Schnider *et al*., 2000, Baker-Herman *et al*., 2004). Reactive oxygen species (ROS) generated by the NADPH oxidase complex are also necessary for respiratory LTF (MacFarlane & Mitchell, 2008). The role of ROS in respiratory LTF is not yet understood but it has been implicated in the pattern sensitivity of LTF, as ROS regulates protein kinase and phosphatase activity. (Wilkerson *et al*., 2008, Parabhakar and Kumar, 2004, MacFarlane and Mitchell, 2008).

Multiple pathways are suggested to be involved in the long-lasting enhancement of respiratory motor output in AIH-induced LTF (Dale-Nagle, *et al*., 2010), with the current model for LTF involving two distinct pathways. The "Q" pathway, mentioned above, is a 5-HT-dependent augmentation of respiratory motor output (phrenic and hypoglossal) following episodic hypoxia that requires intermittent activation of Gq-coupled metabotropic receptors (i.e. 5-HT2 or α1), downstream signaling that leads to activation of PKC, which subsequently initiates new BDNF synthesis and increases the amount of NADPH oxidase (NOX) activity. Synthesis of BDNF results in activation of its high affinity TrkB receptor, and then ERK MAP kinases (pERK). This pathway is necessary and sufficient for AIH-induction of LTF. The alternate pathway giving rise to LTF is initiated by Gs protein coupled metabotropic receptors, such as the adenosine 2A (A2A) (Golder *et al*., 2008). This has been termed the "S" pathway to LTF, and it requires A2A receptor activation coupled to PKA. PKA may induce new

synthesis of an immature TrkB isoform, which auto-phosphorylates and signals from inside the cell via Akt activation (pAkt). Both pathways, pERK and pAkt, phosphorylate glutamate receptors, thereby giving rise to greater synaptic strength and motor plasticity resulting in enhanced phrenic motor output.

Development and Hypoxia-induced Respiratory Plasticity

The potential for a hypoxic stimulus to alter respiration after the stimulus ceases appears to be dependent on various factors that influence the ventilatory response to hypoxia (i.e., degree, duration, maturation, species differences, nature of the exposure) (Powell *et al*., 1998; Nichols *et al*. 2012). The existence and potential underlying mechanisms mediating respiratory neural plasticity have been studied extensively in adult animals; however, much less is known in the neonatal rat. To date, there are only two studies (including one from our lab) aimed at investigating respiratory LTF in response to AIH in the neonatal rat (McKay *et al*., 2004, Reid and Solomon, 2014).

In the developing brain, hypoxia can induce prolonged neural plasticity (both excitatory and inhibitory) in respiratory and non-respiratory neural networks (Gozal and Gozal, 1995). Additionally, there may be certain developmental windows in which plasticity may be most pronounced (Gozal and Gaultier, 2001; Lui and Wong-Riley, 2002; Lui *et al*., 2006; Gozal *et al*., 2011). For example, early postnatal chronic intermittent hypoxia modifies hypoxic respiratory responses and long-term phrenic facilitation in adult rats (Reeves *et al*., 2006).

Clinical Implications involved in Hypoxia-induced Respiratory Plasticity

While evidence supporting respiratory neural plasticity in humans is relatively sparse at present, as our understanding of this phenomenon improves, it may be possible to take advantage of this to alter respiratory responses as a novel treatment for certain respiratory diseases (Gozal and Gaultier, 2001) and neurodegenerative disorders with associated respiratory decline. For example, there is evidence that sustained improvements in sensory and motor function can be induced by repeated sensory and motor cortical excitation (McKay *et al*., 2002; Uy *et al*., 2003; Dale-Nagle *et al*., 2010; Trumbower *et al*. 2012).

The effects of intermittent hypoxia on the CNS and respiration are more variable. For example, intermittent hypoxia produces LTF of respiration rather than depression of ventilation in several mammalian species (Turner and Mitchell, 1997; Gallman and Millhorn, 1998; Fuller *et al*., 2000; Mitchell *et al*., 2001; Olson *et al*., 2001). In awake adult humans, however, ventilation is not enhanced following intermittent hypoxia (McEvoy *et al*., 1996; Jordan *et al*., 2002; Morris and Gozal, 2004).

Instead, similar to the effects of sustained hypoxia, intermittent hypoxia causes ventilatory roll-off or depression rather than LTF of ventilation (McEvoy *et al*., 1996; Jordan *et al*., 2002). During sleep there is some evidence to suggest that LTF occurs in certain individuals in the presence of flow limitation (Babcock and Badr, 1998; Aboubakr *et al*., 2001; Babcock *et al*., 2003). Nonetheless, the marked disparity in the ability to evoke LTF in humans highlights one, of potentially many, species differences concerning hypoxia-mediated effects.

Although numerous studies have examined the mechanisms, timing, and magnitude changes associated with respiratory LTF, there is still a limited amount of information regarding LTF-related changes in inspiratory network complexity. The potential effects of maturation on this phenomenon along with the role of network complexity are therefore also explored in this dissertation.

Most studies investigating respiratory LTF have utilized the anesthetized, mechanically ventilated, paralyzed, vagotomized adult rat model in which phrenic and/or hypoglossal nerve activity are recorded. While two studies (including one from our laboratory) have investigated respiratory LTF in the neonatal rat, these studies were restricted to a narrow range of ages; thus, the influence of maturation on the manifestation of AIH-induced LTF remains to be determined. Therefore, in this study, we investigated the ventilatory responses of postnatal rats to AIH.

RESEARCH DESIGN AND METHODS

General Methods

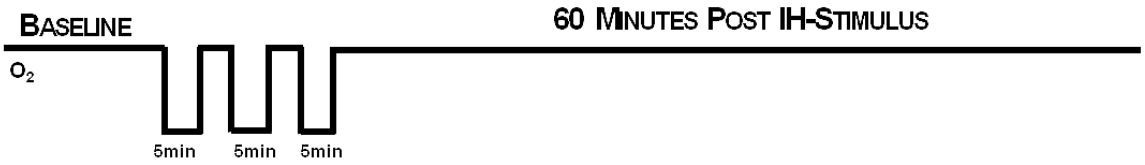
All experiments were performed under protocols approved by the Institutional Animal Care and Use Committee at the State University of New York at Stony Brook in accordance with Public Health Service Policy on Human Care and Use of Laboratory Animals. Experiments were conducted using an in vivo urethane-anesthetized spontaneously breathing P9-P15 neonatal Sprague-Dawley rat (Taconic Farms, Germantown, NY) preparation (n=40).

Animal Subjects

Rats were anesthetized with an intraperitoneal injection of urethane (~1.8 g/kg). The adequacy of anesthesia was regularly verified by absence of withdrawal reflex to a noxious interdigitary pinch and was supplemented (5-10% of initial dose) as necessary. Diaphragm electromyogram (EMG_{Dia}) activity was recorded using a thin twisted platinum wire bipolar electrode inserted into the right side of the rat's diaphragm. Body temperature was measured using a rectal probe and maintained at ~37 °C throughout the experiment, using a heating pad and a heat lamp as needed.

Experimental Protocol

EMG_{Dia} activity was recorded under baseline conditions (40% O_2, 1-4% CO_2 in a balance of N_2) for at least 10 minutes; the inclusion of 4% CO_2 was used to minimize the potential for hypoxic respiratory depression. Following baseline recording, the rats were exposed to three 5-minute trials of intermittent hypoxia (IH) (10% O_2) separated by 5-minute intervals of hyperoxic (40% O_2) recovery. EMG_{Dia} activity was recorded continuously during the IH trials and for 60-70 minutes following the IH protocol. The recording protocol is shown below:



Data Acquisition

EMG_{DIA} discharge signals were amplified (x1k), notch filtered (60 Hz), and filtered to pass frequencies between 10 Hz and 1kHz. Filtered signals were rectified, and a moving average using a 50-ms time constant was obtained. The raw and moving-averaged EMG_{DIAa} discharge signals were recorded on a computer at a sampling rate of 2 kHz (Chart 5.0, PowerLab, ADInstruments) for off-line analyses (MatLab 7.0.1). For all analysis, the data were segmented to obtain data lengths corresponding to inspiratory burst. The data were then re-sampled with a sampling rate of 1 kHz after digital band pass filtering (10-50 Hz) using a 6-pole Butterworth filter.

Data Analyses

Inspiratory burst data were evaluated for temporal characteristics and signal complexity. Temporal analysis included burst duration (T_I), the duration between bursts (T_E), burst frequency (breathes/min), burst amplitude (normalized to percent baseline), and minute activity (MA). Data for these variables were determined for an average of 20 bursts at each of the time points identified below. MA was calculated as the product of bust frequency and burst amplitude, and was normalized to BL levels (which were set to 100%) of discharge for each preparation. ApEn was calculated as a function of the parameters *m*, the embedding dimension, and *r*, the tolerance (threshold) level (Pincus, 1991). For these analysis, *m*=2 and *r*=0.25xSD, (see Analysis for Specific Aim 1). All analyses were performed on 20 bursts obtained under baseline conditions, at the end of each trial of the IH protocol, and at 5-minute intervals during the recovery period following the IH protocol.

Statistical Analyses: All data are reported as either the 20-breath average for the individual experiments or as an average (mean±standard error) for each of the age groups studied. Where appropriate, statistical comparisons were made using either a paired *t-test* or one-way analysis of variance with repeated measures, followed by *Holm-Sidak post hoc* analyses for comparisons against BL levels. For all comparisons, the criterion level was set at $p<0.05$.

RESULTS

Characteristics of EMG_{DIA} *activity in neonatal rats during BL,* CO_2*, IH-1*

In each of the experiments conducted (n=40), stable EMG_{DIA} activity was recorded under baseline (BL) conditions and in response to CO_2 and acute hypoxia. Example recordings showing 30-s of EMG_{Dia} activity for each condition is shown in Fig. 1-A. As noted above, in order to minimize the potential for depression of breathing during the hypoxic exposures, the rats were supplied with CO_2 (range 1-4%) following the initial BL recording. Here, we demonstrate that the inclusion of CO_2 to the inspired gas resulted in an increase in EMG_{Dia} burst amplitude and frequency when compared to the BL condition, and that subsequent hypoxic (10% O_2) exposure further enhanced EMG_{Dia} activity. A similar but more robust response was seen when rats where challenged with 8% O_2 (not shown). For these (and all subsequent) analyses, amplitude of EMG_{Dia} activity with CO_2 added to the inspired gas was set to 100% activity, as this was the condition used for the IH exposures and the subsequent recovery period. Under these conditions, burst amplitude was 94±1.9%, 100±3.6%, and 102±4.1% and burst frequency was 93±2.7 breaths/min, 99±3.6 breaths/min, and 117±4.0 breaths/min for BL, CO_2, and IH-1, respectively. . MA under these conditions is provided in Fig. 1-B. For this figure, MA was set to 100% for the initial BL activity, and Fig. 1B shows that MA was significantly increased during exposure to CO_2 (112±3.8% BL) and the subsequent exposure to IH-1 (135±8.4% BL).

Characteristics of the EMG_{Dia} amplitude response during IH trials and respiratory LTF in the Neonatal Rat

In each of the experiments conducted, repeated exposures to acute hypoxia elicited respiratory LTF. In addition, during the hypoxic trials, there was an increase in burst amplitude (i.e., peripheral chemoreflex), a response that was similar to that reported in phrenic LTF studies (Zabka *et al*., 2001; Bavis and Mitchell, 2003). An example of EMG_{Dia} activity from two experiments is provided in Figure 2. While an increase in EMG_{Dia} burst amplitude was observed during the hypoxic trial, changes in burst amplitude were highly variable, with the peak increase generally occurring during the early part of the 5-min hypoxic trial, after which, the amplitude declined (Figure 2). Thus, by the end of the hypoxic stimulus (which was the time point analyzed in these experiments), the EMG_{Dia} burst amplitude was often reduced from the peak level, such that in many of the rats studied, the amplitude at the end of the IH trial was lower than or at BL levels at this time point. An example of this is seen in the top panel of Figure 2. This hypoxia-induced biphasic amplitude response appears to be consistent with the biphasic ventilatory (inspiratory) response previously reported for neonatal rats (reviewed by Bissonnette, 2000). Thus, at this time point, the average IH EMG_{Dia} amplitude responses for P9, P10, P11, P12, P13, P14, and P15 rats corresponded to 97.7±0.7%, 92.9±0.5%, 92.3±3.2%, 114.3±1.7%, 98.8±0.9%, 101.4±0.7%, and 99.8±1.1%, respectively when compared to BL levels. Immediately after the third and final hypoxic challenge, a subset of rats showed an enhancement of EMG_{Dia} burst amplitude. For these rats, there was a gradual increase in EMG_{Dia} burst amplitude over time to levels that were significantly greater than BL, and this elevation in activity was typically sustained to the end of the 60 min recovery period following removal of the hypoxic stimulus (these data are quantified in Figure 9). In contrast to the typical enhancement of burst amplitude noted in adult rats (and a subset of our neonatal rats), many of the neonatal rats in our experiments exhibited slight or no change in EMG_{Dia} burst amplitude during 60 min recovery from the IH trials (bottom panel of Figure 2) albeit they were exposed to the same AIH protocol.

Although the amplitude response (amp_{LTF}) was variable, ranging from 96% to 109% (mean±SE=101.7±0.9% BL) for BL values and 44% to 150% (mean±SE = 95.2±3.7) at the 60 min time point, respectively, this behavior, which can be seen in the top panel of Figure 2, is representative of 18 of the 40 rats used in this study. In each of the 40 preparations, spontaneous augmented (*i.e.* sigh) bursts were also observed, but they are not included in the data analysis.

Characteristics of EMG_{Dia} frequency response during IH trials and respiratory LTF in the Neonatal Rat

While changes in burst amplitude were only seen in a small subset of the neonatal rats studied, there was an increase in burst frequency during recovery from IH in most of the neonatal rats studied. The frequency LTF response is shown in Figure. 3. During the IH trials, burst frequency was also increased in most of the neonatal rats examined, with the magnitude of the increase ranging from ~30-90% above BL levels (mean±SE=90.9±2.5 bursts/min during BL and 122.1±2.4 bursts/min during IH) . Following the IH trials, respiratory LTF was characterized by predominantly an increase in burst frequency ($freq_{LTF}$) ranging from ~14% to 48%, with most rats exhibiting a 20-40% increase (mean±SE=106.6±2.5 bursts/min).

Temporal characteristics of hypoxia-induced respiratory LTF in the Neonatal Rat

Changes in the timing and duration of EMG_{Dia} bursts was not differentially altered in experiments that exhibited the classic burst amplitude enhancement versus those that did not. Thus, the temporal characteristics represent data from all of the experiments conducted. While data were analyzed for each of the age groups studied, the data are presented as follows: both individual and summary data are provided for each age group, and data for age groups sharing similar temporal characteristics are provided on shared graphs, such that shared graphs show data corresponding to the P9-P11 age groups (n=10), P12 only age group (n=12), and P13-P15 age groups (n=21). In general, EMG_{Dia} burst activities exhibited little variability in T_I and T_E (Figure 4 and Figure 5), with values for T_I of 110.3±1.6 ms, 135.1±5.6 ms, and

120.1±3.3 ms during BL conditions, 109.5±1.6 ms, 134.6±7.0 ms, and 119.4±2.9 ms during IH-1, and 103.7±3.3 ms, 123.3±5.2 ms, and 123.5±3.6 ms at the 60-min recovery time point, for P9-P11, P12, and P13-P15 rats, respectively,. Additionally, an age dependent decrease in the percent change of the peak T_I response was observed (Fig. 4). Values for T_E were significantly decreased during the IH trials in the P9-P11 rats and at the 45-min recovery time point for P13-P15 rats (P=<0.002). Values for T_E were 738.5±50.1 ms, 505.8±29.8 ms, and 514.3±17.8 ms during BL conditions, 602.9±61.1 ms, 598.9.6±7.0 ms, and 405.9±15.3 ms during IH-1, and 717.6±41.2 ms, 536.8±45.0 ms, and 485.7±25.2 ms, at the 60-min recovery time point, for P9-P11, P12, and P13-P15 rats, respectively. Additionally, there was an age-dependent change in the percent change of the peak T_E response.

It should be noted, that significant changes in T_I were not seen during the hypoxic trials or the entire duration of LTF (60 min); however, significant changes in T_E were seen during the same time period, suggesting that this increase in burst frequency was mediated by a significant reduction in the duration between burst.

Response in the neonatal rat during and after AIH trials

On average, there is an increase in burst frequency during the IH trials and the 60 min LTF recovery period. Burst frequency was 75±5.2 breaths/min for BL, 91±7.8 breaths/min for IH-1, and 76±3.7 breaths/min for the 60 min recovery time point in the P9-P11 rats, 97±3.7 breaths/min for BL, 92±8.3 breaths/min for IH-1, and 96±6.7 breaths/min for the 60 min recovery time point in the P12 rats, and 95±2.4 breaths/min for BL, 118±3.4 breaths/min for IH-1, and 102±4.1 breaths/min for the 60 min recovery time point in the P13-P15 rats. The increase in burst frequency was significant during each of the IH trials and at the 45-min recovery time point for P13-P15 rats (P=<0.001). These data are provided in Figure 6.

In contrast to burst frequency, burst amplitude remained at or below BL levels (Figure 7), such that it was 100±0.7% for BL, 98±6.4% for IH-1, and 84±3.5% for the 60 min recovery time point in P9-P11 rats, 100±0.3% for BL, 116±6.8% for IH-1, and 89±9.4% for the 60 min recovery time point in P12 rats, and 100±2.4% for BL, 99±3.3% for IH-1, and 102±5.6% for the 60 min recovery time point in P13-P15 rats. A significant decrease was observed at the 15-min recovery time point for P9-P11 rats (P=<0.016).

Effects of hypoxia-induced LTF on inspiratory neural network complexity in the neonatal rat

To investigate inspiratory neural network complexity, we assessed ApEn of the EMG_{DIA} bursts during BL, IH trials, and at 15-minute increments post-IH stimulus. For each of the age groups studied, there were differences in the ApEn values for BL activity; however, these differences were not statistically significant. Interestingly, however, when we evaluated the change in ApEn values at the peak $freq_{LTF}$ response, there was a developmental trend, such that ApEn values were reduced (~5%) for P9 and P10 rats, followed by a slight increase (~1-2%) in P11 and P12 rats, and a further reduction (~1-4%) for P13-P15 rats. Although IH failed to elicit significant effects on ApEn values, a developmental trend was still observed from the experiments conducted (Figure 8).

Amplitude LTF (amp_{LTF}) vs. Frequency ($freq_{LTF}$)

Summary data showing EMG_{DIA} burst amplitude (amp_{LTF}) and burst frequency ($freq_{LTF}$) responses during BL and 15-min intervals during LTF recovery are provided in Figure 9. Immediately post-IH, EMG_{Dia} amp_{LTF} decreased to below BL values and then reached steady-state or continued to decrease. In contrast, $freq_{LTF}$ exhibited a transient increase post-IH (Figure 9). This increase was significant for P9, P10, P14, and P15 rats. A significant decrease ranging from 22 to 37% was observed post-IH in amp_{LTF} for P11rats and an increase ranging from 22 to 28% was observed at the 50-min, 55-min, and 60-min recovery time points for P13 rats (P≤0.001).

To further characterize burst amplitude and frequency during the IH trials and at the LTF recovery period, we also evaluated these parameters for the shared experimental age groups by examining their relationship relative to BL levels (i.e., %BL). These analyses revealed a reduction in burst amplitude ranging from 3 to 21% (mean±SE= 10.3±5.5%) for P9-P11 rats during IH and 2-6% (mean±SE= 7.1±0.7%) for P13-P15 rats during IH, whereas P12 rats showed an increase of 17% in burst amplitude during IH, with only P13 rats showing an increase in burst amplitude during the LTF recovery period. Interestingly, these effects were reversed when evaluating the burst frequency relative to BL levels (i.e., %BL). In this case, an increase ranging from 1 to 57% (mean±SE=34±7.2%) during IH and 12 to 21% (mean±SE= 16.9±2.6%) at the 60-min recovery time point was seen in P9-P11 rats and 19 to 43% (mean±SE=32±2.6%) during IH and 5 to 15% (mean±SE=6.1±4.7%) at the 60-min recovery time point was seen in P13-P15 rats, while burst frequency remained at a steady-state showing little or no change for P12 rats at either time point.

DISCUSSION

In our investigation, analyses of EMG_{DIA} activity recorded from *in vivo* spontaneously breathing urethane-anesthetized neonatal preparations demonstrated that AIH elicits an enhancement of respiratory output that is predominately seen as an enhancement of burst frequency when using a protocol similar to that previously reported (Mateika and Sandhu, 2011). While breathing remained stable across the experimental period, with neither breathing frequency nor amplitude significantly shifting during the BL period, we propose that the respiratory changes noted were all associated with perturbations in the inspired gas mixture, notably IH, and not changes in the physiological status of the rat. To our knowledge, this study is the first to report respiratory LTF in a spontaneously breathing P9-P15 neonatal rat preparation, and our results demonstrate that IH induces a form of respiratory LTF albeit the precise pattern is not identical to that seen in other mammals (Mitchell *et al*., 2001; Feldman, 2003; Baker and Mitchell, 2004). Previous studies report LTF as an increase in respiratory burst amplitude (Baker and Mitchell, 2004, McKay et al.); however, in the anesthetized spontaneously breathing neonatal rat preparation used in the current investigation, we found that LTF was mainly seen as an enhancement in burst frequency. Moreover, while both burst frequency and amplitude were altered during both the IH challenges and the post-AIH recovery period (*i.e.*, LTF), the dominant change observed in the current study corresponded to an enhancement of burst frequency, with variable effects on burst amplitude. In contrast to amp_{LTF}, which is most often reported in anesthetized adult rats (Baker-Herman and Mitchell, 2008), in anesthetized P9-P15 neonatal rats, respiratory LTF takes the form $freq_{LTF}$ although amp_{LTF} was also noted in some rats studied. In addition, LTF-induced changes in expiratory time (T_E) and ApEn values were noted. The time points for the peak f_{LTF} and amp_{LTF} (if present) responses were, however, variable.

Respiratory LTF burst amplitude versus burst frequency

There are two potential explanations for our findings. In response to a sustained reduction in oxygen availability there is a central accumulation of neuroinhibitory modulators including adenosine, γ-aminobutyric acid (GABA), and endogenous opiods (Neubauer, 1990). The presence of these modulators may act to suppress respiratory sensitivity and repress synaptic transmission thereby decreasing awareness of increased respiratory load. An alternative explanation is to consider that acute hypoxia triggers a "central inhibitory network" within the midbrain and brainstem. This mechanism has been documented (Neubauer, 2004), and suggested to contribute to hypoxic ventilatory depression. Similar to the mechanisms by which those specific neuronal networks may inhibit ventilatory motor output, they could potentially inhibit respiratory sensory afferent pathways and contribute to an impaired response to low oxygen levels.

Many published studies of respiratory LTF have used *in vivo* (Fuller *et al*., 2000; Mahamed and Mitchell, 2007), *in situ* (Tadjalli *et al*., 2007), or *in vitro* (Bocchiaro and Feldman, 2004) preparations, with recordings of efferent motor output from the phrenic and/or hypoglossal (XII) nerves. These studies demonstrate predictable variability across laboratories, preparations, and species, as well as genetic variations between rat strains and sub-strains (Fuller *et al*., 2001). A review of the literature indicates that LTF of efferent phrenic nerve burst amplitude is considerably more robust in the anesthetized and mechanically ventilated preparations as compared to LTF in spontaneously breathing animals (diaphragm EMG activity) and humans (tidal volume) with intact respiratory nerves (Fuller *et al*., 2005). Respiratory LTF in spontaneously breathing animals is often expressed as a persistent increase in breathing frequency rather than tidal volume or EMG burst amplitude (Baker-Herman and Mitchell, 2008; Olson *et al*., 2001), and the difference in LTF expression between spontaneously breathing versus ventilated preparations does not appear to represent an of impact of anesthesia on plasticity (Janssen *et al*., 2000; Mateika and Fregosi, 1997). For example, Janssen and Fregosi (2000) could not induce LTF of EMG_{DIA} burst amplitude in urethane-anesthetized and spontaneously breathing rats despite using an anesthetic and the

IH protocol that elicits robust LTF in anesthetized and mechanically-ventilated rats (Bach and Mitchell, 1996; Fuller *et al.*, 2001). Similarly, LTF of EMG_{DIA} activity is not evident in anesthetized and spontaneously breathing cats following intermittent stimulation of the carotid sinus nerve (Mateika and Fregosi, 1997) although LTF of upper airway muscle activity (*i.e.* genioglossus) can be evoked in spontaneously breathing animals (Mateika and Fregosi, 1997; McKay, 2004; Tadjalli *et al.*, 2008). A similar result was reported from measurements of decreased pulmonary airflow resistance following IH in sleeping humans (Aboubakr *et al*., 2001; Shkoukani *et al*., 2002). Thus, the mechanisms which limit the expression of phrenic/diaphragm LTF may be different than those for the expression o0f LTF in hypoglossal or other upper airway respiratory motor outputs in spontaneously breathing animals.

Another possibility to consider is the potential "ceiling effect" of elevated CO_2. Janssen *et al*. (2000) hypothesized that the (comparative) lack of phrenic/diaphragm LTF expression during spontaneous breathing reflects the relatively higher $PaCO_2$ values observed in spontaneously breathing versus mechanically-ventilated animals. Since even small increases in $PaCO_2$ will increase the overall output of phrenic motoneurons (Kong and Berger, 1986; St John and Bartlett, 1979), elevated $PaCO_2$ may impair burst enhancement following a LTF protocol due to a "ceiling effect". In other words, if phrenic motor output is relatively high during baseline (pre-IH) conditions, there may be a reduced capacity for further increased motoneuron recruitment in the subsequent post-hypoxic period (Doperalski and Fuller, 2006). Consistent with this idea, phrenic LTF is difficult to evoke in phrenic neurograms recorded contralateral to cervical spinal cord hemisection injury (Doperalski and Fuller, 2006), a condition which results in robust compensatory increases in contralateral phrenic output (Miyata *et al*., 1995). In contrast, Lee and colleagues (2009) recently demonstrated that raising $ETCO_2$ by approximately 4 mmHg above eupneic values is a prerequisite for induction of ventilatory LTF (including both increased frequency and tidal volume) in spontaneously breathing humans. Thus, in some circumstances $PaCO_2$ elevations appear to be necessary for respiratory LTF, and factors other than $PaCO_2$ levels may be responsible for differences in LTF between spontaneously breathing and ventilated preparations. Based on our data, we suggest that the

maturational age of the animals along with very precise conditions (vagal afferent input, mechanical ventilation, arterial blood pressure, arterial blood gases) contribute to the observed LTF differences because at the end of each of our experiments, the rats were sacrificed using severe hypoxia and in all experiments, they exhibited a robust (increase in both burst amplitude and frequency) peripheral chemoreflex response. Previous work suggests that the activation of vagal pathways in studies on awake spontaneously breathing animals may mask or override the manifestation of LTF (Mateika and Fregosi, 1997). In addition, the magnitude of the LTF response has been shown to differ for different respiratory outputs. In this case, intercostal nerve LTF has been reported to be significantly greater than phrenic LTF, and hypoglossal (XII) nerve LTF has been shown to be far more robust than phrenic LTF in certain sub-strains of anesthetized Sprague-Dawley rats (Bach and Mitchell, 1996). Thus, differences in the magnitude of the LTF response among different preparations and laboratories may be partially explained by these factors. In the current study, which was conducted in urethane-anesthetized, spontaneously breathing, vagal intact neonatal rats, a number of the variables identified above could have influenced the magnitude of the observed LTF response. We did not, however, evaluate the specific contributions of these factors; thus, future experiments could be conducted in neonatal rats to assess their contributions, including the influence of postnatal development on the manifestation of respiratory LTF.

EMG_{Dia} Expression of Respiratory LTF in the neonatal rat

Previous work, mostly perfiormed in anesthetized, vagtomized and mechanically-ventilated adult rats has demonstrated that hypoxia-induced LTF is mainly expressed as an increase in burst amplitude (Baker and Mitchell, 2000; Feldman *et al*., 2003; Fuller, 2005; Olson *et al*., 2001). However, we found that respiratory LTF was manifested primarily as an increase in respiratory burst frequency with little or no change in amplitude. Our findings are similar to those from previous studies in the developing rat (McKay *et al*., 2004; McGuire and Ling, 2005 and Tadjalli *et al*., 2007) in that respiratory LTF is observed following IH.

For example, previous work in P3-P7 neonatal rats has demonstrated manifestation of respiratory LTF that took the form of amp_{LTF} (McKay et al., 2004). Similar to our study, McKay *et al*. used three 5-minute episodes of hypoxia separated by 5-miutes of normoxia; however, in their study, they examined genioglossus EMG activity. Whether differences between the observations of McKay *et al*. (2004) and the current study result from differences in the respiratory-related muscles examined (*i.e.*, genioglossus *vs*. diaphragm), the age of the rats studied (*i.e.*, P3-P7 *vs*. P9-P15), and/or the different anesthetic agents used (*i.e*., ketamine-xylazine *vs*. urethane) is unclear.

We are not the first to report the lack of amp_{LTF} during the recovery period following repetitive bouts of acute hypoxia in a rodent model nor are we the first to report the AIH-induced manifestation of respiratory LTF as exclusively f_{LTF} in a rodent model. In awake 2-month-old rats (McGuire and Ling, 2005) and unanesthetized adult mice (Hickner et al., 2014), for example, LTF has been described as an increase in respiratory frequency with minor or no changes in inspiratory amplitude. Similarly, in the *in situ* arterially-perfused working-heart brainstem decerebrate P15-P25 rat preparation, Tadjalli and colleagues, reported that episodic hypoxia elicited respiratory LTF that was seen primarily as an enhancement of burst frequency, but not amplitude, over a 60-min period following the last bout of episodic hypoxia (Tadjalli *et al*., 2007). While these studies were performed in unanesthetized rodent

preparations, they clearly demonstrate that the augmentation of respiratory motor output evoked by AIH (*i.e.*, respiratory LTF) may correspond to exclusively an increase in respiratory frequency.

Hypoxia-induced LTF on inspiratory neural network complexity in the neonatal rat

We have previously reported that ApEn values increase with maturation, suggesting a maturational change in the central respiratory network. In the current study, we found that ApEn values were not significantly changed in response to IH or post-IH although a maturational trend was observed when we evaluated ApEn at the peak $freq_{LTF}$ response. This observation is somewhat surprising since our observations in Specific Aim 1 clearly demonstrated a developmental trend in ApEn of basal EMG_{Dia} bursts as well as the response to severe hypoxia (i.e., gasping), suggesting a re-organization of the inspiratory neural network with increasing age as well as with hypoxic stimulation. While we would expect to see changes in ApEn during IH, the timing of our measurements may have masked an effect since we evaluated the end of the 5-min hypoxic stimulus and not the peak response. Additionally, our inability to detect a developmental shift under basal conditions may be related to the fairly small sample sizes and limited developmental (P9-P15) window examined. Additionally, changes in ApEn may have been absent due to two factors previously discussed: (1) the severity of the hypoxic stimulus used in this study and (2) the biphasic ventilatory response previously reported for neonatal rats (reviewed by Bissonnette, 2000). Additional analyses of more time points (*i.e.* each minute of the IH trials) may provide insight into whether or not changes are occurring in the biphasic respiratory response to hypoxia that may have been overlooked by our current analyses.

Clinical Relevance

Although the physiological significance of LTF in spontaneously breathing anesthetized animals is not yet understood, we believe that the study of this phenomenon will have important implications from several perspectives. For one, it can be therapeutically beneficial for patients with upper airway obstruction during sleep or persons suffering from a neurodegenerative disorder or pathology in which the airway has been compromised (Ryan and Nolan, 2009, Vinit et al., 2009, Trumbower et al., 2012).

Obstructive sleep apnea (OSA) is a devastating sleep disorder that is at the root of numerous health complications (Horner, 1996). Intermittent airway obstruction is characteristic feature of OSA, and it is possible that the body is compensating physiologically through LTF to promote a steady-state of oxygen supply when the system is challenged by respiratory challenges (Mahamed and Mitchell, 2007). Homeostasis is promoted by LTF through increasing the upper airway patency which then leads to the prevention of decreased airflow associated with sleep apnea (Mahamed and Mitchell, 2007). Preventing the reductions in airflow resistance will reduce the incidence of apneas (Remmers *et al*., 1978).

Currently, there are no effective pharmacological FDA approved drugs to treat obstructive sleep apnea (Smith and Quinnell, 2004) and surgical intervention does not show an effective success rate (Pang, 2005). Moreover, the time associated with the process of fixing OSA (surgery then recovery) and the low success rate may lead patients to be hesitant about the surgical process (Pang, 2005, Won *et al*., 2008). The most effective and common form of treatment is through the use of CPAP (continuous positive airway pressure) (Harsch *et al*., 2004). Unfortunately, due to the low compliance with this device, individuals often fail to see results (Sullivan *et al*., 1981, Harsch *et al*., 2004). Therefore, the best course of action would be the use of pharmacological drugs, which would have potential advantages in both the ease of use and compliance amongst patients.

Further, by investigating the cellular and synaptic mechanisms responsible for the induction and maintenance of LTF, further insights may be gained concerning this important, homeostatic control system once believed to be fixed following maturation. Thus, studies investigating LTF in neonates may yield insight into mechanisms for non-ventilated breathing during maturation (Fuller et al., 2000). To this end, Gozal and colleagues (2002) suggest that, at least in neonates, IH may impact an animal's ability to autoresuscitate, further stressing the importance of studying the influence of early postnatal development to delineate the mechanism(s) associated with this adaptive behavior.

Specifically, understanding postnatal LTF during the critical period is important because it may help explain various aspects underlying abnormal ventilatory drive in a common pathology known to many parents, Sudden Infant Death Syndrome (SIDS). Neurochemical studies of the rat brain have suggested that a critical period of respiratory development might be related to SIDS because of its occurrences at a very specific time (Wong-Riley and Liu 2008, Lui and Wong-Riley 2010, Wong-Riley *et al*. 2013), with 90% of SIDS occurrences being experienced within the first six months of an infant's life (Kinney *et al*. 1992). Maturational modifications in expression levels of various neurotransmitter/neuromodulator systems involved in central respiratory control (*e.g.*, receptor subunit expression, neurotransmitter abundance, neurotrophic factors (Gozal and Torres, 1997 Harris et al. 2007, Wong-Riley and Liu 2008, Lui and Wong-Riley 2010, Toppin et al., 2007) are often associated with developmental changes in intrinsic membrane properties and synaptic transmission within the CNS; thus, studies aimed at identifying developmentally regulated differences in the mechanisms of respiratory rhythm generation, pattern formation, and central and peripheral chemoreception may help us to understand the ventilatory dysfunction associated with SIDS.

This is why understanding the mechanisms that underlie the phenomenon of LTF will contribute to the development of novel pharmaceuticals. When research has led to a better understanding of key players at all levels, then pharmaceuticals aimed at enhancing this mechanism could prove to be beneficial. However, before pharmaceuticals can be developed, the neural substrates mediating LTF along with the manifestation of LTF in more physiological conditions must be known. Once we understand the critical processes mediating LTF at different maturational ages, we can start to develop drugs which stimulate or enhance the pathways during sleep to prevent decreases in both upper airway patency and respiratory motor output in a variety of respiratory pathologies.

LIMITATIONS/FUTURE DIRECTIONS

The purpose of this study in this thesis work was to characterize the influence of IH in eliciting LTF in the spontaneously breathing neonatal rat and investigate changes in inspiratory network complexity. We found that IH elicits respiratory LTF in neonatal rats at all ages examined (P9-P15), and that this respiratory LTF takes the form of primarily an enhancement of burst frequency although an increase in amplitude was noted in some of the neonatal rats studied. There are, however, a number of limitations associated with the presented body of work that could influence breathing, and as a result, affect the responses that are reported. These include: (1) the use of anesthesia, (2) the use of the spontaneously breathing animal model, and (3) the use of an afferent intact preparation. While these factors could have influenced our observations, they are not unique to our study, as most studies reporting respiratory LTF have used anesthetized preparations and both spontaneously breathing preparations and afferent intact preparations have been shown to express some form of respiratory LTF. There are, however, many open avenues of study that could proceed from this direction.

Further, in this thesis work, we investigated whether IH played any role in producing respiratory plasticity in the developing rat. We did not attempt to identify CNS regions and their role(s) in the induction or maintenance of hypoxia-induced plasticity; thus, future studies could be constructed to evaluate this in the neonatal rat. Moreover, since respiratory LTF is influenced by numerous factors, including but not limited to, neuromodulatory synaptic transmission (Fuller *et al*., 2001, Baker-Herman and Mitchell, 2002, Baker-Herman *et al*., 2004), which is also known to exhibit developmental changes, especially in the developmental window examined in the current investigation, additional studies could be directed at examining the roles or contributions of such systems in this behavior..

The anesthetic used in our experiments exerts minimal cardiorespiratory depression; however, there are still caveats to this approach. Anesthetics alter the modulation of neural transmission that can affect motor neuron excitability, and of relevance to this work is serotonergic neurotransmission (Franks and

Lieb, 1994, Yost *et al*., 1998, Whittington and Virag, 2006) since serotonin receptor activation is required for the induction of hypoxia-induced LTF (Fuller *et al*., 2001).

Effects of hypoxia on respiratory drive and ventilation have been studied extensively. Compared with hypercapnia, hypoxia is a lesser respiratory stimulus. Although hypoxia has a high affinity to hemoglobin under usual ambient conditions, the PO_2 must decrease substantially to approximately 60 mmHg before hypoxia becomes a major stimulus to breathe (Marieb, 1998); thus, the degree of hypoxia may be an important predictor of the magnitude of the LTF response (Nichols *et al*., 2012; Devinney *et al*., 2014). Further, since hypoxia acts to modulate oxygen-hemoglobin affinity, during disease states, changes in pH, PaO_2, and $PaCO_2$ are important determinants of hypoxia-induced ventialtory drive. The hypoxic ventilatory response in healthy men and women is also attenuated during NREM sleep and declines further during REM sleep compared to wakefulness (Douglas, 1982). Thus, experimentally-induced hypoxia sufficient to increase ventilation is associated with accompanying decreases in $PaCO_2$. Therefore, to study the effects of hypoxia, it is essential that $PaCO_2$ is controlled, in addition to the degree of hypoxia and sleep state.

FIGURE LEGENDS

Figure 1. Inspiratory motor output in response to CO_2 and acute hypoxic challenges. A) Example traces of integrated (Int) diaphragm EMG activity from a P14 neonatal rat. Traces show a 30-s record for baseline (BL), CO_2, and acute hypoxia (first IH trial, IH-1). Both CO_2 and acute hypoxia increased amplitude and burst frequency above BL levels. **B)** Summary data (mean±SE) showing increase in minute activity (MA) elicited by CO_2 and acute hypoxia (IH-1). * *represent a significant difference ($P<0.05$) vs BL; # significant difference ($P<0.05$) CO_2.*

Figure 2. Integrated EMG_{DIA} recording showing the development of LTF over a 60-miute period following three hypoxic episodes (5-minute duration each) in a urethane-anesthetized spontaneously breathing neonatal rat P13. The pattern of progressive increase in EMG_{DIA} burst amplitude in the top panel is similar to that seen in anesthetized, mechanically ventilated adult rat phrenic motor output following intermittent hypoxia (Baker and Mitchell, 2000). However example two (bottom tracing) shows response to three IH trails following by no changes in burst amplitude over the 60-minute post IH stimulus. In both experiments, IH increase d burst amplitude and frequency, with the peak response being noted early in the IH trail, after which a marginal decline in burst amplitude is observed. After the IH trails, the effects on burst amplitude in both examples shown varied however; an increase in frequency was observed during the 60-minutes post IH for each experiment.

Figure 3. Example traces showing burst frequency LTF ($freq_{LTF}$) of EMG_{DIA} activity. 30-second traces recordings corresponding to BL, IH-1, and LTF responses at 15- and 45-min post-IH stimulus. Bottom panel traces showing expanded time scale for time points indicated in the top panel. Shown are 5-s traces of both Int and raw diaphragm EMG activity demonstrating the increase in ($freq_{LTF}$) following IH trials. Data were obtained from a P15 neonatal rat. Dotted line represents burst amplitude of baseline recordings.

Figure 4. Group data showing the effects of hypoxia-induced LTF inspiratory burst timing and peak EMG_{Dia} activity. Top panel: Data acquired from each individual experiment conducted. Open circles-P9, Open Triangle-P11, Open square-P11, Closed hexagon-P12, Closed circles-P13, Closed Triangle-P14, Closed square-P15. Middle panel: Summary data for group averages (Mean±SE) for P9-P11 (right), P12 (middle) and P13-P15(left) for burst duration (T_I) mean animal age groups. Bottom panel: percent change of peak T_I response at each developmental age. Generally changes were seen examined however there was no significance detected. Peak response demonstrated a maturational trend.

Figure 5. Group data showing the effects of hypoxia-induced LTF on duration between burst and peak EMG_{Dia} activity. Top panel: Data acquired from each individual experiment conducted. Open circles-P9, Open Triangle-P11, Open square-P11, Closed hexagon-P12, Closed circles-P13, Closed Triangle-P14, Closed square-P15. Middle panel: Summary data for group averages (Mean±SE) for P9-P11 (right), P12 (middle) and P13-P15 (left) for the duration between burst (T_E) mean animal age groups. Bottom panel: percent change of peak T_E response at each developmental age. Generally changes were seen for all ages however there was only a significant decrease in T_E was for P9-P11 and P13-P15 age groups (also significant was 45-miunte mark for P13-P15 age group). Percent change establishes a maturational trend in T_E at the peak response for each developmental age. *Asterisks represent a statistically significant difference (P<0.002) from BL.*

Figure 6 . Summary data showing the effects of hypoxia-induced LTF on burst frequency of EMG_{Dia} activity. Top panel: Data acquired from each individual experiment conducted. Open circles-P9, Open Triangle-P11, Open square-P11, Closed hexagon-P12, Closed circles-P13, Closed Triangle-P14, Closed square-P15. Middle panel: Summary data for group averages (Mean±SE) for P9-P11 (right), P12 (middle) and P13-P15 (left) for burst frequency response at each developmental age. Similar to T_E changes were seen for all ages however there was only a significant increase in burst frequency for P9-P11 and P13-P15 age groups (also note the significance of the 45-miunte mark for P13-P15 age group). *Asterisks represent a statistically significant difference (P<0.001) from BL.*

Figure 7 . Summary data showing the effects of hypoxia-induced LTF on burst amplitude of EMG_{Dia} activity. Top panel: Data acquired from each individual experiment conducted. Open circles-P9, Open Triangle-P11, Open square-P11, Closed hexagon-P12, Closed circles-P13, Closed Triangle-P14, Closed square-P15. Middle panel: Summary data for group averages (Mean±SE) for P9-P11 (right), P12 (middle) and P13-P15 (left) for burst amplitude in response to hypoxia and post-IH for animal ages. Significant decrease was detected for P9-P11 at the 15-minute mark post IH. *Asterisks represent a statistically significant difference (P<0.001) from BL.*

Figure 8. Inspiratory neural network complexity during IH and post-IH stimulus in the *in vivo* anesthetized spontaneously breathing neonatal rat preparation. (Top and Bottom) The effect of maturation on ApEn underlying EMG_{Dia} burst. Top panel: Open circles-P9, Open Triangle-P11, Open square-P11, Closed hexagon-P12, Closed circles-P13, Closed Triangle-P14, dark square-P15. Middle panel: Summary data (Mean±SE) acquired from experimental age groups P9-P11, P12, and P13-P15. Bottom panel: ApEn values at peak response resulted in a developmental percent change. Overall, ApEn values were not significantly different although a clustering of symbols could be seen.

Figure 9. Summary data showing burst changes in burst amplitude and frequency under BL and at 5-miute intervals post-IH in P9-P15 animal ages. Mean (±SE) percent burst amplitude and frequency is plotted against time. Following BL and IH trails respiratory frequency (closed circles) significantly increased throughout the 60-minutes post-IH whereas in most animal ages burst amplitude (open circles) did not. All points are percent change from BL values. *Asterisks represent a statistically significant difference (P<0.001) from BL.*

Figure 10. Group mean (±SE) burst amplitude versus burst frequency in EMG_{Dia} activity in neonatal rat ages P9-P15. The change in burst amplitude (right panel) and burst frequency (left panel) expressed as a percentage of baseline activity (dashed line), during IH-1, IH-2, IH-3, and 15-minue intervals of LTF.

Figure 1

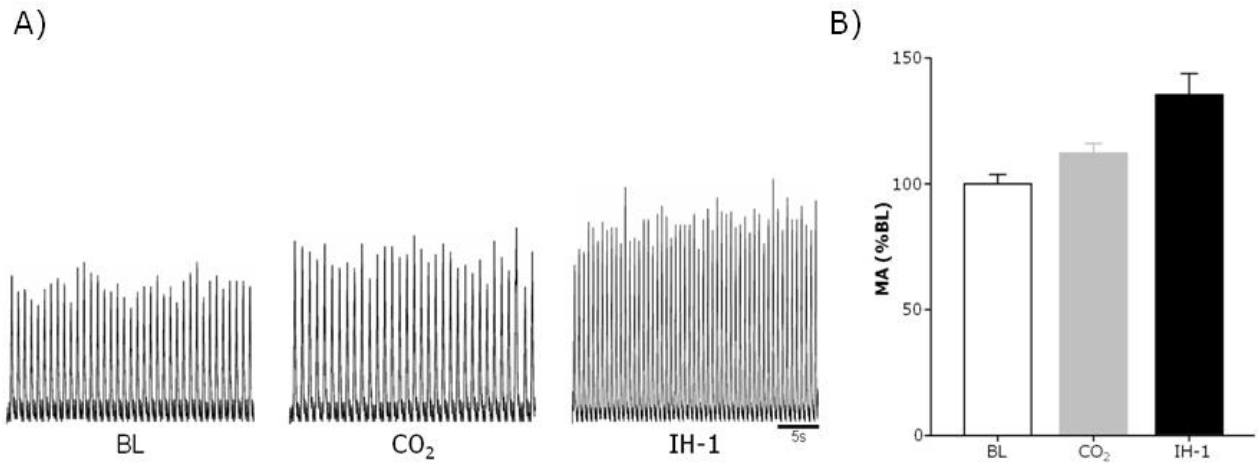


Figure 2

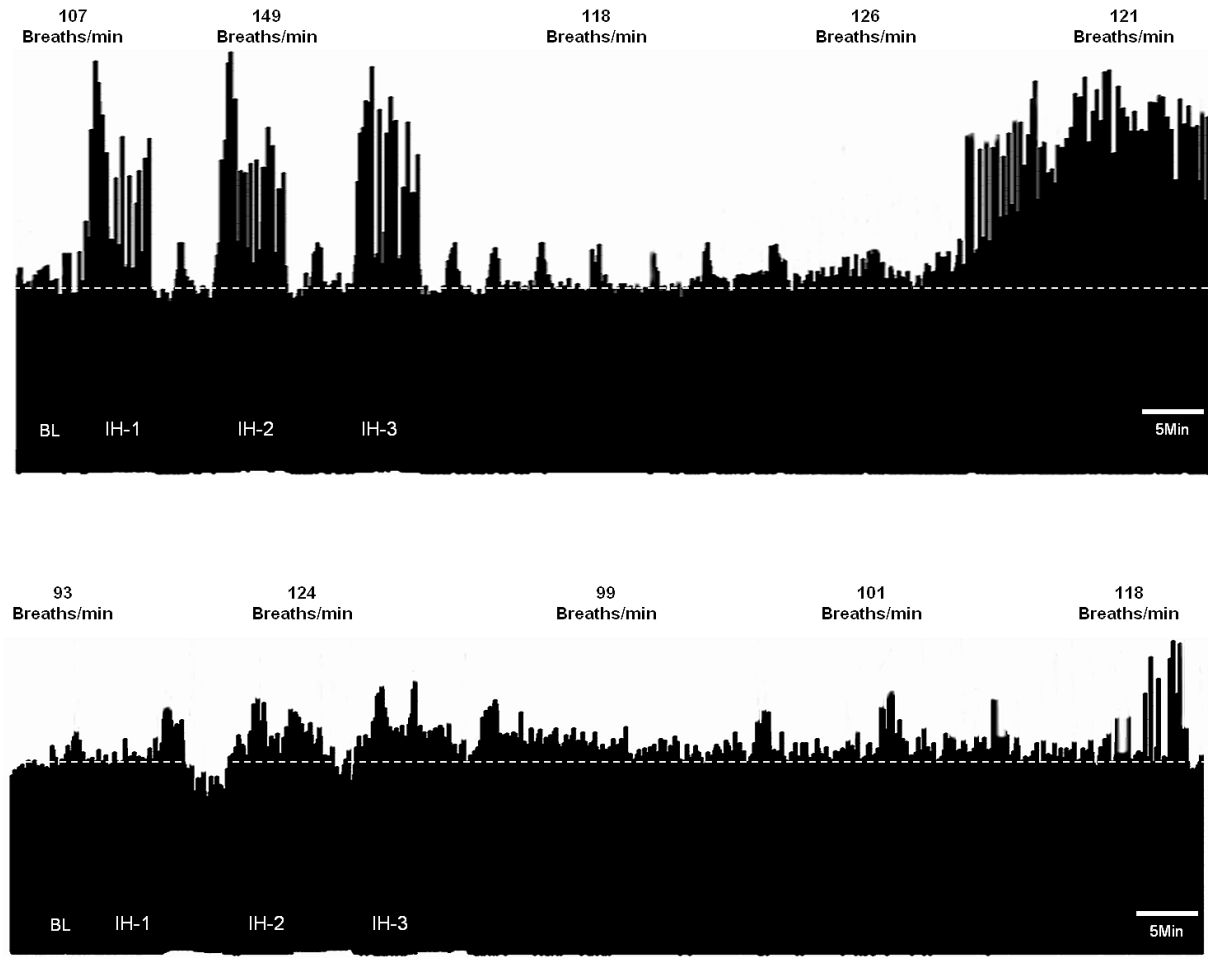

107
Breaths/min
149
Breaths/min
118
Breaths/min
126
Breaths/min
121
Breaths/min
BL
IH-1
IH-2
IH-3
5Min
93
Breaths/min
124
Breaths/min
99
Breaths/min
101
Breaths/min
118
Breaths/min
BL
IH-1
IH-2
IH-3
5Min

Figure 3

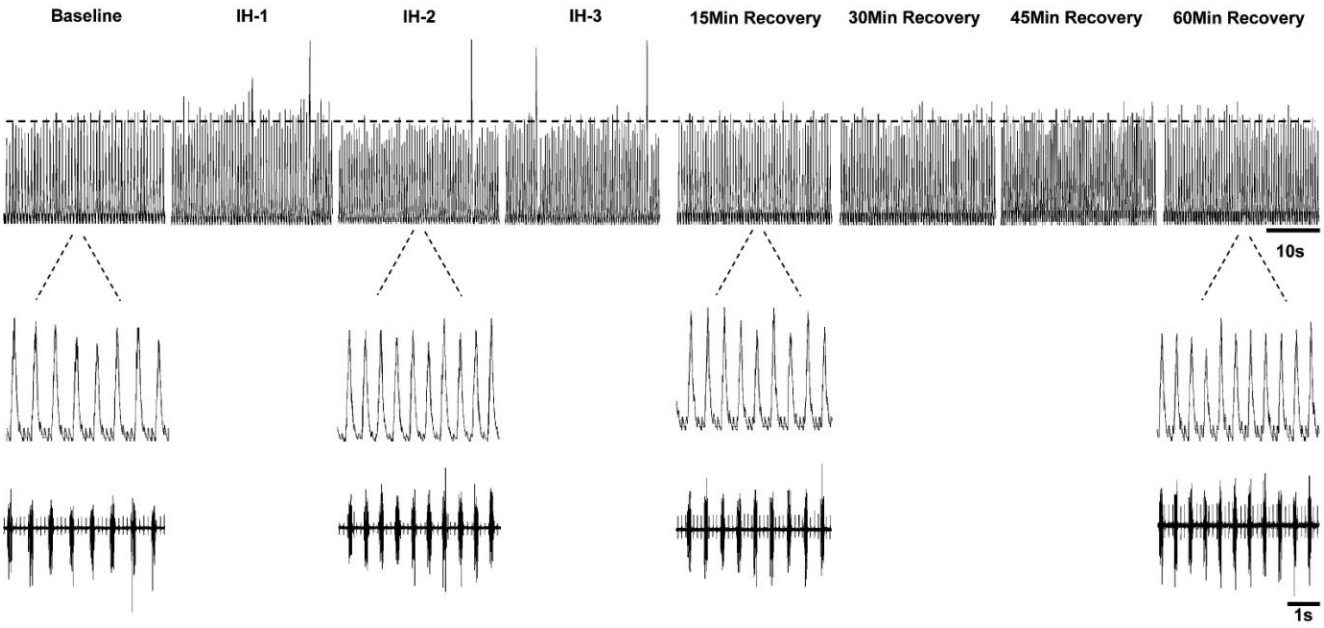

Baseline
IH-1
IH-2
IH-3
15Min Recovery
30Min Recovery
45Min Recovery
60Min Recovery
10s
1s

Figure 4

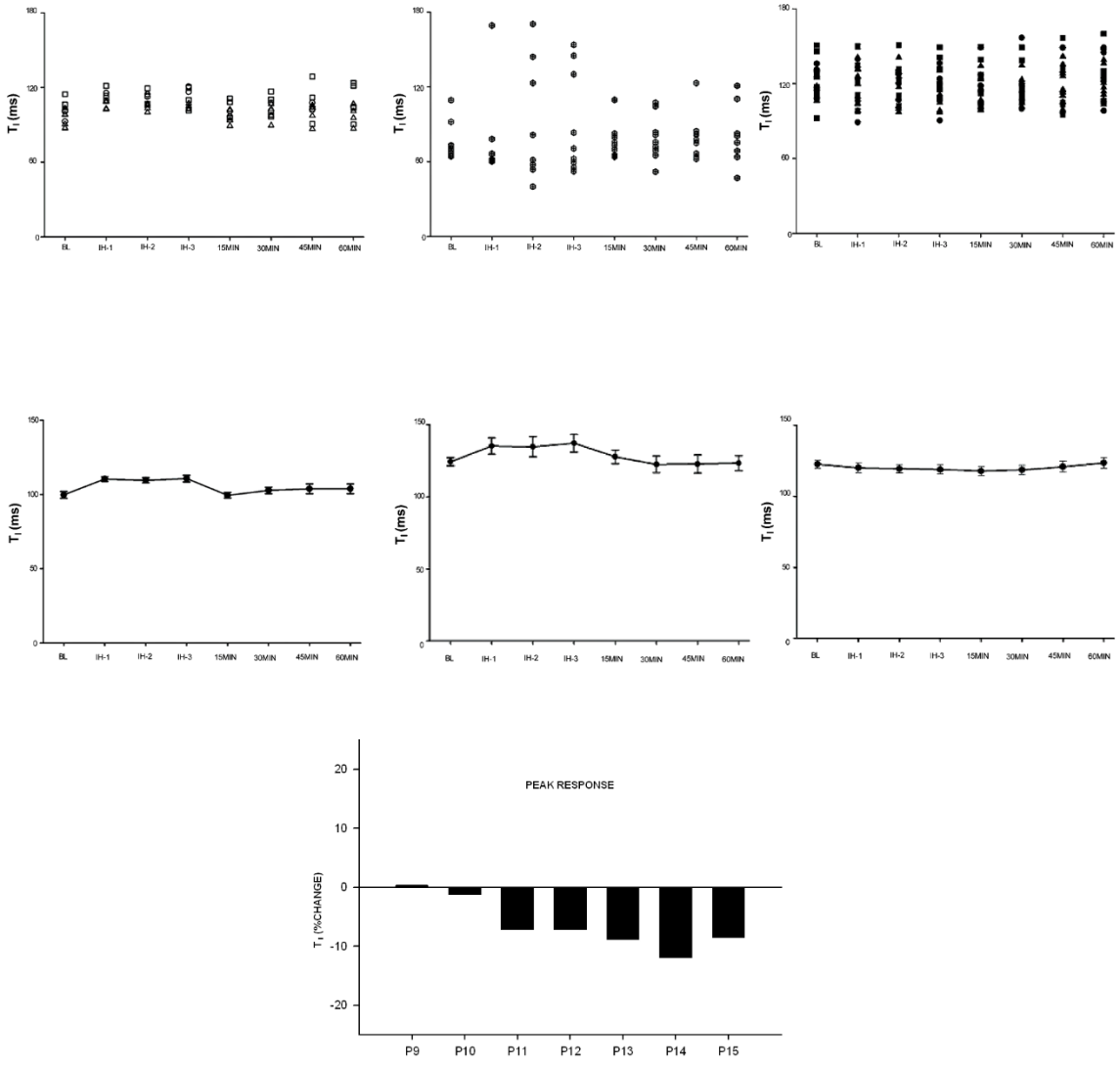

T1 (ms)
BL
IH-1
IH-2
IH-3
15MIN
30MIN
45MIN
60MIN
PEAK RESPONSE
T1 (%CHANGE)
P9
P10
P11
P12
P13
P14
P15

Figure 5

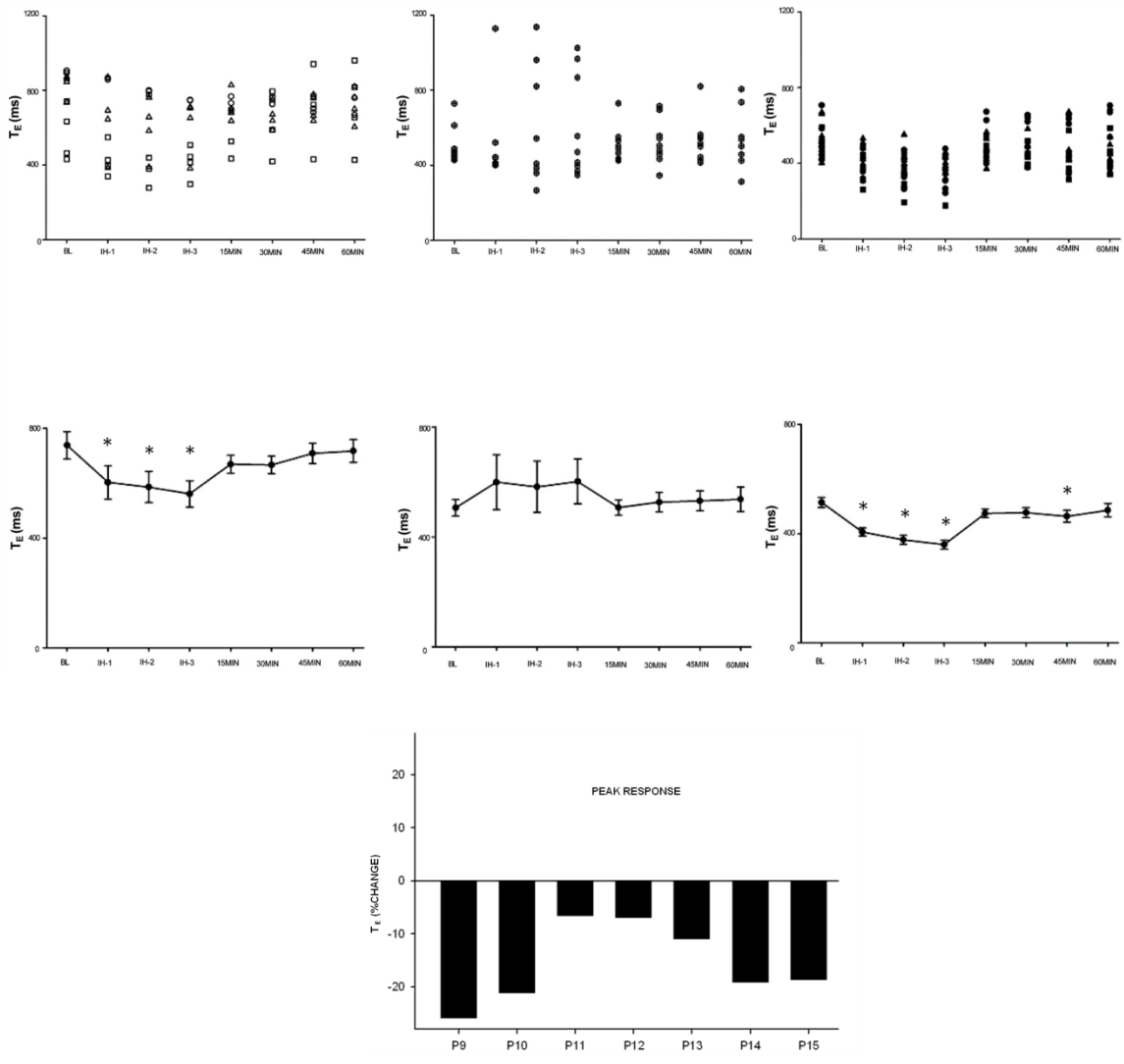

T_E (ms)
1200
800
400
0
BL
IH-1
IH-2
IH-3
15MIN
30MIN
45MIN
60MIN
*
PEAK RESPONSE
T_E (%CHANGE)
20
10
0
-10
-20
P9
P10
P11
P12
P13
P14
P15

Figure 6

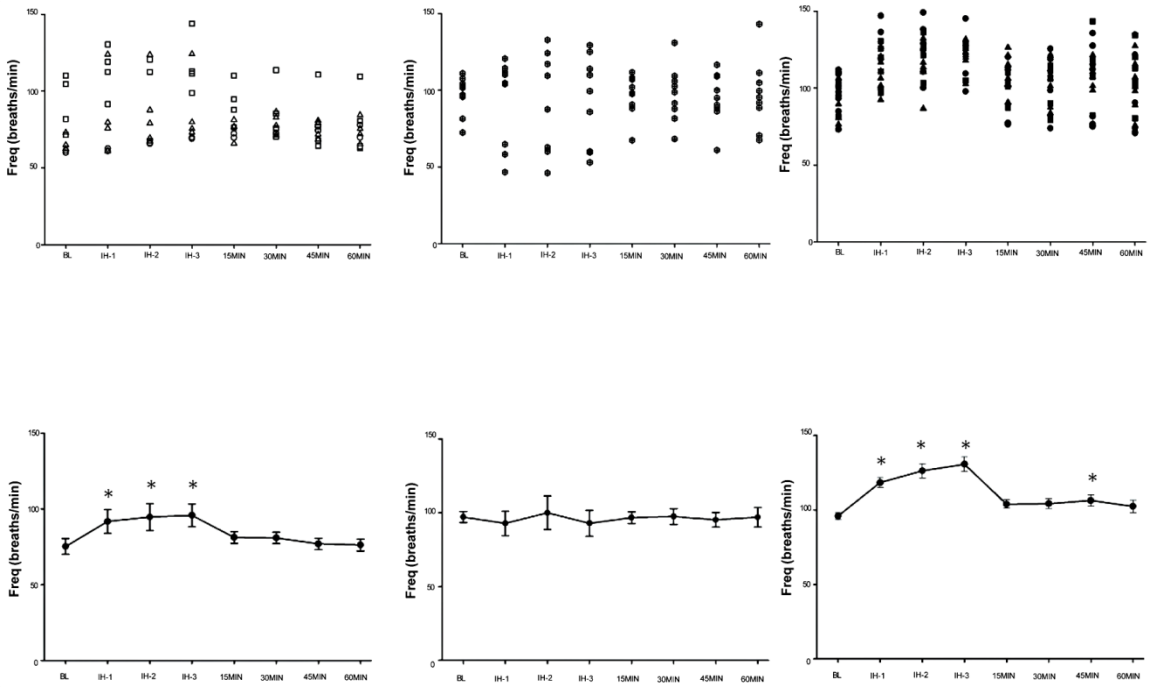

Freq (breaths/min)
150
100
50
0
BL
IH-1
IH-2
IH-3
15MIN
30MIN
45MIN
60MIN
*

Figure 7

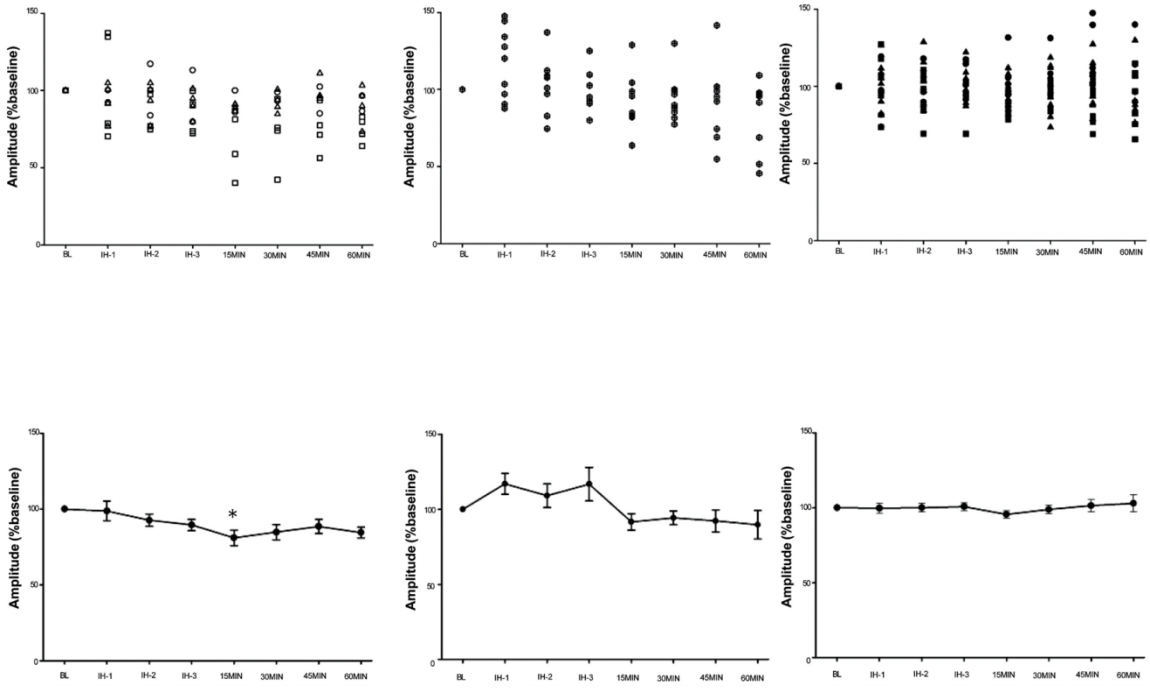

Amplitude (%baseline)
150
100
50
0
BL
IH-1
IH-2
IH-3
15MIN
30MIN
45MIN
60MIN
*

Figure 8

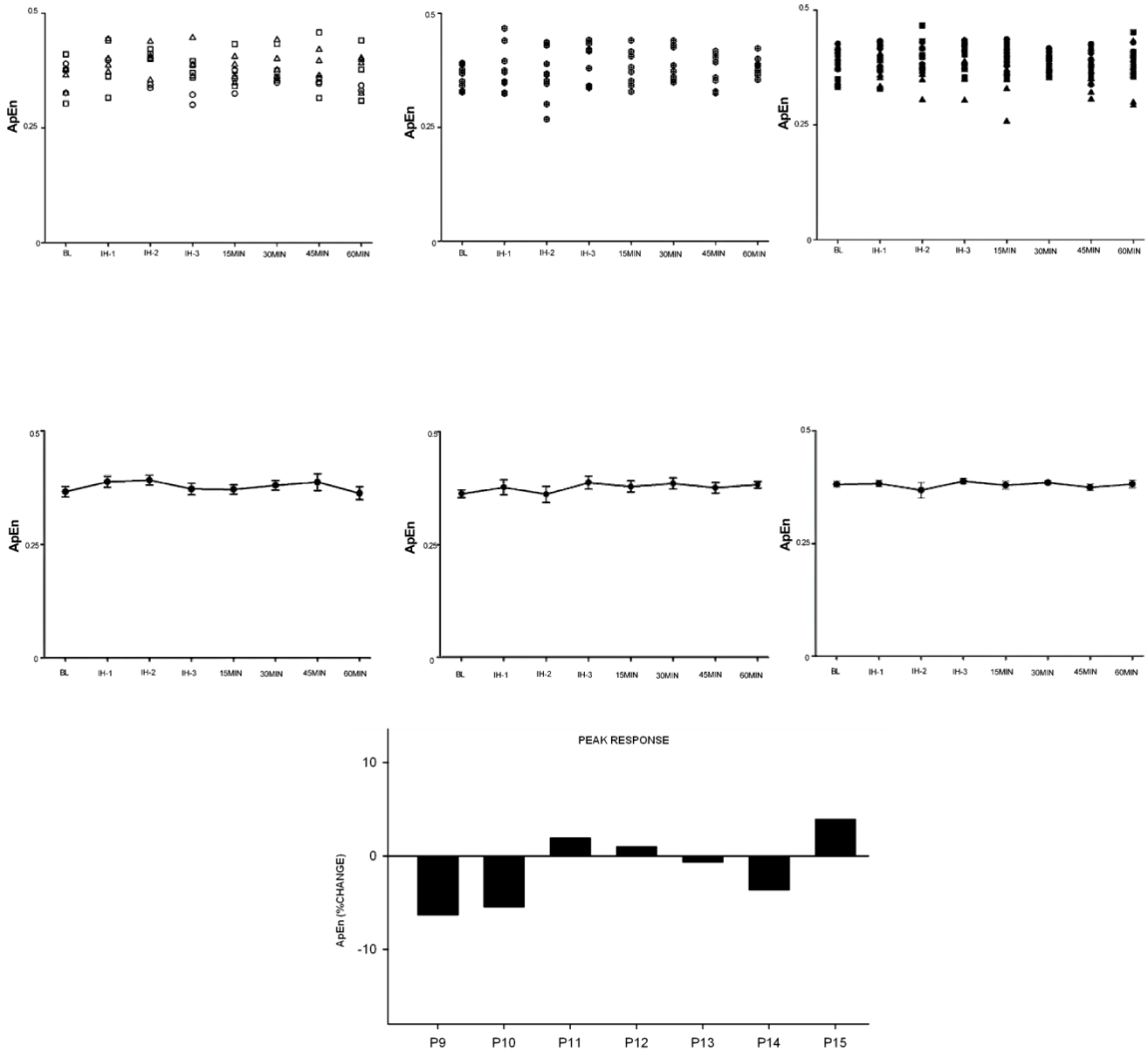

ApEn
0.5
0.25
0
BL
IH-1
IH-2
IH-3
15MIN
30MIN
45MIN
60MIN
PEAK RESPONSE
ApEn (%CHANGE)
10
0
-10
P9
P10
P11
P12
P13
P14
P15

Figure 9

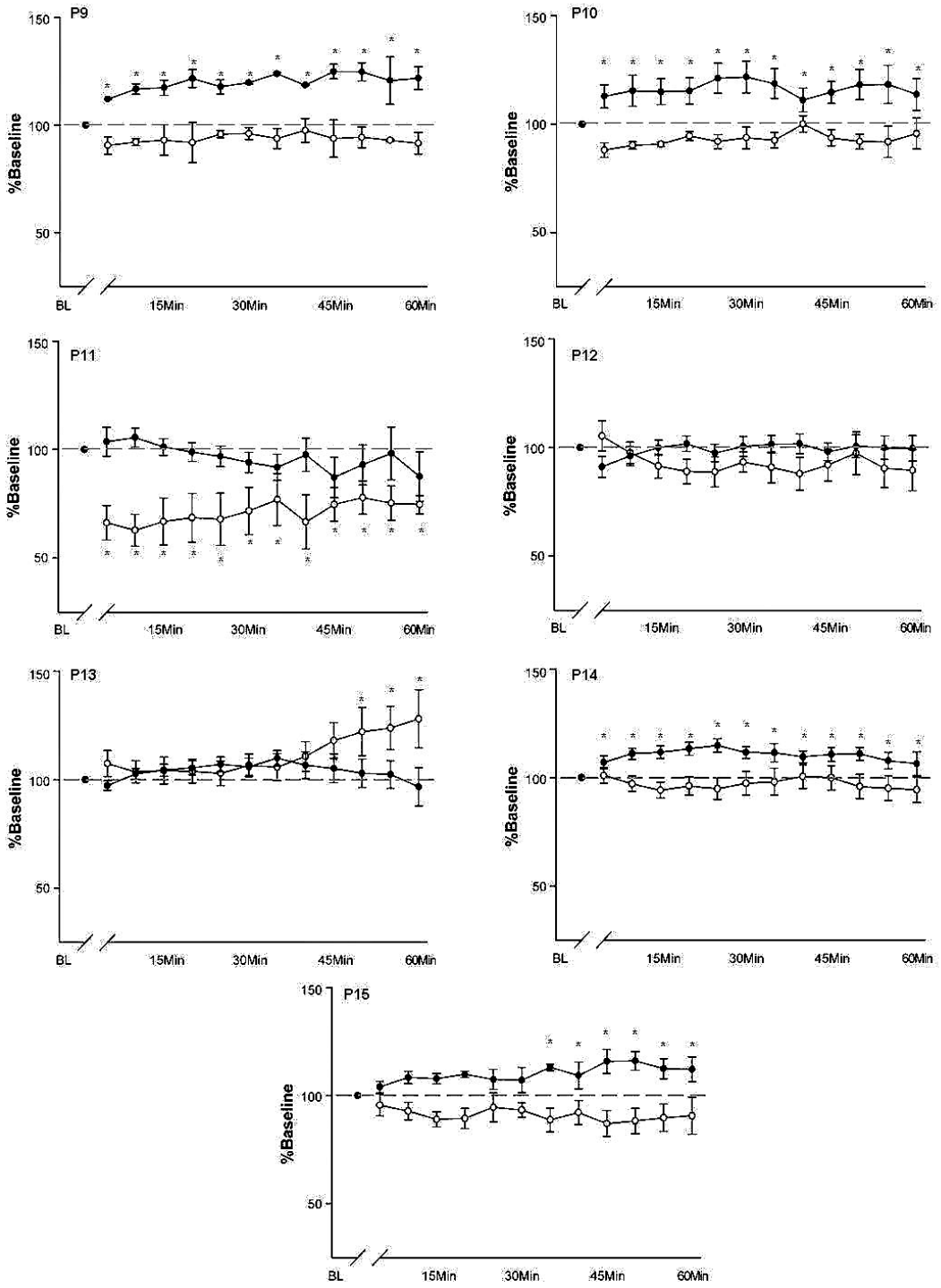
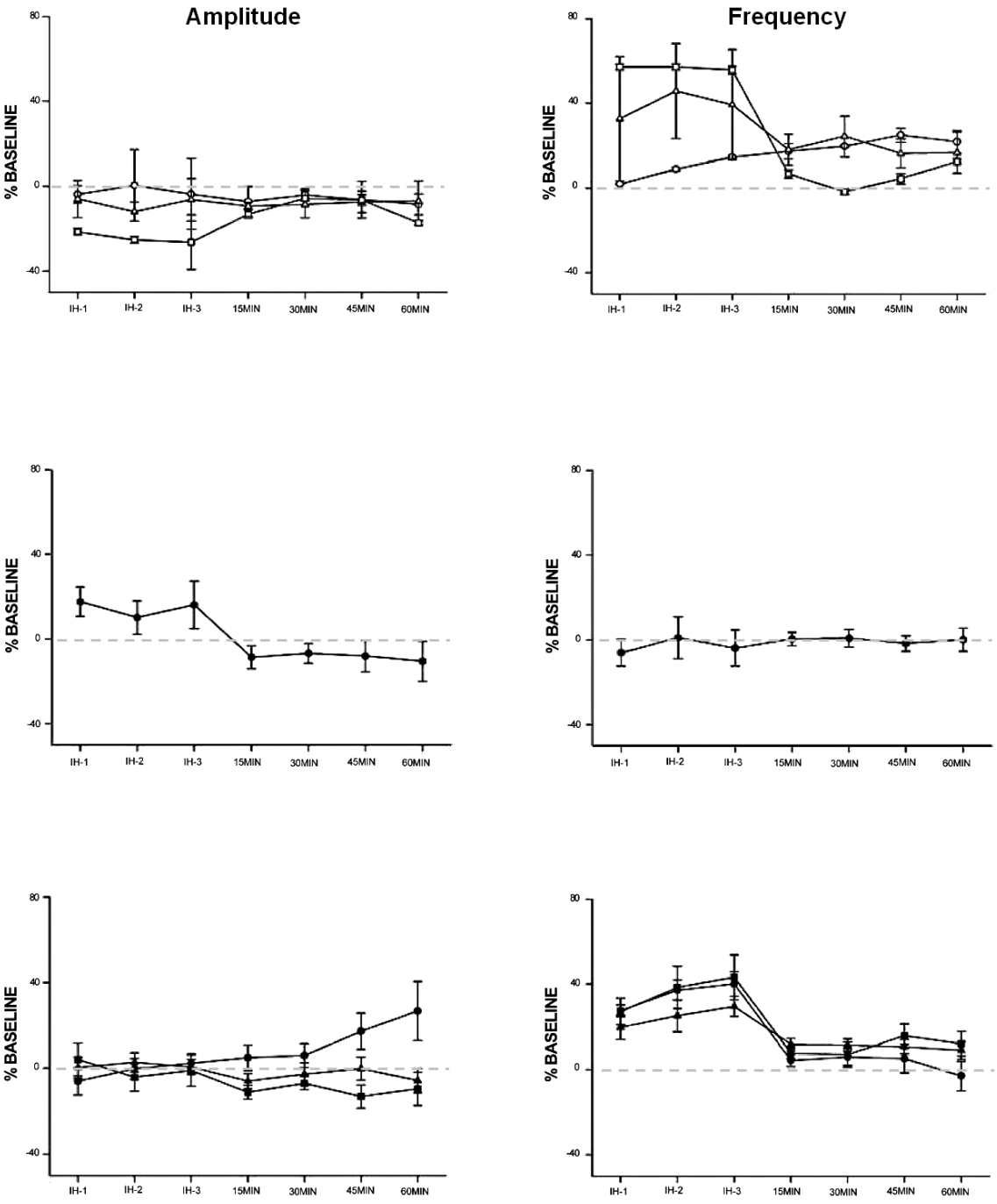

P9
P10
P11
P12
P13
P14
P15
150
100
50
%Baseline
BL
15Min
30Min
45Min
60Min

Figure 10


Amplitude
Frequency
% BASELINE
80
40
0
-40
IH-1
IH-2
IH-3
15MIN
30MIN
45MIN
60MIN

CHAPTER 5

SUMMARY AND CONCLUSION

OVERVIEW

The function of the central nervous system is to produce appropriate actions in response to different physiological conditions in order to maintain homeostasis. These actions is largely determined by current and past capabilities. This phenomenon, a modification in neuronal control that changes behavioral patterning, is the hallmark of neural control of breathing. The neuronal network undergoes maturational changes and neuroplasticity and has many mechanisms put in place to maintain homeostasis throughout life. This doctoral dissertation provides insights into respiratory neuronal plasticity during maturation in response to different levels of hypoxia. The current study have addressed the role of developmentally-regulated changes in neurotransmitter systems and corresponding alterations in inspiratory motor output in response to hypoxia.

Developmental gasping in the neonatal rat: This thesis describes age-dependent changes in the incidence and characteristics of gasping during the early postnatal period in neonatal rats. In our study, we identified a developmental correlation in that younger neonatal rats exhibit a higher likelihood of gasping in response to severe hypoxia as compared to older neonatal rats. Further, they show that the gasping response is not identical, such that both the duration of gasping and number of gasps decline with maturation.

In general both inspiratory burst duration (T_I) and network complexity (ApEn) are reduced during gasping (except in the very young rats). Changes in inspiratory central network not only reflect the developmental reconfiguration of the central respiratory network but also provide a quantitative insight to the extent of reconfiguration. Changes were also observed in the timing between burst (T_E) which substantially decreased during gasping in all ages examined.

Multiple gasping patterns in the neonatal rat: This work is the first to characterize multiple gasping patterns in the spontaneously breathing EMG_{Dia} in the neonatal rat. Our results provide insight into development contributions in the expression of multiple gasping patterns, and their role in understanding SIDS. Our data demonstrate that neonatal rat are capable of producing both doublet and triplet gasps in response to severe hypoxia and that there is a greater likelihood of eliciting doublet rather than triplet gasps.

With this we demonstrate that rats P6 or younger are more susceptible to alternate gasping patterns in comparison with neonatal rats P7 and older.

Developmental AIH-induced LTF in the neonatal rat: Repeated bouts of hypoxia triggers a form of respiratory plasticity that functions to strengthen the respiratory motor output. This type of plasticity is known as long-term facilitation (LTF) serves to deepen breathing and improve lung ventilation. Our findings demonstrate that IH elicits respiratory LTF in the neonatal rat at all ages examined (P9-P15), and while time points of peak response varies, compared with baseline respiratory LTF primarily takes the form of an enhancement of burst frequency rather than burst amplitude,. In addition there were maturational changes also noted in temporal characteristics, our results suggest that LTF may have also induced a reconfiguration of the central inspiratory network which was dependent on developmental stage.

Typically, hypoxia-induced LTF is studied in ventilated, anesthetized, vagotomized rats, and chemical feedback is tightly controlled by maintaining the levels of arterial O_2 and CO_2 throughout the experiment there is still much insight to be gained from studying LTF in spontaneously breathing animals. For example, spontaneously breathing better mimics human psychological conditions. Although the physiological significance of LTF in spontaneously breathing anesthetized animals is not yet understood, we believe that the study of this phenomenon will have important implications from several perspectives. For one, it can be therapeutically beneficial for patients with upper airway obstruction during sleep or persons suffering from a neurodegenerative disorder or pathology in which the airway has been compromised (Ryan and Nolan, 2009, Vinit et al., 2009, Trumbower et al., 2012). Further, by investigating the cellular and synaptic mechanisms responsible for the induction and maintenance of LTF, further insights may be gained concerning this important, homeostatic control system once believed to be fixed following maturation. Thus, studies investigating LTF in neonates may yield insight into mechanisms for non-ventilated breathing during maturation (Fuller et al., 2000). To this end, Gozal and colleagues (2002) suggest that, at least in neonates, IH may impact an animal's ability to autoresuscitate, further stressing the importance of studying the influence of early postnatal development to delineate the mechanism(s) associated with this adaptive behavior.

Together these studies have highlighted the complex interaction between hypoxia and maturation.

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