RESPIRATORY LOAD COMPENSATION RESPONSES IN CONSCIOUS ANIMALS

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2010

To my loving parents

ACKNOWLEDGMENTS

I would first like to thank my mentor, Dr. Paul Davenport, for his guidance during my Ph.D. career. He took a chance on me, giving me the opportunity to pursue a doctorate in respiratory physiology without my having extensive research experience or knowing with conviction my ultimate goals. He has been infinitely patient over the past years, supporting my various interests and going to bat for me when others might not. I would also like to thank those on my supervisory committee, including Dr. Donald Bolser, Dr. Linda Hayward, Dr. Christine Sapienza, and Dr. David Fuller, whose wisdom has shaped me in innumerable ways and without whom none of this would have been possible.

This body of work was enhanced by the assistance of individuals who were generous with their time: Mark Hotchkiss for his aid in the laboratory and with animal surgeries, Dr. Roger Reep for the use of his laboratory space and troubleshooting with imaging software, Margaret Stoll for her knowledge of histochemical techniques, and Dr. Deborah Scheuer for her background in cardiovascular physiology and expertise in stress. I have learned that collaborations and teamwork do not just make the science behind research stronger, they make the experience richer.

Dr. Floyd Thompson, Dr. Kevin Anderson, and Dr. Davenport all deserve special recognition for showing me the joys of teaching. My time spent as a teaching assistant in their neuroscience, gross anatomy, and respiration courses were some of my most memorable during my Ph.D. These individuals gave me the priceless gift of knowing that academia is where I belong. I would also like to thank Dr. Anderson for his friendship over the years and for bringing me into the world of cycling, which has become an integral part of my life. He will be greatly missed.

The highest and lowest points during the past years of my doctoral career have all been shared with my many laboratory members and friends, as only they could understand the happiness and pain that accompany this process. Dr. Joslyn Ahlgren, Dr. Carie Reynolds, Dr. Karen Porter, Dr. Pei-Ying Sarah Chan, Dr. Teresa Pitts, Mark Hotchkiss and soon-to-be-doctor Vipa Bernhardt have all made this such a worthwhile journey. Every success was celebrated with these amazing individuals, no matter how insignificant. They have always been available to offer advice and keep me centered during so many times when I felt off balance.

My family has also been an integral part of my success. Their love and support have continually been the rock in my life, but especially during the completion of my Ph.D. I thank my parents and brothers for listening whenever I wanted to share my excitement or frustration, even when they may not have wanted to hear it again, and again. I cannot express what it means to have had such unconditional support. Additionally, I want to express gratitude to my brother, Matthew, who has had the unfortunate luck of living with me during the last, stressful semester of my Ph.D., and who has remained a peaceful force in my life during these difficult times.

Finally, I would like to acknowledge Dr. David Julian, who piqued my interest in physiology as an undergraduate and opened my eyes to the possibility of pursuing a Ph.D. Without his guidance I would not have discovered the path I am on today.

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RESPIRATORY LOAD COMPENSATION RESPONSES IN CONSCIOUS ANIMALS

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August 2010

Chair: Paul W. Davenport
Major: Veterinary Medical Sciences

The respiratory load compensation reflex is well-characterized in anesthetized animals, but the load compensation response pattern to mechanical respiratory stimuli in conscious animals is variable and appears to be influenced by behavior. This is relevant to our understanding of respiratory obstructive diseases, which evoke load compensation responses in individuals on a regular basis. These studies were undertaken to determine the behavioral response pattern to repeated, transient, tracheal occlusions in conscious rats, understand how stress may contribute to the pattern, and elucidate neural structures underlying these responses.

The first study was designed to test the hypothesis that intrinsic, transient, tracheal occlusions (ITTO) would elicit reflexive respiratory and neural load compensation, without changing blood gases, lung compliance, or tracheal integrity. It was known that resistive loads and airway occlusions applied to either inspiration or expiration separately evoked compensation responses including changes in breath volume-timing and increases in respiratory muscle activation. It was also known that previous models used to study load compensation responses to mechanical respiratory stimuli changed blood gases or lung compliance. ITTO was sustained for 2-3 breaths and elicited

reflexive respiratory load compensation responses including a prolongation of inspiratory duration (Ti), expiratory duration (Te), an increase in diaphragm activity (EMG_{dia}), and decrease in esophageal pressure (P_{es}), which is an indication of changes in pleural pressure. Additionally, neural activation in response to repeated ITTO, determined by c-Fos staining, was found in respiratory brainstem and suprapontine brain nuclei, suggesting that there is a neural compensation response to tracheal occlusions, including both sensory and motor components.

The second study investigated the hypothesis that the respiratory load compensation response pattern in conscious animals is variable and changes as a result of conditioning. The variability, also observed in humans, has been attributed to between-individual differences in perception. Previous studies applying mechanical loads to breathing acutely in conscious humans and animals showed inconsistent changes in breath-timing but somewhat consistent respiratory motor responses to loads. The initial respiratory load compensation response pattern to ITTO in conscious rats consisted of a prolongation of Te and increase in EMG_{dia} amplitude during occlusions. The change in Te did not habituate. The augmentation of EMG_{dia} during occlusions was greater after ITTO conditioning, suggesting a sensitized behavioral response. These results suggest that the volume-timing reflex may be suppressed by behavioral mechanisms in conscious animals, which includes breath-holding and increases in respiratory muscle activation.

The third study investigated the hypothesis that 10 days of ITTO causes stress and anxiety responses in conscious animals. We had evidence from human studies conducted by our laboratory that mechanical loads to breathing were stressful stimuli,

and it is known that repeated stressful stimuli cause changes in an animal's basal and acute stress responses. We were also aware that individuals with respiratory obstructive diseases often have affective disorders such as anxiety and depression. After 10 days of ITTO rats had increased basal corticosterone levels, greater adrenal weights, and elevated anxiety levels, determined by the Elevated Plus Maze, compared to animals not receiving tracheal occlusions. Thus, healthy animals develop stress and anxiety responses to repeated, severe airway loading.

The final study investigated the hypothesis that the behavioral, stress, and anxiety responses to 10 days of ITTO were mediated by plasticity within respiratory brainstem, stress-related, and suprapontine discriminative and affective neural regions. It was known that areas within the central nervous system were involved in the acute sensory and motor responses to respiratory loading, but how activity in these regions may be modified as a result of repeated loading was unknown. We conditioned animals to 10 days of ITTO or 10 days of handling, and removed brain tissue the following day. The tissue was stained for cytochrome oxidase (CO), which is used as an indicator of changes in neural activity. Significant increases in activity were found in the rostral nucleus of the solitary tract (nTS), caudal periaqueductal gray (PAG), dorsal raphe (DR), ventroposteromedial thalamus (VPM), and anterior insular cortex (AI), supporting our hypothesis and suggesting that respiratory load conditioning causes state changes in the brain that may lead to modulation of subsequent responses to respiratory loads.

The results of these studies collectively indicate that ITTO elicits behavioral related neural load compensation responses in conscious animals, and that the pattern of these responses is altered through ITTO conditioning. Repeated ITTO leads to stress and

anxiety, supporting the link between respiratory obstructive diseases, stress, and affective disorders.

CHAPTER 1
RESPIRATORY LOAD COMPENSATION AND THE NEURAL CONTROL OF BREATHING

Respiratory Load Compensation

Respiration is a complex physiological process that is carried out continually for the lifetime of an animal. The system is able to respond to voluntary input and a variety of mechanical and metabolic stimuli in a way that allows the animal to continue ventilation through changes in breathing pattern. Early respiratory studies focused on defining normal respiratory patterns and control mechanisms and were primarily conducted in anesthetized animals where reflexes and metabolic needs drive the system. It wasn't until experiments were regularly carried out in conscious humans and animals that researchers became aware that consciousness plays a major role in modulating breathing. Voluntary control of breathing is capable of overriding the expected responses to metabolic needs. It has even been determined that adaptive responses to stimuli, if experienced repeatedly, can develop into regular breathing pattern changes through conditioning and learning. Unfortunately, this also means that if the respiratory system responds to a repeated stimulus in a maladaptive manner, aberrant ventilatory patterns may arise. In conscious animals, the voluntary and conscious behavioral changes to breathing are driven by respiratory afferents that cause suprapontine activation, which is suppressed in anesthetized animals. Cortical activation leads to respiratory sensations and emotional responses to those sensations, and it is this combination that ultimately controls breathing in conscious humans and animals.

Anesthetized Responses

The two components of the respiratory pattern that contribute to ventilation are breath frequency and tidal volume. Clark and von Euler (Clark & von Euler, 1972) showed that inspiratory time (Ti) is dependent upon inspiratory volume (Vi) in anesthetized cats, but that this relationship can change depending upon the type of anesthesia used. They reported a weak volume-timing (Vt-T) modulation of breathing pattern in conscious humans. With the respiratory depressant pentobarbitone, the Vt-T relationship is maintained for eupneic volumes and those well above eupneic values. When using urethane, however, animals maintained a constant Ti for changing values of Vi in the eupneic range, and Ti only became volume-dependent for large volumes, mimicking the pattern observed in the conscious man. The subsequent expiratory duration (Te) was thought to be dependent upon the preceding Ti. These volume-timing relationships were abolished after vagotomy in anesthetized animals, indicating that respiratory Vt-T requires intact vagal afferents.

Zechman and colleagues (Zechman *et al.*, 1976) investigated the role of breath phase in the Vt-T relationship during eupneic volumes. Extrinsic mechanical loads to breathing allow for studying respiratory reflex control in response to mechanical stimuli. Resistive (R) and elastic (E) loads are flow- and volume- dependent, respectively (Zechman & Davenport, 1978). Zechman and colleagues (Zechman *et al.*, 1976) hypothesized that the dependence of Te upon Ti and the constant Ti seen during eupneic volumes would be altered if a R load was applied separately to either the inspiratory or expiratory breath phase. The response they observed is called the respiratory load compensation reflex. Applying a R load to one phase of a single breath causes decreased volume, increased duration, and a return to baseline of volume and

timing parameters for subsequent unloaded breaths in animals (Zechman *et al.*, 1976) and human infants (Kosch *et al.*, 1986). These responses were vagal-dependent. Koehler and Bishop (Koehler & Bishop, 1979) confirmed this volume-timing relationship during the expiratory phase of breathing. For inspiration and expiration, there is a volume threshold at which the breath phase is terminated that decreases with time. During a complete occlusion, known as an infinite R load or functional vagotomy, a change in lung volume is prevented, causing the system to rely upon its inherent brainstem timing pattern to end one phase of the breath and begin another. The increase in breath duration associated with occlusion approaches the values obtained after vagotomy which are prolonged relative to intact animals and unaffected by volume changes (Zechman *et al.*, 1976).

It was hypothesized that slowly adapting pulmonary stretch receptors (PSR) are the vagal afferents mediating the volume-dependent reflex control of breath phase duration (Koehler & Bishop, 1979). Results from Davenport et al. (Davenport *et al.*, 1981a; Davenport *et al.*, 1984) supported that PSR's were the vagal afferent involved in mediating the Vt-T response to respiratory loading. Further investigation of PSR-mediated responses led to the understanding that PSR input modulates Ti through changes in action potential (spike) frequency and Te through changes in spike number (Davenport *et al.*, 1981a; Davenport & Wozniak, 1986), suggesting separate brainstem regulatory mechanisms for Ti and Te. Additionally, Davenport and colleagues (Davenport *et al.*, 1981c) showed that PSR's respond to airway muscle tension, and it is the change in tension associated with differing lung volumes that activates PSR's, not the actual lung volume itself (Davenport *et al.*, 1981b). Thus, the respiratory Vt-T

relationship can also be thought of as an airway transluminal pressure-timing relationship, with the pressure referring to transmural pressure across the airways during breathing. The transluminal pressure is further modulated by the smooth muscle tone of the airways (Davenport *et al.*, 1981b). In addition to volume-timing changes, load compensation is also characterized by increased respiratory motor output measured using electromyography (EMG), primarily in the diaphragm (EMG_{dia}) during inspiratory loading (Lopata *et al.*, 1983) and also in the abdominal muscles during expiratory loading (Koehler & Bishop, 1979).

PSR's are distributed throughout the smooth muscle of airways and respond to airway pressure differently depending upon their location. Intra-thoracic PSR's in the smooth muscle of bronchi and bronchioles transduce information to the central nervous system (CNS) in phase with lung volume, whereas extra-thoracic receptors in the smooth muscle of the trachealis muscle transduce information in phase with airflow (Sant'Ambrogio & Mortola, 1977; Davenport *et al.*, 1981b; Sant'Ambrogio, 1982). Upper airway afferents have been shown to contribute to the load compensation response during inspiratory (Webb *et al.*, 1994) but not expiratory (Webb *et al.*, 1996) resistive loading in anesthetized animals. PSRs in the upper airways send their afferent fibers to the brainstem via the cervical vagus, superior laryngeal (SLN), and recurrent laryngeal nerves (RLN) (Sant'Ambrogio *et al.*, 1977; Lee *et al.*, 1992) depending upon their location. These afferents differentially terminate on separate areas of the nucleus of the solitary tract (nTS) (Kalia & Mesulam, 1980b, a; Kalia, 1981b). The nTS is part of the dorsal respiratory group (DRG), a medullary area involved in inspiratory activity. Preventing PSR input from reaching the brainstem by cutting the vagi abolishes the

modulation of Vt-T during loading and hyperinflation even in neonatal animals (Webb *et al.*, 1994, 1996). Afferents from PSR's in the trachea have been found to influence cardiovascular activity (Traxel *et al.*, 1976; Barthelemy *et al.*, 1996) and respiratory reflexes (Traxel *et al.*, 1976; Remmers & Bartlett, 1977; Citterio *et al.*, 1985). Thus, both intra- and extra-thoracic vagal PSR's are necessary for the reflexive load compensation pattern observed in anesthetized animals.

Conscious Responses

Studies in conscious humans and animals are less numerous. Results from experiments in anesthetized animals provide information about reflexive and neural load compensation, but do not add to our understanding of behavioral load compensation, or voluntary modulation of the reflexive patterns. In a study in conscious neonatal lambs exposed to a single expiratory R load, researchers observed decreased airflow and prolonged Te which resulted in elevated end-expiratory volume (Watts *et al.*, 1997). Subsequent unloaded breaths showed decreased Vi, increased expiratory volume (Ve), and the integrated EMG activity in the larynx and diaphragm remained active as a compensatory mechanism to help restore lung volume to baseline (Watts *et al.*, 1997). In conscious goats presented with two consecutive inspiratory loads, the integrated diaphragm and external abdominal oblique muscle responses were augmented and Ti increased during the first breath (Hutt *et al.*, 1991). These values remained the same for the second loaded breath but returned to baseline during unloaded breaths, unlike the responses seen during expiratory R loading in lambs (Watts *et al.*, 1997). This difference could be due to the fact that the magnitudes of the R loads were different (370 $cmH_2O/L/sec$ in lambs vs. 18 $cmH_2O/L/sec$ in goats), animal ages were different (neonates vs. adults), or perhaps that the inspiratory and expiratory phases of breathing

are under separate control mechanisms (Davenport *et al.*, 1981a; Davenport & Wozniak, 1986; Webb *et al.*, 1994, 1996).

When multiple consecutive inspirations are loaded in conscious ponies, Ti is prolonged while Te and Vt are decreased during the first breath (Forster *et al.*, 1994). During the second through fifth loaded breaths, small changes were seen in volume and timing parameters which stabilized after the fifth breath for the subsequent 2-4 minutes of loading. The time of EMG_{dia} activation mirrored Ti, and mean EMG_{dia} activity was elevated during the first loaded breath to a value that remained augmented for subsequent loaded breaths. During the first recovery breath following loading, Vt and mean EMG_{dia} activity increased above values during loading but timing parameters did not change. All parameters progressively returned to control values over the course of subsequent unloaded breaths. Surprisingly, the loaded and recovery breath compensation effect persisted even after pulmonary and diaphragmatic deafferentation, indicating that these afferents quantitatively but not qualitatively modulate the load compensation response (Forster *et al.*, 1994). These researchers hypothesized that intercostal afferents and cortical input likely contributed greatly to the observed response.

In the conscious man, some studies have shown that R loading decreases volume, prolongs breath duration, and enhances motor output (Axen *et al.*, 1983; Daubenspeck & Bennett, 1983; Hudgel *et al.*, 1987; Nishino & Kochi, 1994; Daubenspeck & Rhodes, 1995). In addition, an augmented recovery response to loading has been documented (Altose *et al.*, 1979), as have greater respiratory responses to loads of larger magnitudes (Altose *et al.*, 1979; Nishino & Kochi, 1994;

Calabrese *et al.*, 1998). However, there seems to be great variability in response patterns to respiratory loading and occlusion in conscious humans (Axen *et al.*, 1983; Daubenspeck & Bennett, 1983; Davenport *et al.*, 1986; Hudgel *et al.*, 1987; Nishino & Kochi, 1994; Daubenspeck & Rhodes, 1995). Others have documented increases in volume and decreases in frequency in response to inspiratory R loading (Iber *et al.*, 1982). Interestingly, load compensation responses that differed between individuals remained qualitatively similar within the same individual in response to various load magnitudes (Daubenspeck & Rhodes, 1995). Differing responses may be due to perceptual differences between individuals (Daubenspeck & Rhodes, 1995). Respiratory afferents other than those in the airways and in the main inspiratory muscles must play a role in load compensation in conscious humans and animals. These other afferents most likely transduce respiratory sensations associated with loading and airway occlusion. The way a conscious human or animal feels (affective component) about its breathing may further lead to modulation of the reflexive pattern of load compensation.

Respiratory Brainstem Network

An intact brainstem respiratory neural network includes multiple nuclei in the dorsal, ventral, and ventrolateral medulla and dorsolateral pons. These nuclei are essential for normal breathing rhythm and contribute to respiratory reflexes, including reflexive load compensation mediated by vagal afferents (Ezure, 1990; Bianchi *et al.*, 1995; St-John, 1998). Respiratory vagal afferents primarily terminate within the caudal nTS but can also directly influence pontine respiratory centers to evoke motor responses (Kalia & Mesulam, 1980b, a). Pontine respiratory centers include the parabrachial (PBN) and Kolliker-Fuse (KF) nuclei, both of which have reciprocal

connections with the nTS (Herbert *et al.*, 1990). The lateral PBN (lPBN) receives projections from the nTS and is involved in sympathetic drive (Hayward, 2007). The extensiveness of the respiratory neural network highlights the important ability of the respiratory system to respond and adapt to a variety of different stimuli.

Dorsal periaqueductal gray (dPAG) stimulation leads to c-Fos expression in lPBN subnuclei (Hayward & Castellanos, 2003). The PAG plays a major role in the descending pain pathway (Cunha *et al.*, 2010), and is a mediator of defensive behaviors such as freezing, escape, and fear-potentiated startle (Brandao *et al.*, 1994; Cunha *et al.*, 2010). Electrical stimulation of the dPAG elicits a progression of behaviors characteristic of an animal exposed to a threat, and these behaviors are accompanied by increases in mean arterial pressure, heart rate, and respiration (Hilton & Redfern, 1986; Brandao *et al.*, 1990). Hayward and colleagues (Hayward *et al.*, 2003) disinhibited the dPAG and elicited changes in ventilation, which they further determined was mediated by the lPBN in part by glutamate receptor activation (Hayward *et al.*, 2004). The observed increase in respiratory frequency was a result of a decrease in both inspiratory and expiratory durations. Zhang and colleagues (Zhang *et al.*, 2007) observed the same increase in respiratory frequency with dPAG stimulation, along with an increase in EMG_{dia} activity and cardiovascular responses. Stimulation of the caudal PAG evoked greater respiratory responses than in the rostral PAG, but there was no difference in cardiovascular responses depending upon region (Zhang *et al.*, 2007). This same group found that chemical activation of the dPAG potentiated the load compensation reflex with an increase in breath phase duration and diaphragm activity associated with inspiratory and expiratory occlusions (Zhang *et al.*, 2009).

Vianna and colleagues (Vianna *et al.*, 2001) showed a progression of behaviors from alertness and freezing to escape with increased electrical stimulation of the PAG. The dorsal and ventral regions of the PAG differentially regulate these responses, such that electrical stimulation of the dorsolateral PAG causes unconditioned freezing in rats and the ventral PAG (vPAG) mediates conditioned responses (Vianna *et al.*, 2001). The amygdala shares a critical link with the PAG in the fear circuitry of the brain and is also involved in both types of aversive responses (Brandao *et al.*, 1994; Davis *et al.*, 1994; LeDoux, 2000; Martinez *et al.*, 2006). The central nucleus of the amygdala (CeA) is the site of many convergent inputs and is an important part of fear pathways (LeDoux *et al.*, 1988; Phillips & LeDoux, 1992; Davis *et al.*, 1993; Davis *et al.*, 1994; Campeau & Davis, 1995; Martinez *et al.*, 2006). The basolateral and lateral amygdala act as the regulators of information received from aversive stimuli reaching the CeA, the motor output subnucleus of the amygdala (Campeau & Davis, 1995; Saha, 2005). Our laboratory has shown (Shahan *et al.*, 2008) that electrical stimulation of the CeA leads to an increase in respiratory rate in anesthetized rats, likely through output to the PAG. The locus coeruleus (LC), an area involved in the body's noradrenergic response to stressful stimuli (Bremner *et al.*, 1996; Van Bockstaele *et al.*, 2001), receives afferent projections from the CeA, the nTS, and the PAG (Van Bockstaele *et al.*, 2001). It is evident that respiration is intricately linked with a number of different neural regions, many of which are involved in defensive, stress, and fear responses.

Respiratory-Related Neural Activation Techniques

There are a number of different methods available for determining neural activation responses to stimuli. The primary non-invasive techniques for determining brain activity in response to respiratory stimuli include functional magnetic resonance

imaging (fMRI) (Gozal *et al.*, 1995; von Leupoldt & Dahme, 2005a) and cortical evoked potentials (CEP) (Davenport *et al.*, 1985; Davenport *et al.*, 1986; Davenport *et al.*, 1993; Davenport & Hutchison, 2002; Davenport *et al.*, 2006). Inspiratory occlusions lead to activation in the somatosensory cortex in normal humans (Davenport *et al.*, 1986), double lung transplant patients (Davenport *et al.*, 2006) and conscious lambs (Davenport & Hutchison, 2002). Intercostal (Davenport *et al.*, 1993) and phrenic (Davenport *et al.*, 1985) nerve stimulation also leads to increased activity in the sensorimotor cortex.

Although these studies have added to our understanding of how the brain processes respiratory stimuli, they do not provide information about activity at the individual neuronal level within discrete brain nuclei. Analysis of neural tissue is one way to obtain more specific information about central nervous system activity patterns in response to a stimulus, but it requires removal of brain tissue after experimentation and can therefore only be done in animals. One method that achieves this end is c-Fos immunohistochemistry. c-Fos is a protein expressed in neurons recently activated and is used as a marker to determine areas of the brain that have an excitatory response to specific stimuli (Dragunow & Faull, 1989); however, it cannot differentiate between sensory or motor activation and indicates little about neural inhibition. c-Fos expression has been noted in the PAG in response to laryngeal afferent stimulation (Ambalavanar *et al.*, 1999), and throughout the brain and spinal cord in response to phrenic nerve stimulation (Malakhova & Davenport, 2001). Another histochemical method is staining neural tissue for cytochrome oxidase, (CO) an enzyme involved in oxidative metabolism in the electron transport chain. CO is used to indicate changes in brain steady state

activity levels in response to stimuli (Wong-Riley, 1979; Wong-Riley, 1989; Gonzalez-Lima & Garrosa, 1991; Hevner *et al.*, 1995), and is therefore best used in studies of prolonged rather than acute duration. CO can reveal adaptation within brain nuclei, and neural adaptation has been documented in response to inspiratory resistive loading in humans (Gozal *et al.*, 1995). Thus, c-Fos immunohistochemistry can be used to determine neural areas mediating an animal's acute response to a respiratory stimulus, and CO staining can elucidate neural modulation resulting from repeated respiratory stimuli.

Respiratory Discriminatory Sensory Processing and Detection

During normal respiration a barrage of sensory information is transmitted by respiratory afferents about each breath, but rarely do conscious individuals become aware of these sensations. When presented with a R load to breathing, humans and animals become aware of their respiration *(*Zechman & Davenport, 1978; Davenport *et al*., 1991). The qualities of breathing are relayed by various respiratory afferents such as the phrenic (Davenport *et al*., 1985; Zechman *et al*., 1985) and intercostal nerves (Davenport *et al*., 1993), which ultimately reach the somatosensory cortex, the neural substrate for discriminative sensations. When respiratory afferents activate this cortical area in humans and animals, the resulting sensations may cause behavioral changes in order to enhance or diminish those sensations depending upon their quality (affective sensations). The activation of the cortex produces CEP's, which have been elicited in humans and animals in response to respiratory stimuli (Davenport & Hutchison, 2002), classifying them as respiratory-related evoked potentials (RREP). RREP's are similar in all species, suggesting respiratory information is processed similarly in humans and animals.

Afferent information related to respiration may not reach the cortex if it does not exceed a certain threshold (Chou & Davenport, 2007), is temporally too close to a prior signal (Chan & Davenport, 2008), or if attention is manipulated (Chan & Davenport, 2009). Additionally, the time required for detecting a load to breathing decreases as the load magnitude increases (Zechman & Davenport, 1978; Zechman *et al*., 1985), and is also dependent upon background respiratory resistance (Zechman *et al*., 1985). Detection and magnitude estimation of respiratory loads in the conscious human do not require vagal feedback (Guz *et al*., 1966; Zhao *et al*., 2002a, 2003), nor is cortical activation during a respiratory load dependent upon the vagi (Zhao *et al*., 2002b), supporting the hypothesis that other respiratory afferents contribute to respiratory sensations and may contribute to the behavioral load compensation response in conscious animals. Similarly, anesthetizing the glossopharyngeal nerve (Guz *et al*., 1966) or the upper airway (Chaudhary & Burki, 1978, 1980; Fitzpatrick *et al*., 1995) does not alter load detection thresholds, and upper airway and mouth afferents are not required for cortical activation during inspiratory occlusions (Davenport *et al*., 2006). These studies collectively suggest that although lung, upper airway and mouth afferents may contribute to load compensation in animals, they are not vital for evoking RREP's, detecting loads, or estimating the magnitude of loads. Sensory feedback from respiratory muscles must play a role in these processes *(Killian et al., 1980; Killian et al*., 1982; Davenport & Hutchison, 2002), and ultimately influence the load compensation response in conscious animals.

Respiratory Affective Sensory Processing

Breathing through increased airway resistance causes sensations of discomfort while breathing, a subjective experience called dyspnea (O'Donnell *et al.*, 2007).

Dyspnea is a primary symptom in respiratory obstructive diseases like chronic obstructive pulmonary disease (COPD) and asthma (O'Donnell *et al.*, 2007; Bernstein, 2008). Resistive loads are commonly used to experimentally induce and study dyspnea. Dyspnea includes both a discriminative and affective component (von Leupoldt & Dahme, 2005b). The discriminative component is relayed to the somatosensory brain network, which is processed in a similar fashion in both humans and animals (Davenport *et al.*, 1985; Davenport *et al.*, 1991; Davenport *et al.*, 1993; Davenport & Hutchison, 2002). The affective component is relayed to parts of the limbic neural network and shares similarities with the affective component of pain, mostly via processing in the insular and anterior cingulate cortices (Aleksandrov *et al.*, 2000; von Leupoldt & Dahme, 2005a; Schon *et al.*, 2008). Individuals diagnosed with COPD have dyspnea and are often concomitantly diagnosed with affective disorders such as anxiety and depression (Karajgi *et al.*, 1990; Brenes, 2003; Wagena *et al.*, 2005). Patients diagnosed with respiratory obstructive diseases lead sedentary lifestyles (ZuWallack, 2007; Bourbeau, 2009) and sedentary behavior can be a symptom of depression (Roshanaei-Moghaddam *et al.*, 2009). Because dyspnea is such an aversive sensation, individuals modify their behavior in order to adapt or avoid experiencing the sensation. It is therefore plausible that repeated respiratory obstructions cause intense sensations of discomfort, leading to an experience of negative affect. Over time, this negative affect may result in disorders such as anxiety or depression. Until the link between respiration, sensations, behavior and mental state is determined we will not fully understand the multitude of respiratory disease phenotypes, disease progression, or proper treatment methods.

Experimental Approach

It has been demonstrated that:

Respiratory load compensation responses are seen in anesthetized and conscious humans and animals. Although the pattern in conscious humans and animals is less consistently defined, increased respiratory muscle recruitment is always apparent.

Load compensation relies upon afferent and efferent projections relayed throughout the central nervous system that have many connections to pathways involved in stress and defensive behaviors.

Resistive loading and airway occlusion cause dyspnea, which has discriminative and affective components, and respiratory obstructive diseases are associated with affective disorders such as anxiety and depression.

Based on these previous studies, this dissertation investigated the following hypotheses:

Hypothesis 1: Intrinsic, transient tracheal occlusions (ITTO) will evoke respiratory and neural load compensation responses in anesthetized rats without changing lung compliance or tracheal integrity.

Hypothesis 2: ITTO in conscious rats will elicit variable respiratory pattern responses that change after 10 days of ITTO conditioning.

Hypothesis 3: Ten days of ITTO in conscious rats will lead to stress and anxiety responses.

Hypothesis 4: Respiratory brainstem nuclei, areas involved in stress responses, and suprapontine regions involved in discriminative and affective respiratory information processing will show altered activity in response to 10 days of ITTO conditioning.

The overall goal of this dissertation is to determine the pattern of the respiratory load compensation response to tracheal occlusions in conscious rats. Urethane-anesthetized and conscious, vagal-intact, adult, male Sprague-Dawley rats were used. The behavioral, stress, and neural components of load compensation were investigated. These results provide a new understanding of the conscious modulation of respiratory load compensation reflexes.

CHAPTER 2
RESPIRATORY LOAD COMPENSATION AND NEURAL ACTIVATION IN ANESTHETIZED RATS

Introduction

Respiratory load compensation in anesthetized animals has been characterized as reflexive and vagal-dependent (Zechman *et al.*, 1976), specifically mediated by pulmonary stretch receptors (Davenport *et al.*, 1981a; Davenport *et al.*, 1984; Davenport & Wozniak, 1986). Load compensation has been observed in response to mechanical challenges to breathing such as resistive or elastic loads applied to the respiratory circuit (Zechman & Davenport, 1978). The stereotypical response to a resistive load applied to one phase of the breath included a decrease in volume inspired (Vi) or expired (Ve) for the duration of the load and an increase in the loaded breath phase duration (Zechman *et al.*, 1976). The volume-timing (Vt-T) parameters of the unloaded phase of the breath were unchanged. The Vt-T values obtained from complete respiratory occlusion approached those seen after vagotomy, including long loaded phase duration (Zechman *et al.*, 1976). Load compensation is also characterized by increased respiratory motor output, measured from respiratory muscle electromyography (EMG) primarily in the diaphragm (Lopata *et al.*, 1983), although abdominal muscle responses are also observed (Koehler & Bishop, 1979).

Load compensation has brainstem and suprapontine neural components. The primary non-invasive techniques for imaging brain activity in response to respiratory stimuli includes functional magnetic resonance imaging (fMRI) (Gozal *et al.*, 1995; von Leupoldt & Dahme, 2005a) and cortical evoked potentials (CEP) (Davenport *et al.*, 1985; Davenport *et al.*, 1993; Davenport & Hutchison, 2002; Davenport *et al.*, 2006), but these methods can be too general and do not provide information about activity at the

individual neuronal level. Analysis of neural tissue is one way to obtain more specific information about activity patterns in response to a stimulus, but it requires removal of brain tissue after experimentation and can therefore only be done in animals. One method that achieves this end is c-Fos immunohistochemistry. c-Fos is a protein expressed in neurons recently activated and is used as a metabolic marker to determine areas of the brain that respond to specific stimuli (Dragunow & Faull, 1989). c-Fos expression has been noted in the periaqueductal gray (PAG) in response to laryngeal afferent stimulation (Ambalavanar *et al.*, 1999), and throughout the brain and spinal cord in response to phrenic nerve stimulation (Malakhova & Davenport, 2001).

Determining mechanistically how increased resistances during breathing affect the respiratory pattern, motor output, and neural response is vital to understanding aspects of respiratory obstructive diseases, which are characterized by elevated airway resistance. These diseases include chronic obstructive pulmonary disease (COPD), asthma, and obstructive sleep apnea (OSA). Researchers have utilized two primary methods for increasing airway resistance. Bronchoconstriction, such as methacholine challenge, is used to elicit intrinsic, non-specific, sustained mechanical loading. Bronchoconstriction can also reduce lung compliance, induce lung inflammation and change blood gases (Wiester *et al.*, 1990), preventing the specific mechanical effects of loading from being evaluated. Alternatively, R loads can be applied extrinsically at the mouth or via a tracheal stoma, enabling load duration and intensity to be controlled. However, extrinsic loading does not model the increase in airway resistance associated with intrinsic obstructions occurring in individuals with COPD, asthma, or OSA.

Our laboratory has developed a model of tracheal occlusions in anesthetized rats that produces increases in airway resistance without changing lung compliance. These intrinsic, transient tracheal obstructions (ITTO) elicit load compensation Vt-T and diaphragm activity changes. The neural substrates involved in mediating the load compensation response were investigated using immunohistochemistry. It was hypothesized that ITTO in the rat would elicit a load compensation respiratory motor pattern response and neuronal activation, and the neurons activated by the mechanical load will express c-Fos within brainstem and suprapontine brain nuclei.

Methods

Animals

These experiments were performed on male Sprague-Dawley rats (360.5 ± 77.4 g). The animals were housed two to a cage in the University of Florida animal care facility where they were exposed to a 12 h light/12 h dark cycle. The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the University of Florida.

Surgical Procedures

All animals were anesthetized with urethane (1.3g/kg, ip) and anesthesia was supplemented as needed (20 mg/ml). Anesthetic depth was verified by the absence of a withdrawal reflex to a rear paw pinch. Body temperature was measured with a rectal probe and maintained at 38°C using a heating pad. Animals were spontaneously breathing room air.

The right femoral artery was cannulated using a saline-filled catheter (Micro-Renathane, 0.033 outer diameter, Braintree Scientific, Inc.) connected to a calibrated differential pressure transducer (Konigsberg). A saline-filled tube (PE90) was passed

through the mouth into the esophagus and connected to a second calibrated pressure transducer to measure esophageal pressure (P_{es}). Both pressure signals were amplified (Stoelting; Wood Dale, IL), digitized at 1kHz [Cambridge Electronics Designs (CED) 1401 computer interface; Cambridge, UK], computer processed (Spike2, Cambridge Electronics Design) and stored for subsequent analysis. Pleural pressure changes were inferred from relative changes in P_{es}.

Diaphragm EMG (EMG_{dia}) was recorded with Teflon-coated wire electrodes, threaded through 25 gauge needles. The distal end of the wires were bared and bent to form a hook. With the animal in a supine position, the right ribcage was lifted and the needle was inserted in a rostral and dorsal direction through an incision in the skin. The needle was retracted and the hooked tip of the electrode remained in the costal portion of the right side of the diaphragm. Two electrodes were inserted for bipolar EMG_{dia} recording. The external ends were bared and connected to a high-impedance probe. The signal was amplified (P511, Grass Instruments) and band-pass filtered (0.3-300 Hz). Analog output was digitized and processed as described above.

The trachea was exposed by a ventral incision. The trachea was freed from surrounding tissue and an inflatable cuff (Fine Science Tools) was sutured around the trachea, two cartilage rings caudal to the larynx. The actuator tube of the cuff was connected to an air-filled syringe. The extent to which the bladder of the cuff was inflated was dependent upon the amount of pressure applied with the syringe. When fully inflated, the trachea was compressed to completely occlude the airway. Removal of the pressure resulted in full recovery of the trachea with no interference to breathing. Tracheal integrity was anatomically confirmed at the end of the experiment.

There were 4 groups of animals. The experimental group (n=11) was prepared and received tracheal occlusions as described above. Control group 1 (n=4) were prepared as described above with a tracheostomy 0.5 cm below the tracheal cuff. A five cm endotracheal tube (PE240) was inserted into the tracheostomy with the tube length approximately equal to the distance between the oral cavity and incision to maintain dead space volume. These animals received tracheal compression but breathing was not obstructed. Control group 2 (n=4) were prepared as described above but did not receive a tracheostomy or tracheal obstructions. Control group 3 (n = 6) consisted of animals that were anesthetized with urethane and left undisturbed for 90 min before being sacrificed and perfused.

Protocol

All animals were surgically prepared and maintained under anesthesia for 90 minutes. The experimental group and control group 1 animals were presented a 10 min experimental occlusion trial. The tracheal cuff was inflated at end-expiration to completely obstruct the airway. The occlusion was applied for 2-3 breaths and then removed for 5-10 unobstructed breaths (confirmed by P_{es}). This was repeated for the duration of the 10 min experimental trial. Animals in control groups 2 and 3 were maintained without obstructions during the 10 min period.

Following completion of the experimental trial, the animal was maintained under anesthesia for 90 minutes. Following this 90 min period the animal was sacrificed with an overdose of anesthetic, transcardially perfused with 0.5 ml heparin into the left ventricle, followed by 200 ml perfusion saline and 200 ml 4% paraformaldehyde in 0.4 M phosphate buffered saline (PBS). The brain was removed and placed in 4% paraformaldehyde for 24 h. The brain was then transferred to a solution of 30% sucrose

in PBS. The fixed tissue was frozen, sectioned with a cryostat (Carl Zeiss, HM101) into coronal slices 40 μm thick, and placed in PBS for storage.

Immunohistochemical Analysis

For each brain, every third section of tissue was used for processing. Slices were incubated for 1 h in a 1:30 solution of goat serum in PBS + Triton X-100 (GS-PBS-T). The tissue then sat overnight in a solution of rabbit anti-c-Fos primary antibody (1:2000, sc-52r; Santa Cruz Biotechnology). The following day the tissue was washed for 1 h in a solution of 1% GS-PBS-T, and then incubated for 2 h in a solution of goat anti-rabbit biotin (1:500, Jackson ImmunoResearch). Following another 1 h wash in 1% GS-PBS-T, all slices were treated with avidin-biotin peroxidase complex (ABC Vectastain Kit; Vector; Burlingame, CA), and washed again (1% GS-PBS-T for 1 h). To complete the staining process, a chromagen solution (0.05% diaminobenzidine hydrochloride, 2.5% ammonium sulfate, 0.033% hydrogen peroxide in 0.05 M Tris·HCl (DAB); Sigma) was applied for roughly 3 min until the tissue changed to a darker brown color, indicative of Fos-like immunoreactivity (FLI). The reaction was halted with distilled water, and then all tissue was washed 3 times in PBS. Following staining, slices of tissue were mounted on glass slides and were allowed to air-dry for 3 days. All slides were dehydrated with a series of washes in alcohol and CitraSolv, then coverslipped (Vectamount).

Data Analysis

Breathing pattern

Breathing pattern analysis was performed with Spike 2 software on six experimental animals and four control group 1 animals. Raw EMG_{dia} signal recordings were rectified and integrated (50 ms time constant). The amplitude of the integrated EMG_{dia} signal was measured from baseline to peak amplitude (relative units).

Inspiratory time (Ti) was the duration of EMG_{dia} burst; expiratory time (Te) was the duration between EMG_{dia} bursts; total breath time (Ttot) was the sum of Ti and Te. P_{es} was measured from end expiration to peak (Figure 2-1). These parameters were analyzed for the complete unobstructed control breath (C) prior to occlusion, the occluded breaths (O1-O3), and the complete unobstructed recovery breath (R) immediately following occlusion (Figure 2-1). Ti, Te, and Ttot were averaged for each rat and for each group. P_{es} and EMG_{dia} amplitude were normalized by dividing O1-O3 and R by the control breath, C. The normalized P_{es} and EMG_{dia} values were compared for each rat and for each group.

c-Fos

Images of brain sections to be analyzed were viewed using light microscopy (Zeiss Axioskope) and captured with imaging software (AIS). Masks delineating areas to be counted were drawn on the images using Adobe Photoshop according to brain regions (Paxinos & Watson, 1997), and the masked images were saved. Neurons expressing c-Fos were identified by the round, dark staining of their nuclei (Figure 2-7). Size and shape limits were set to exclude spots that were too lightly stained, or were outside the diameter range of 7-10μm. The number of c-Fos-labeled neurons in the area of interest was counted (MetaMorph). For each brain, 3 sections per nucleus were counted bilaterally. If no bilateral differences were seen, counts for that nucleus were averaged. These values were then combined according to group, and used for between-group comparisons (Table 2-1).

Statistics

All breathing parameters for C, O1, O2, O3, and R were averaged within each rat. These values were used to conduct a two-way ANOVA for each parameter with

breath number and group as factors. Differences were statistically significant if $p<0.05$. A one-way ANOVA was performed comparing c-Fos expression in all 4 groups for each nucleus. If statistical significance was achieved ($p<0.05$), post-hoc analysis was obtained via multiple t-tests with Bonferroni's correction to determine specific group differences. All data are reported as means ± SEM.

Results

Breathing Pattern

In obstructed animals with an intact trachea, Ttot was significantly greater during O1-O3 compared to preceding C and subsequent R breaths ($p<0.002$) (Figures 2-1 & 2-4). Prolongation of Ti ($p<0.03$) (Figure 2-2) and to a greater extent Te ($p<0.008$) (Figure 2-3) contributed to this Ttot difference. The Ttot of R breaths returned to pre-obstruction, C breath values. The rectified and integrated EMG_{dia} signal progressively increased in amplitude over the course of the obstruction ($p<0.001$), and remained significantly elevated above C values during R breaths ($p=0.02$) (Figure 2-5). Similarly, inspiratory P_{es} became increasingly negative during the obstruction ($p<0.001$) and also remained significantly more negative during R breaths compared to C ($p=0.004$) (Figure 2-6). During all obstructions in tracheostomized animals no changes in breath timing or EMG_{dia} were observed (Figures 2-1 to 2-5). Pre-surgical baseline recordings for P_{es}, EMG_{dia}, and breath timing did not differ from end-experiment recordings. Implantation of the tracheal cuff had no effect on P_{es}, EMG_{dia} or breathing pattern when it was sutured in place and deflated.

Fos-like Immunoreactivity

Fos-like immunoreactivity (FLI) was found in all nuclei analyzed for all groups (Table 2-1). In the medulla, the nucleus ambiguus (nA) and caudal nucleus of the

solitary tract (cnTS) (Figure 2-7) had significantly greater c-Fos expression in the experimental group compared to all other groups (p-values < 0.002). There was also a significant difference between groups in the rostral nTS (rnTS) (p=0.04) but post-hoc analysis revealed no differences.

In the locus coeruleus (LC) the experimental group had the greatest amount of FLI compared to controls. In the various parabrachial subnuclei the external lateral (elPBN) area showed the greatest number of Fos-expressing cells. In the elPBN, dorsal lateral (dlPBN), and central lateral (clPBN) subnuclei the experimental group had greater FLI compared to all control groups, and the difference was significant in the dlBPN (p=0.03, comparing Exp and Ctrl 3). The lateral crescent (lcrPBN) subnucleus was the only pontine region analyzed where the tracheostomized animals had the greatest c-Fos expression.

In the midbrain periaqueductal gray (PAG), the greatest amounts of FLI were observed in the caudal ventral region (cvPAG). The tracheostomized control group 1 had the most stained cells in all PAG regions, and the difference in group means was significant in the caudal dorsal (cdPAG) and cvPAG for this group compared to control group 3 (p<0.007). The experimental group had significantly more c-Fos expression in the cvPAG compared to control group 3 also (p=0.001). No differences between any groups were seen in the rostral dorsal PAG (rdPAG). In addition, the experimental group and control group 1 had significant (p<0.05) rostral-caudal differences in the dorsal PAG; the experimental group showed significant dorsal-ventral (p<0.05) differences in the PAG.

Discussion

Our method of ITTO for 2-3 breaths in anesthetized rats elicited respiratory load compensation, including a prolongation of Ti and Te, increased EMG_{dia} activity, and more negative inspiratory P_{es}. ITTO in anesthetized rats also induced neural activation in respiratory brainstem nuclei, and nuclei involved in cardiovascular, stress, and fear responses. End-expiratory P_{es} did not change with obstruction or recovery in any animals, indicating that ITTO does not change lung compliance (Figure 2-1). The short duration of occlusions ensured minimal changes in blood gases, supporting our hypothesis that ITTO elicits a load compensation respiratory motor pattern response and neuronal activation within respiratory related brain nuclei. In the tracheostomized control group 1, tracheostomy decreased baseline P_{es} to a value that remained stable during obstructions and recovery. Because Ti, Te, EMG_{dia}, and P_{es} were not altered in control group 1 during ITTO, the changes in these parameters observed in the experimental group result from the load compensation response of the respiratory neural control system to a breathing effort against a closed airway. Tracheal compression alone was not sufficient to evoke altered breath timing and respiratory motor responses.

The decreased breathing frequency during ITTO was primarily due to an increase in the duration of Te. Because Te is normally longer than Ti for a given breath, there could be more potential for breath-timing modulation during Te than Ti. Many previous studies on the responses to respiratory loading used loads applied to inspiration (Zechman *et al.*, 1976; Chaudhary & Burki, 1978; Davenport *et al.*, 1981a; Lopata *et al.*, 1983; Davenport *et al.*, 1991; Forster *et al.*, 1994; von Leupoldt & Dahme, 2005b) and expiration (Zechman *et al.*, 1976; Davenport *et al.*, 1981a; Davenport *et al.*,

1984; Davenport & Wozniak, 1986; Webb *et al.*, 1996; Watts *et al.*, 1997) separately, and this study was unique in that airway occlusion was maintained for both phases during several breaths. Zechman and colleagues (Zechman *et al.*, 1976) showed that expiratory loading resulted in an upward shift in functional residual capacity (FRC). It is the accumulated volume above FRC that contributes to the termination of the breath, not the Vi or Ve during only that cycle. If the tracheal occlusion were to occur at FRC, as was the aim in this study, both Vi and Ve would be restricted, preventing a net increase in lung volume. A tracheal occlusion at end-inspiration would prevent full expulsion of inspired air and a return to FRC, so subsequent breaths would occur upon inflated lungs.

Lung inflation activates respiratory afferents, the primary group of which project to the brain via the vagus nerve (Clark & von Euler, 1972; Zechman *et al.*, 1976). An important type of vagal afferent that contributes to the volume-timing relationship is the pulmonary stretch receptor (PSR). These receptors lie within the smooth muscle of bronchi and respond to increases in transpulmonary pressure (P_{tp}) which is influenced by lung volume (Davenport *et al.*, 1981b). As lung volume increases with inspiration, the discharge rate of PSR's increases until a frequency threshold is reached that terminates inspiration (Davenport *et al.*, 1981a). Te is also influenced by PSR activity, although the threshold for terminating expiration is dependent upon PSR discharge number rather than frequency (Davenport *et al.*, 1981a; Davenport & Wozniak, 1986). During ITTO at FRC in anesthetized rats, the resistance caused by occlusion decreases Vi and Ve, which subsequently decreases the frequency and number of PSR discharge, lengthening Ti and Te, respectively. If ITTO occurs at end-inspiration, Ve decreases as

Te lengthens, but the subsequent inspiration will begin before the lungs can return to FRC. This will result in a shorter Ti and Vi than if the occlusion were applied at FRC since accumulated lung volume causes P_{tp} and PSR discharge frequency to remain elevated. Thus, the Vt-T threshold that terminates Ti and Te does not change, but accumulated volume history may modulate their durations for a specific breath.

P_{es} and EMG_{dia} follow a similar pattern of activity during occluded breaths, with P_{es} becoming more negative as EMG_{dia} amplitude increases from O1 to O3. These values remain elevated above control breaths during the recovery breaths, although they are less than during obstructions. Forster and colleagues (Forster *et al.*, 1994) found that sustained inspiratory resistive loading in conscious ponies caused an augmentation of Vt and respiratory motor – but not breath timing – responses during recovery breaths. The augmentation of parameters during recovery breaths after ITTO is likely a result of the respiratory system acting to restore ventilation after prolonged perturbation. Loading or occluding one breath at a time does not affect subsequent breaths (Zechman *et al.*, 1976). The exact mechanisms underlying these responses are unknown, but it is clear that consecutive obstructions to breathing necessitate a recovery response that exists in both the anesthetized and conscious state. Although the augmented recovery response in the conscious ponies has a reflexive component, it cannot be ruled out that it might also be a behavioral response to the sensations associated with the loading.

Medulla

The rnTS and cnTS were activated with acute ITTO, indicated by enhanced FLI in those regions compared to control groups. The rnTS and cnTS had more stained cells compared to most other nuclei, which was expected considering the greater amount of convergent input to these areas. The load compensation response in anesthetized

animals is reflexive and dependent upon PSR activation (Zechman *et al.*, 1976; Davenport *et al.*, 1981a; Davenport *et al.*, 1984; Webb *et al.*, 1994, 1996). The nTS, part of the medullary dorsal respiratory group, is the primary target of these vagal afferents (Kalia & Mesulam, 1980a, b; Kalia, 1981a). Although vagal afferents from the lung play a large role in controlling the breath Vt-T response during load compensation , upper airway vagal afferents also contribute to that response during inspiratory (Webb *et al.*, 1994) but not expiratory (Webb *et al.*, 1996) resistive loading. Upper airway tracheal stretch receptors lie within the trachealis muscle (Davenport *et al.*, 1981c) and send their afferent fibers to the brainstem via the cervical vagus, superior laryngeal (SLN), and recurrent laryngeal nerves (RLN) (Sant'Ambrogio *et al.*, 1977; Lee *et al.*, 1992). The afferents from these receptors also terminate in the nTS (Kalia, 1981b) and were likely activated by the squeeze of the cuff during ITTO. Thus, both pulmonary and tracheal receptors were activated by ITTO, sending convergent input to the nTS where a compensatory motor response could be initiated. The nA, a nucleus within the ventral respiratory group (VRG), also showed significant increases in FLI in experimental animals compared to controls. This nucleus is activated by the nTS (Loewy & Burton, 1978), phrenic, vagus, carotid sinus, and SLN stimulation, bronchoconstriction, and inspiratory occlusions (Davenport & Vovk, 2009). The increase in FLI in medullary respiratory nuclei results from tracheal afferents stimulated by the squeeze of the cuff, pulmonary afferents activated by lung inflation, and efferents mediating changes in respiratory pattern. Thus, respiratory load compensation has a neural component that includes both sensory- and motor-activated medullary neurons.

Pons

ITTO also evoked significant neural activation in the dlPBN. The dlPBN responds to various respiratory stimuli including inspiratory occlusions (Davenport & Vovk, 2009), and is also an important part of the descending pathway from the PAG involved in cardiovascular (Hayward *et al.*, 2004; Hayward, 2007) and respiratory (Hayward *et al.*, 2004) control. Neurons in the nTS that are activated by vagal afferents send projections to pontine respiratory groups, including to the lPBN (Loewy & Burton, 1978; Ezure *et al.*, 1998). Specifically, the respiratory (ventrolateral) nTS has reciprocal connections with the dlPBN (Herbert *et al.*, 1990). The FLI seen in the lPBN after ITTO could be a response to afferent projections to that nucleus, but it could also be a result of projections from the lPBN to other neural areas such as the limbic system (Saper & Loewy, 1980). No differences were seen between groups in the LC, despite receiving input from the medullary dorsal and ventral respiratory groups (Herbert *et al.*, 1990). The LC is the main source of norepinephrine in the brain and is greatly involved in stress responses (Berridge & Waterhouse, 2003). The lack of group differences in the LC appears to be a result of substantial neural activation across all group conditions, possibly resulting from global urethane activation or an elevated and sustained stress response from handling the animals before bringing them to the laboratory. Neural activation seen in the PBN is likely primarily the result of integrating ascending medullary and descending PAG projections into both respiratory and cardiovascular responses to ITTO. The PBN is also involved in defensive, fear, and limbic pathways and although animals in this study were anesthetized and non-behaving, those roles should also be considered.

Midbrain

The caudal region of the midbrain PAG was activated by ITTO, with the ventral region more than the dorsal. The PAG is known to be involved in defensive behaviors (Bandler & Carrive, 1988; Brandao *et al.*, 1994; Vianna *et al.*, 2001), pain modulation (Mayer *et al.*, 1971; Behbehani, 1995), respiratory activation (Hayward *et al.*, 2003; Zhang *et al.*, 2005), and modulation of the respiratory load compensation response (Zhang *et al.*, 2009). The results of Zhang et al., (Zhang *et al.*, 2007) showed greater respiratory activation in response to cdPAG compared to rdPAG stimulation. SLN stimulation activates the PAG (Ambalavanar *et al.*, 1999), and the PAG and nTS have reciprocal projections (Loewy & Burton, 1978; Farkas *et al.*, 1998). The dPAG and vPAG have been implicated in panic and anxiety, respectively (Carrive, 1993; Bandler & Shipley, 1994; Brandao *et al.*, 1994; Vianna *et al.*, 2001; Cunha *et al.*, 2010), and the vPAG appears to be involved in conditioned fear responses (Vianna *et al.*, 2001). It is possible that ITTO causes respiratory-specific activation in the cdPAG and cvPAG, but that ITTO also stimulates stress or fear pathways that additionally contribute to PAG activation. In addition, one cannot rule out the potential contribution of other sensory afferents to respiratory Vt-T, motor, and neural responses to ITTO.

Any lack of significant differences in FLI expression between control group 1 and the experimental group in certain nuclei might signify that tracheal compression is a powerful stimulant of afferents that project to those specific areas. Although FLI is a useful indicator of neural activity in response to acute stimuli, many control groups are needed to ensure activation is not a result of handling, anesthesia, noise, scent, or another factor. Urethane was used to anesthetize animals for this study, and it is known that urethane causes neural activation throughout the brain, so differences between

treatments may have been difficult to determine above an already elevated pattern of activation. Furthermore, cells stained positively for the c-Fos protein cannot be divided into sensory or motor activation, and it cannot give any indication of inhibition. If proper control groups are designed, however, c-Fos immunohistochemistry can lay the foundation for understanding which neural substrates should be investigated further.

The results of this study suggest that airway resistance can be increased intrinsically without changing lung compliance, evoking respiratory load compensation indicated by breath timing, diaphragm activity, and esophageal pressure changes. This model has potential applications in studying respiratory obstructive diseases characterized by increased upper airway resistance. Individuals with asthma, OSA, and COPD all experience some degree of airway mechanical changes, including increased resistance, that are maintained over the course of many breaths. Using our model of ITTO to investigate the neural mechanisms mediating the load compensation response has important implications for elucidating the neural mechanisms modulated by these diseases.

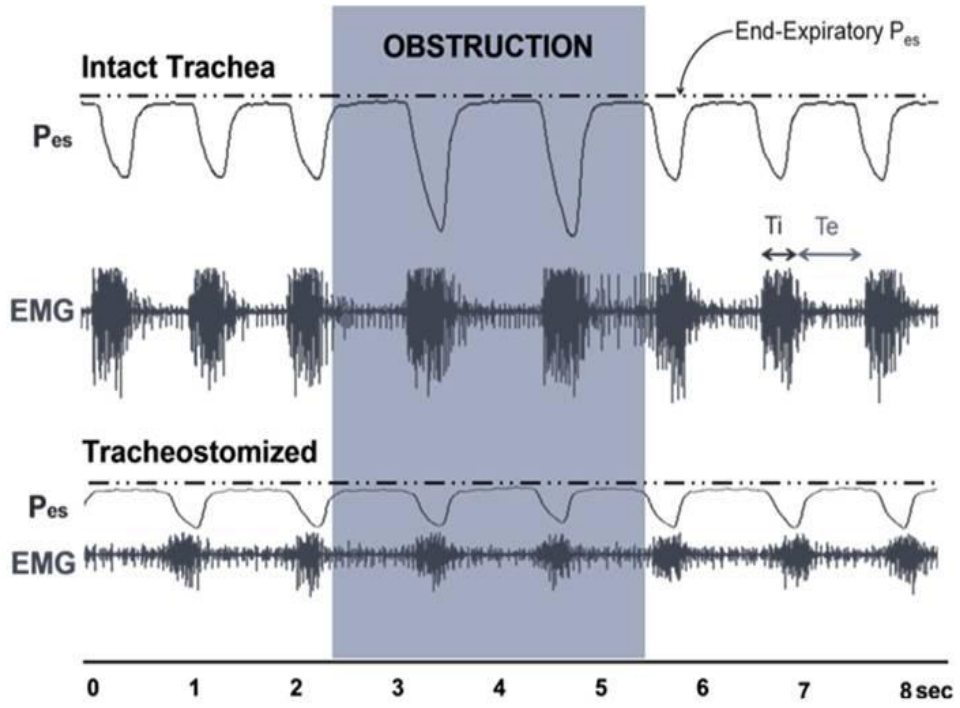

Figure 2-1. EMG_{dia} and P_{es} traces during tracheal occlusion in an intact (experimental) and tracheostomized (control group 1) animal. Indications of how Ti and Te were measured are displayed in the trace second from the top. There was no change in end-expiratory P_{es} during the course of the experiment.

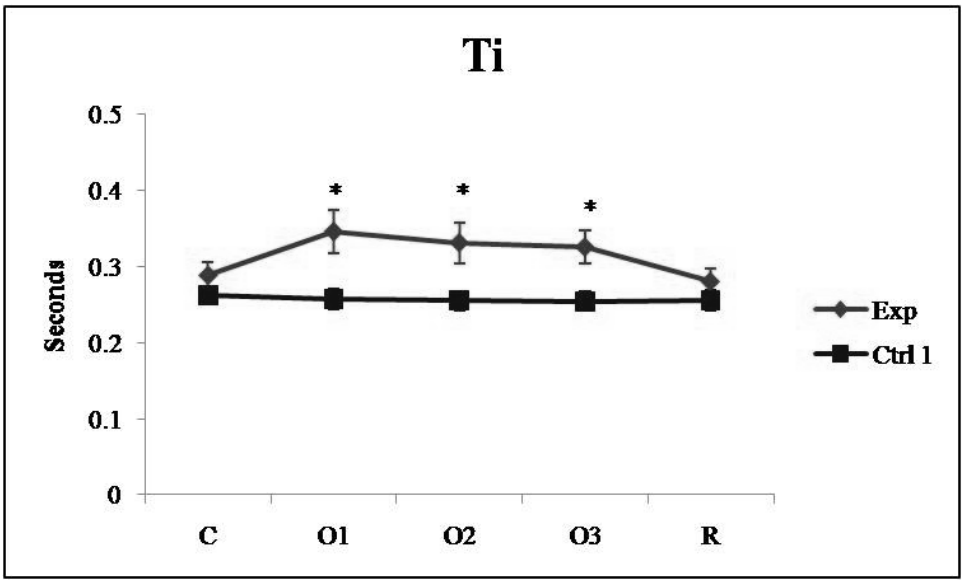

Figure 2-2. Inspiratory duration during tracheal occlusions in intact (Exp) and tracheostomized (Ctrl) animals. O1-O3 values were significantly different from C for the Exp animal (*p=0.03).

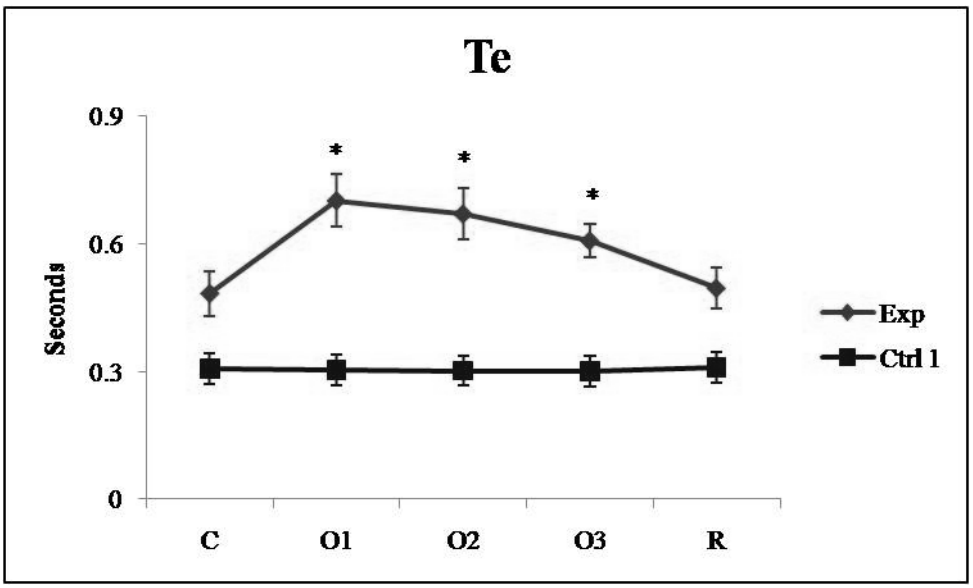

Figure 2-3. Expiratory duration during tracheal occlusions in intact (Exp) and tracheostomized (Ctrl) animals. O1-O3 values were significantly different from C for the Exp animal (*p=0.008).

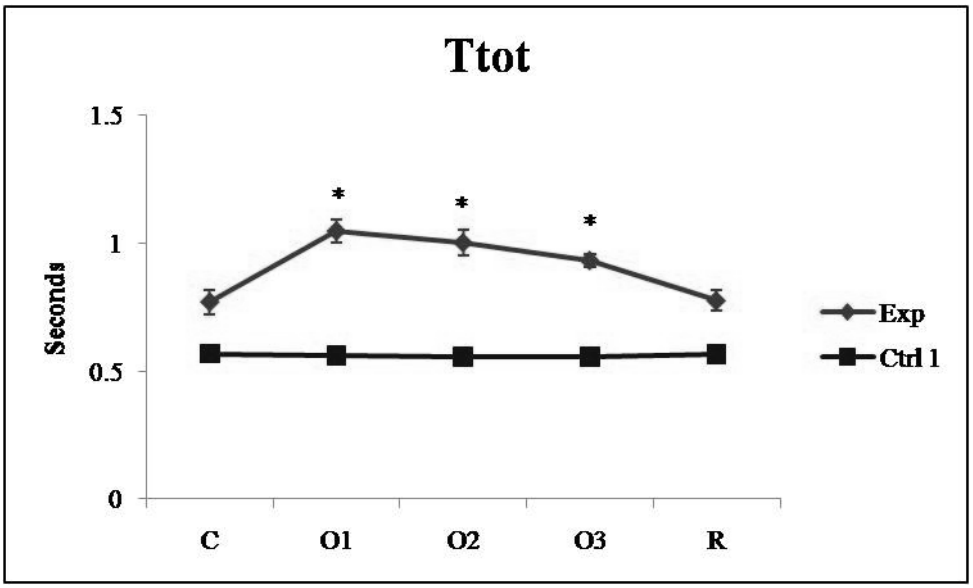

Figure 2-4. Total breath duration during tracheal occlusions in intact (Exp) and tracheostomized (Ctrl) animals. O1-O3 values were significantly different from C for the Exp animal (*p=0.002).

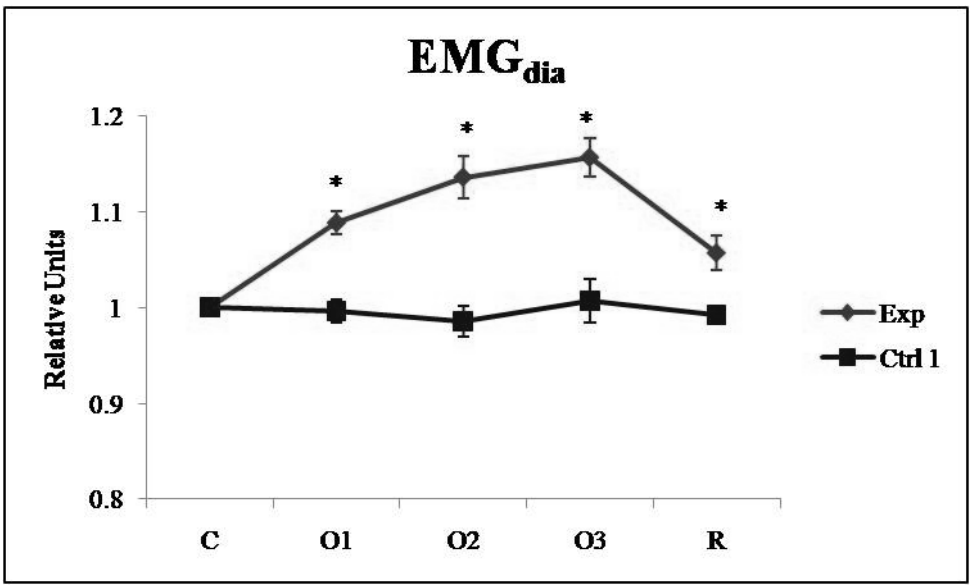

Figure 2-5. Peak amplitude of the integrated EMG_{dia} trace during tracheal occlusions in intact (Exp) and tracheostomized (Ctrl) animals. Values were significantly different from C for the Exp animal (*p=0.001, O1-O3; *p=0.02, R).

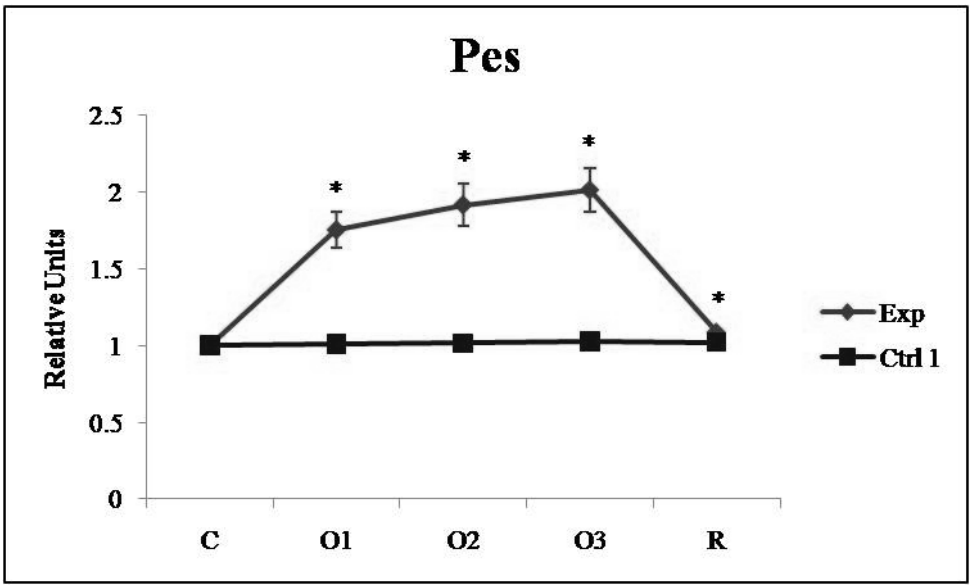

Figure 2-6. Peak negative esophageal pressure during tracheal occlusions in intact (Exp) and tracheostomized (Ctrl) animals. Values were significantly different from C for the Exp animal (*p=0.001, O1-O3; *p=0.004, R).

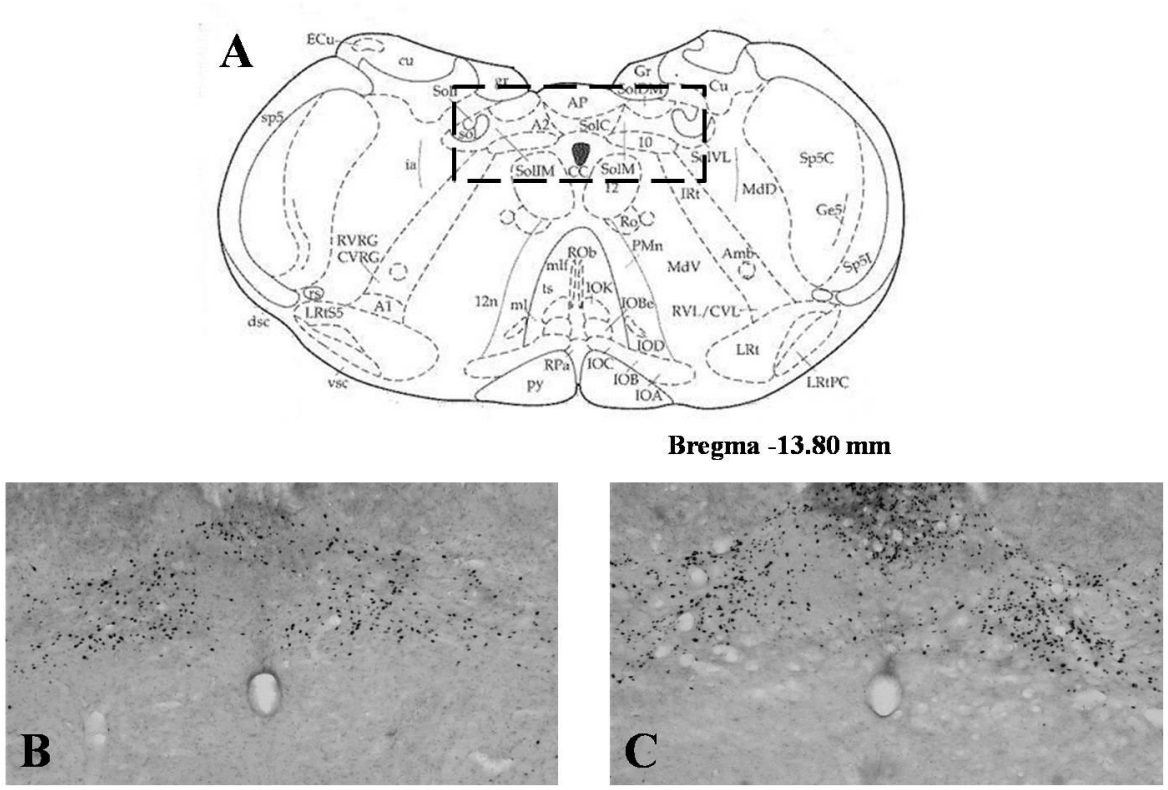

Figure 2-7. c-Fos in the caudal nTS of anesthetized animals after repeated tracheal occlusions in B) a tracheostomized and C) trachea-intact animal. The tracheostomized animal did not experience occlusions to breathing because the tracheostomy was distal to the cuff. The dashed line in part A shows the area of the brain atlas (Paxinos & Watson, 1997) corresponding to the images in B and C.

Table 2-1. c-Fos Expression in brainstem and suprapontine nuclei.

Nucleus	Naïve	Sx Only	Trach'd	Intact Obs	p-value
cdPAG	40.8 (6.4)	70.3 (22.2)	131 (33.7)*	88.7 (17.6)	0.007
cvPAG	57.6 (17.0)	153 (31.4)	236.7 (46.7)*	176.5 (39.2)*	0.001
rdPAG	59.5 (18.9)	58 (9.8)	69.2 (19.4)	52.8 (39.2)	n/a
nA	1.0 (0.2)	0.5 (0.2)	1.4 (0.3)	4.9 (0.5)**	0.001
cnTS	61.9 (16.0)	37.6 (10.5)	66.6 (19.7)	169.5 (21.5)**	0.002
rnTS	44 (0)	79.7 (26.5)	53.3 (6.5)	203.3 (44.3)	0.040
LC	42.5 (12.3)	37.8 (13.8)	42.7 (16.2)	44.9 (10.2)	n/a
clPBN	13.2 (3.0)	26.8 (7.3)	15.1 (6.8)	29.8 (5.1)	n/a
elPBN	38.0 (4.9)	30.1 (7.0)	20.7 (7.8)	38.1 (3.9)	n/a
lcrPBN	17.1 (2.7)	17.9 (2.7)	18.1 (3.1)	16.4 (2.4)	n/a
dlPBN	6.1 (1.3)	6.7 (0.8)	9.1 (1.3)	12.4 (1.4)*	0.007

The number of cells stained positively for c-Fos in each region of interest. SEM shown in parentheses. *Values were significantly different from the Naïve group; **values are significantly different from all other groups. Naïve (control group 3), Sx Only (control group 2), Trach'd (control group 1), Intact Obs (experimental group).

CHAPTER 3
RESPIRATORY LOAD COMPENSATION AND BEHAVIORAL CONDITIONING IN CONSCIOUS RATS

Introduction

Respiratory load compensation has been observed in response to mechanical challenges to breathing such as resistive or elastic loads applied to the respiratory circuit (Zechman & Davenport, 1978). The response to a resistive (R) load applied to one phase of the breath included a decrease in volume inspired (Vi) or expired (Ve) for the duration of the load and an increase in the loaded breath phase duration (Zechman *et al.*, 1976). The volume-timing parameters of the unloaded phase of the breath were unchanged. Respiratory load compensation in anesthetized animals has been characterized as reflexive and vagal-dependent (Zechman *et al.*, 1976), specifically mediated by pulmonary stretch receptors (PSR) responding to transmural pressure changes across the airways (Davenport *et al.*, 1981a; Davenport *et al.*, 1981b; Davenport *et al.*, 1984; Davenport & Wozniak, 1986). Modulation of Ti occurs through changes in PSR spike frequency and Te through changes in spike number (Davenport *et al.*, 1981a; Davenport & Wozniak, 1986). There is also a muscle response during load compensation which includes increased respiratory motor output, measured from respiratory muscle electromyography (EMG), primarily in the diaphragm (Lopata *et al.*, 1983). The volume-timing values obtained from complete respiratory occlusion approached those seen after vagotomy, including long loaded phase duration (Zechman *et al.*, 1976). Occlusion at end-expiration, or FRC, causes a prolongation of Ti and increased EMG_{dia} activity (Zechman *et al.*, 1976; Kosch *et al.*, 1986; Zhang & Davenport, 2003; Zhang *et al.*, 2009), and occlusion at end-inspiration causes a prolongation of Te (Gautier *et al.*, 1981; Zhang *et al.*, 2009).

Studies in conscious humans and animals are less numerous. Results from experiments in anesthetized animals provide information about reflexive and neural load compensation, but do not add to our understanding of behavioral load compensation, or voluntary modulation of the reflexive patterns. Single bouts of R loads or tracheal occlusions have been applied during expiration in lambs via a facemask (Watts *et al.*, 1997) and inspiration in dogs through a tracheal stoma (Davenport *et al.*, 1991). Expiratory R loading causes a decrease in airflow and prolonged expiratory time (Te) (Watts *et al.*, 1997). Subsequent unloaded breaths showed decreased Vi, increased Ve, and the integrated EMG activity in the larynx and diaphragm remained active as a compensatory mechanism to help restore lung volume to baseline (Watts *et al.*, 1997). When inspiratory R loads are added to two consecutive breaths in conscious goats, the integrated diaphragm and external abdominal oblique muscle responses were augmented and inspiratory time (Ti) increased during both inspirations (Hutt *et al.*, 1991). These values returned to baseline during unloaded breaths, unlike the responses seen during single bouts of expiratory R loading in lambs (Watts *et al.*, 1997). This difference could be due to the fact that the magnitudes of the R loads were different (370 cmH_2O/ l/sec in lambs vs. 18 cmH_2O/ l/sec in goats), animal ages were different (neonatal lambs vs. adult goats), or perhaps that the inspiratory and expiratory phases of breathing are under separate control mechanisms (Davenport *et al.*, 1981a; Davenport & Wozniak, 1986; Webb *et al.*, 1994, 1996).

When multiple consecutive inspirations are loaded, Ti is prolonged while Te and tidal volume (Vt) are decreased during the first breath in conscious ponies (Forster *et al.*, 1994). During the second through fifth loaded breaths, small changes were seen in

volume and timing parameters which stabilized after the fifth breath for the subsequent 2-4 minutes of loading. The time of EMG_{dia} activation mirrored Ti, and mean EMG_{dia} activity was elevated during the first loaded breath to a value that remained augmented for subsequent loaded breaths. During the first recovery breath following loading, Vt and mean EMG_{dia} activity increased above values during loading but timing parameters did not change. These responses differed from responses documented in conscious animals in response to single- (Watts *et al.*, 1997) or two-breath (Hutt *et al.*, 1991) loading. All parameters progressively returned to control values over the course of subsequent unloaded breaths. Surprisingly, the loaded and recovery breath compensation effect persisted even after pulmonary and diaphragmatic deafferentation, indicating that these afferents quantitatively but not qualitatively modulate the load compensation response (Forster *et al.*, 1994). The authors of this study hypothesized that intercostal afferents and cortical input likely contributed greatly to the observed response.

In the conscious man, some studies have shown that R loading decreases volume, prolongs breath duration, and enhances motor output (Axen *et al.*, 1983; Daubenspeck & Bennett, 1983; Hudgel *et al.*, 1987; Nishino & Kochi, 1994; Daubenspeck & Rhodes, 1995). In addition, an augmented recovery response to loading has been documented (Altose *et al.*, 1979) similar to some aspects of conscious animal studies (Forster *et al.*, 1994; Watts *et al.*, 1997). Greater respiratory responses to loads of larger magnitudes have also been recognized (Altose *et al.*, 1979; Nishino & Kochi, 1994; Calabrese *et al.*, 1998). However, there seems to be great variability in response patterns to both respiratory loading and occlusion in conscious humans (Axen

et al., 1983; Daubenspeck & Bennett, 1983; Davenport & Wozniak, 1986; Hudgel *et al.*, 1987; Nishino & Kochi, 1994; Daubenspeck & Rhodes, 1995). Others have documented increases in volume and decreases in frequency in response to inspiratory R loading (Iber *et al.*, 1982). Interestingly, load compensation responses that differed between individuals remained qualitatively similar within the same individual in response to various load magnitudes (Daubenspeck & Rhodes, 1995). Differing responses may be due to perceptual differences between individuals (Daubenspeck & Rhodes, 1995). Respiratory afferents other than those in the airways and in the main inspiratory muscles must play a role in load compensation in conscious humans and animals. These other afferents most likely transduce respiratory sensations associated with loading and airway occlusion. The way a conscious human or animal feels (affective component) about its breathing may further lead to modulation of the reflexive pattern of load compensation.

Our laboratory has developed a method to study the load compensation reflex in conscious rats via intrinsic, transient and reversible tracheal occlusions (ITTO). ITTO is performed by inflating a rubber cuff around the trachea with enough pressure to reversibly close the lumen of the trachea. We designed a 10 day ITTO protocol in conscious rats that would elicit and behaviorally condition the animal to respiratory load compensation. It was hypothesized that ITTO in conscious rats would cause variable respiratory load compensation pattern responses that are altered after 10 days of ITTO conditioning.

Methods

Animals

These experiments were performed on four male Sprague-Dawley rats (387.5 ± 71.3 g). The animals were housed two to a cage in the University of Florida animal care facility where they were exposed to a 12 h light/12 h dark cycle. The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the University of Florida.

Surgical Procedures

Animals were initially anesthetized using isoflurane gas (2-5% in O_2) administered in a whole-body gas chamber. Anesthetic depth was verified by the absence of a withdrawal reflex from a rear paw pinch. Buprenorphine (0.01-0.05 mg/kg body weight) and carprofen (5mg/kg body weight) were administered preoperatively via subcutaneous injection. Incision sites were shaved and sterilized with povidine-iodine topical antiseptic solution. Body temperature was maintained at 38°C by a heating pad, and anesthesia was maintained by isoflurane gas via a nose cone.

Diaphragm electrodes

The anesthetized animal was placed in a supine position and a 4 cm vertical incision was made in the abdomen. The skin and muscular abdominal wall were pulled laterally in order to expose the diaphragm. Diaphragm EMG (EMG_{dia}) electrodes were made using stainless steel, Teflon-coated wire (AS631, Cooner Wire, Chatsworth, CA, USA), threaded through the tip of a 22 gauge needle. Two of these electrodes were implanted into the exposed right diaphragm, parallel to one another and roughly 4 mm apart. The wires were bared where they contacted the diaphragm and were secured

into place with knots. The insulated, distal ends of the electrode wires were routed through the abdominal incision and directed subcutaneously to the dorsal surface of the animal, between the scapulae, where they were externalized and secured in place with sutures.

Tracheal cuff

The trachea was exposed by a ventral neck incision. The trachea was freed from surrounding tissue and a saline-filled inflatable cuff (Fine Science Tools) was sutured around the trachea, two cartilage rings caudal to the larynx. The actuator tube of the cuff was plugged with a blunt needle and routed subcutaneously to the dorsal surface of the animal, externalized via an incision between the scapulae, rostral to the EMG_{dia}. The tissue exposed in the ventral incision was pulled over the cuff and the skin was sutured closed. The skin at the dorsal incision was sutured closed and the tube was secured in place by tying the ends of the suture around a bead on the tube. Rats were then administered warm normal saline (0.01-0.02 ml/g body weight) and isoflurane anesthesia was gradually reduced. The animal was placed in a recovery cage on a heating pad and was returned to the Animal Care Facilities once fully mobile. Postoperative analgesia was provided for two to three days using buprenorphine (0.01-0.05 mg/kg body weight) and carprofen (5mg/kg body weight) given every 24 hours. Animals were allowed a full week of recovery before experiments began.

Protocol

The animals were retrieved from the Animal Care Facility on the morning of Experiment Day 1 and were taken to the laboratory. They were placed in a recording chamber for an acclimatization period of 15 minutes. During this time, the externalized actuator tube of the tracheal cuff from the animal was connected to a saline-filled

syringe outside the chamber but no pressure was applied to the syringe. The external ends of the EMG_{dia} were bared and connected to a high-impedance probe. The signal was amplified and band-pass filtered (0.3-3.0 kHz) (P511, Grass Instruments). Analog output was sampled (PowerLab, ADInstruments), computer processed (LabChart 7 Pro, ADInstruments), and stored for offline analysis. At the end of the 15 min protocol the animal was returned to its home cage and the chamber was cleaned with alcohol wipes. Once all animals had completed the acclimatization protocol they were returned to the Animal Care Facility.

The animals were retrieved from the Animal Care Facility on the morning of Experiment Day 2 and were brought to the testing laboratory. One animal was placed in the recording chamber and the actuator tube was connected to a saline-filled syringe. EMG_{dia} were connected to the recording equipment. Diaphragm activity was recorded and stored for later analysis. The animal was allowed to rest undisturbed in the chamber for 2.5 minutes, followed by a series of cuff inflations. The syringe and cuff were pressure calibrated so a known amount of fluid movement in the syringe would result in the amount of pressure required to fully compress and occlude the trachea. Removing the pressure allowed for full recovery of the trachea with no interference to breathing. The cuff of the animal was inflated 3-6 seconds at a time with ~35 trials in a 15 minute period. The trials were applied in a random time pattern. After the last trial, the animal was allowed to rest undisturbed in the recording chamber for 2.5 minutes. At the end of the 20 minutes, the animal was removed from the chamber and returned to its cage. The recording chamber was cleaned with alcohol wipes between animals. The next animal was placed in the recording chamber and the 20 minute protocol was repeated.

This was continued for all animals. All animals were returned to the Animal Care Facility at the end of Experiment Day 2. This procedure was repeated daily for Experiment Days 3-11. Animals were sacrificed on Experiment Day 12.

Analysis

Breathing pattern analysis was performed offline (LabChart 7 Pro, ADInstruments). Raw EMG_{dia} signal recordings were rectified and integrated (50 ms time constant). The integrated trace was integrated further to obtain total EMG_{dia} activation ($intEMG_{dia}$) during the 3 seconds prior to (C) and the first 3-4 seconds of each occlusion (O). This analysis was completed for the first and last day of ITTO conditioning. Additionally, the number of EMG_{dia} peaks (efforts) was counted during C and O. The ratio of $intEMG_{dia}$ to effort was obtained and averaged during C and O for each animal and for each day. The relative contribution of Ti and Te to Ttot during C and O were determined. Ti was defined as the duration of EMG_{dia} activation, Te as the duration of EMG_{dia} quiescence, and Ttot as Ti + Te. Ti, Te, and Ttot were averaged during C and O for each animal and for each day. A one-way repeated measures analysis of variance (RMANOVA) was performed comparing breath (C vs. O) and day (first vs. last), separately for the following parameters: Ti, Te, Tot, Ti/Te, Ti/Ttot, Te/Ttot, $intEMG_{dia}$/effort. Differences were significant when $p<0.05$, and post-hoc comparisons were performed using Tukey's HSD with overall significance of $p<0.05$. Additionally, a paired t-test was performed comparing the ratio of O to C for $intEMG_{dia}$/effort for the first and last day of ITTO with significance at p-values < 0.05. All data are reported as means ± SEM.

Results

Timing

An example of the raw and integrated EMG_{dia} signal (from the same animal) during the initial segment of one tracheal occlusion at FRC on the first (A) and last (B) day of the ITTO protocol is shown in Figure 3-1. Ti non-significantly increased during O compared to C on both the first and last day of ITTO (Figure 3-2A). Te was significantly prolonged during O compared to C on both the first and last day of ITTO (Figure 3-2B, *p=0.03). Ttot was non-significantly longer during O compared to C on the first or last days of ITTO (Figure 3-2C). Normalized breath timing values (O/C) for Ti, Te, Ttot are shown in Figure 3-2D, and no difference in O/C ratios for any timing variable were seen between the first and last day of ITTO (Figure 3-2A to 3-2D).

There was no significant change in relative contribution of Ti (Figure 3-3A, D) and Te (Figure 3-3B, D) to Ttot during O or C on either the first or last day of ITTO, although during O there was a trend for greater contribution to Ttot by Te. The Ti/Te ratio did not change between C and O, or between the first and last day of ITTO (Figure 3-3C, D).

EMG_{dia} Activity

The $intEMG_{dia}$ activity is illustrated in Figure 3-4. $intEMG_{dia}$/effort was not greater during either O or C on the last compared to the first day of ITTO (Figure 3-4A, p=0.07 for difference between O on first compared to last day of ITTO). There was no difference in values during O and C on the first day of ITTO, however, $intEMG_{dia}$/effort was significantly greater during O compared to C on the last day of ITTO (Figure 3-4A, *p=0.03). The difference between relative values of $intEMG_{dia}$/effort on the first and last day of ITTO was significant (Figure 3-4B, *p=0.05).

Discussion

The results of this study show that repeated tracheal occlusions elicit a load compensation response in conscious rats, and that the pattern of this response can be altered as a result of repeated days of ITTO conditioning. The first day response to ITTO consisted of a prolongation of Te with no significant changes in Ti, Ttot or diaphragmatic activation. After 10 days of conditioning, conscious rats showed lengthened Te and increased diaphragmatic activation during O compared to C breaths, which were not accompanied by increases in Ti. There were no significant changes in timing parameters from the first day of ITTO to the last day; however, there was a significant increase in the O/C ratio of diaphragmatic activity per effort. This suggests that after 10 days of ITTO, conscious animals preferentially change respiratory drive rather than timing in response to the occlusions, and that the typical volume-timing reflex seen in anesthetized animals is modulated by behavioral compensation in conscious rats.

Previous studies investigating timing reflexes in response to airway occlusions applied the occlusions to inspiration (Zechman *et al.*, 1976; Kosch *et al.*, 1986; Zhang & Davenport, 2003; Zhang *et al.*, 2009) or expiration (Zhang *et al.*, 2009) separately. The prolongation of Ti seen with occlusions at FRC is a response to functional vagotomy, which does not allow changes to occur in PSR input and depends upon the internal respiratory rhythm generator to establish timing parameters (Feldman *et al.*, 2003). The prolongation of Te seen with end-inspiratory occlusions results from an elevated number of activated PSR's that delay inspiration from beginning (Gautier *et al.*, 1981). The ITTO protocol used in this study is unique in that airway occlusions were sustained for both inspiration and expiration over the course of several consecutive breaths. We

tried to apply the tracheal occlusions at FRC in order to maintain a consistent method of application and to avoid PSR-mediated first breath responses to each occlusion. We expected that both Ti and Te would be prolonged during O compared to C since the occlusions were sustained for both breath phases. We noticed a significant prolongation of Te, but only a trend toward an increase in Ti during O. The increase in Te during O on both the first and last day of the ITTO protocol could be explained by lung hyperinflation. Applying the occlusion at end-inspiration or during expiration would lead to sustained lung inflation, causing continuous PSR activation and producing prolonged Te (Gautier *et al.*, 1981; Zhang *et al.*, 2009). Te would likely remain elevated for subsequent breaths as long as the elevated lung volume was maintained. This could explain why there was not a significant increase in Ti during O compared to C on either the first or last day of ITTO. Although we attempted to prevent this response by applying each occlusion at FRC, the rapid respiratory rate and movement of the animals hindered precise timing with the occlusions. To control for the occlusion at lung volumes above FRC, we evaluated parameters for occlusions that occurred near end-expiration, most likely during the end-expiratory pause phase of the breath.

Another explanation for the prolongation of Te during O is behavioral breath holding. Large resistive loads are known to cause multi-dimensional sensations including air hunger and breathlessness (Simon *et al.*, 1989; Simon *et al.*, 1990; ATS, 1999), and these sensations are collectively referred to as the symptom dyspnea. Dyspnea is a highly aversive sensation (von Leupoldt & Dahme, 2005b; von Leupoldt *et al.*, 2008) and is linked with the fear that arises in response to the threat of suffocation (Lang *et al.*, 2010) or asphyxiation (Campbell, 2007). Threat of asphyxiation is produced

when a stimulus leads to hypoxemia, hypercarbia, or increased inspiratory efforts (Campbell, 2007). In the current study, only mechanical stimuli were used to elicit responses. Furthermore, results from our laboratory (Van Diest *et al.*, 2006) suggest that respiratory loading is more distressing than hypercapnic stimulation of breathing. Taken together, we conclude that the sensations associated with trying to breathe through the tracheal occlusions were highly aversive to conscious rats. The prolongation of Te observed during tracheal occlusions was likely the attempt of the animals to hold their breath and avoid the sensations rather than breathe against the closed airway. There was an increase in Te on both the first and last day of ITTO training but those values were not different from each other. This suggests that even with conditioning, animals continued to behaviorally adjust their load compensation breathing pattern to avoid the aversive sensations associated with ITTO. Aversive sensations associated with respiratory stimuli may not habituate, which is beneficial when considering the survival of the animal.

Experimentally-induced voluntary breath holding responses in humans are variable, mediated by conscious suppression of the central respiratory rhythm and involuntary mechanisms which eventually restore respiration before loss of consciousness (Parkes, 2006). The central respiratory rhythm does not stop, and only its expression is suppressed (Agostoni, 1963). The eventual override of voluntary suppression appears to be related to diaphragmatic afferents (Parkes, 2006). We have observed (unpublished observations) that animals may initially respond to ITTO by voluntary breath holding, but if the occlusion is maintained for 10-15 seconds the animals will reach a breaking point where intense, ballistic respiratory efforts begin.

Future studies using 15 second occlusions and comparing the first 3 seconds with the last 3 seconds would allow us to characterize the respiratory load compensation response to sustained ITTO. In the present study, the short duration ITTO avoided changes in blood gases and minimized chemical activation of respiration.

We observed no change in the breath timing pattern between the first and last day of ITTO. The lack of alterations in breath timing is consistent with respiratory studies in humans (Clark & von Euler, 1972; Axen *et al.*, 1983). The only significant change we noticed was the increased duration of Te during O breaths compared to C on both days. The contribution of Ti and Te to Ttot were not different between O and C breaths or between the first and last day of ITTO. The lack of significance was likely due to the small, non-significant increase in Ti during O breaths that abolished any differences in Te/Ttot. The Ti/Ttot ratio, or duty cycle, during C on the first and last day of ITTO in our rats ranged from 0.44-0.48, consistent with other reports in Sprague-Dawley rats (Walker *et al.*, 1997). Ti/Ttot non-significantly decreased during O due to the prolonged Te. Thus, Te increased in response to ITTO but there were no other pattern changes due to O or resulting from conditioning, and the respiratory timing pattern in our animals remained in the reported normal range.

The respiratory load compensation timing response is extremely variable in conscious humans and animals (Axen *et al.*, 1983). Increased respiratory muscle activation appears to be a more consistently reported response in both the conscious and anesthetized human and animal (Axen *et al.*, 1983; Lopata *et al.*, 1983; Hutt *et al.*, 1991; Frazier *et al.*, 1993; Xu *et al.*, 1993a; Xu *et al.*, 1993b; Osborne & Road, 1995; Zhang *et al.*, 2009). The intEMG_{dia}/effort in conscious rats was increased during O

compared to C in this study. This means that either the EMG_{dia} was active for a longer period of time or the amplitude of the signal was greater. A longer activation period would correspond with an increase in Ti, which was not observed. Thus, the increase in muscle activation per effort results from larger EMG_{dia} amplitude, suggesting that more motor units were recruited during Ti. The preferential response to ITTO is an increase in respiratory motor drive rather than a change in timing. Taken together with our timing data, the response of conscious rats to tracheal occlusions appears to be breath holding and stronger inspiratory efforts. The increased $intEMG_{dia}$/effort during O compared to C was observed on both the first and last day of ITTO, and the ratio of O/C for this measure was significantly increased on the last compared to the first day of ITTO. This suggests a conditioning response characterized by increased diaphragmatic recruitment during O after 10 days of ITTO.

Increasing inspiratory muscle activity would generate a greater driving force to move air into the lungs if the airways were open; however, a complete tracheal occlusion closes off the airways and prevents airflow. On the first day of ITTO the animal has no experience with the occlusions and the increased EMG_{dia} response during O compared to C may reflect an attempt to re-establish airway patency and restore ventilation. After 10 days of ITTO, however, we expected that the animals would cease intense inspiratory efforts as a result of learning. The reverse was actually observed: animals not only maintained a ratio of O/C greater than 1 for $intEMG_{dia}$/effort, the ratio was significantly greater on the last day of ITTO compared to the first day. In another study done by our laboratory (unpublished results) we observed that this 10 day ITTO protocol leads to diaphragm and intercostal muscle hypertrophy, indicated by

increased cross-sectional area in type IIx/b fibers. This suggests a potential increase in muscular strength and force production, which would in turn require the recruitment of fewer motor units to do the same work load. In the present study, the behavioral load compensation reflex response was characterized by an increase in diaphragm muscle recruitment during each inspiratory effort on the last day of ITTO, counter to what we expected with muscle hypertrophy. Both the learning and muscle hypertrophy effects should have resulted in less EMG_{dia} activity per breath after 10 days of ITTO, hence the observed increase implies that this type of respiratory stimulus drives the behavioral load compensation response to make strong inspiratory efforts in an attempt to defend minute ventilation.

The increased O/C ratio for $intEMG_{dia}$/effort on the last day of ITTO compared to the first day could be explained by a sensitized behavioral response to the occlusions. We have shown that ITTO produces stress responses in conscious rats (unpublished results), and others have reported fear potentiation in response to uncontrollable stressors (Korte *et al.*, 1999), of which ITTO is one. Also, our animals were trained in the same context each day, and due to the aversive nature of the ITTO stimulus, it is possible that affective conditioning occurred. Fear-potentiated behaviors are observed as a result of contextual affective conditioning (Risbrough *et al.*, 2009). The increased O/C $intEMG_{dia}$/effort response seen on the last day of ITTO compared to the first day may also be due to a heightened affective state from contextual conditioning, which could result in alterations in breath timing and EMG_{dia} parameters during C as well. However, resting behaviors in the context are known to habituate (Beck & Fibiger, 1995). Furthermore, if the animals did have potentiated breathing responses during C

we would have expected to see increased diaphragmatic activation and increased breath frequency as a typical fear response (Van Diest *et al.*, 2009). This would have resulted in greater O/C ratios for timing parameters and an O/C ratio closer to 1 for the EMG_{dia} activity per breath which was not what was observed. It is possible that the increase in inspiratory effort during O after 10 days of ITTO is a fear-potentiated behavior.

In conclusion, the results of this study suggest that there is a behavioral respiratory load compensation response to tracheal occlusions in conscious rats that is different from the anesthetized reflexive response reported in animals exposed to respiratory loads. We observed an increase in Te during O, attributable to breath holding, and an increase in diaphragmatic activity per breath, attributable to a sensitized affective behavioral response. Thus, the volume-timing reflex may be suppressed by behavioral mechanisms in conscious animals where a voluntary increase in respiratory drive is the preferential compensation response.

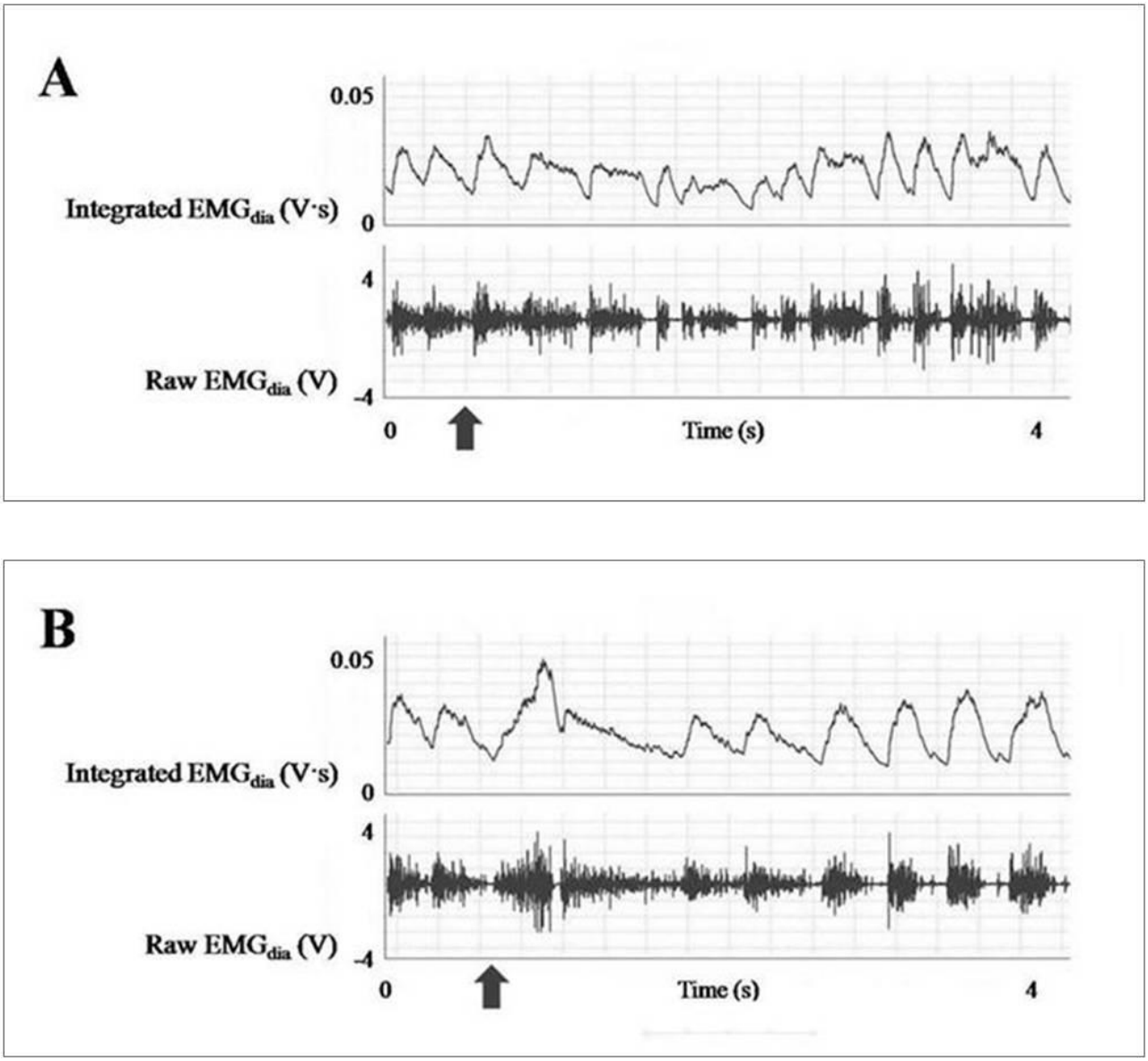

Figure 3-1. EMG_{dia} during the initial segment (4 s) of one tracheal occlusion at FRC on the first (A) and last (B) day of ITTO conditioning. Traces are from the same animal. Arrows indicate when cuff inflation was performed. Inflation was maintained over the rest of each segment shown in A and B.

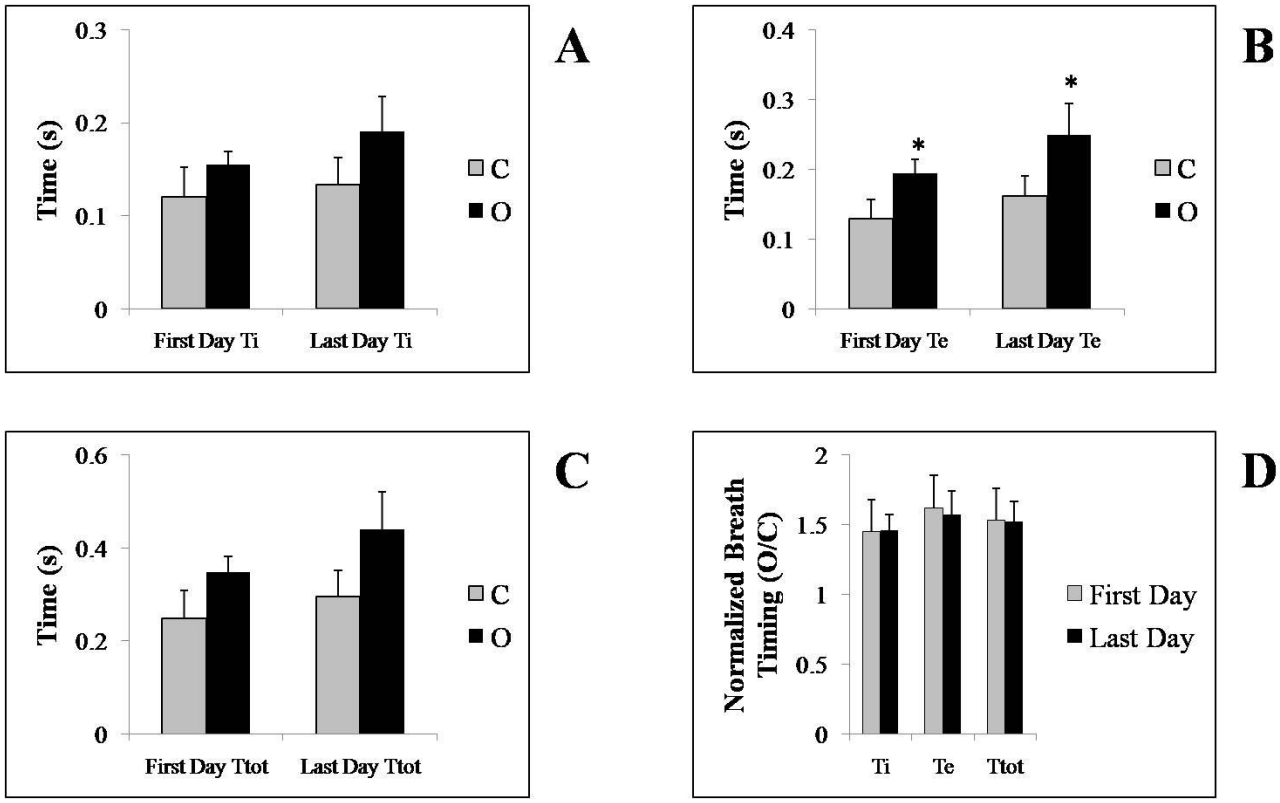

Figure 3-2. Timing parameters during control (C) and occluded (O) breathing. C from the 3 s prior to occlusion, and the first 3-4 s of O are shown for Ti (A), Te (B), and Ttot (C) on the first and last day of ITTO conditioning. Differences in Te during C and O were significantly different on the first and last day (*p=0.03). Normalized breath timing data (O/C) for Ti, Te, Ttot are also shown (D).

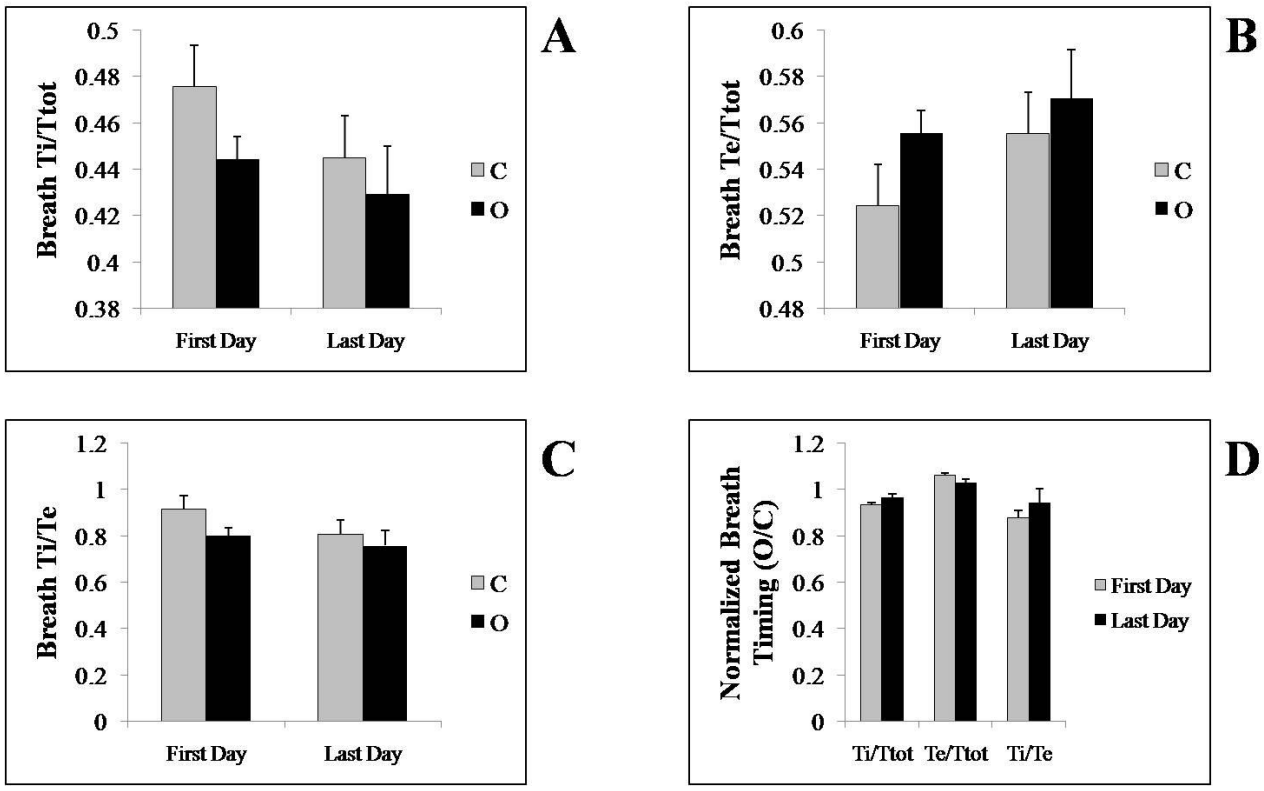

Figure 3-3. Relative timing parameters during control (C) and occluded (O) breathing. The contribution of Ti to Ttot (A) and Te to Ttot (B) are shown for C from the 3 s prior to occlusion, and the first 3-4 s of O on the first and last day of ITTO. The ratio of Ti/Te during C and O are also shown (C). These ratios are expressed as normalized values (O/C; part D).

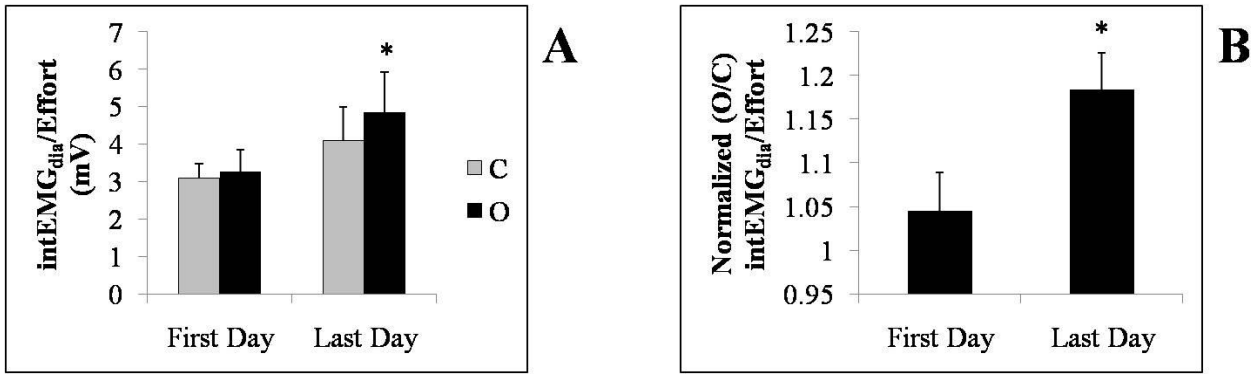

Figure 3-4. Integrated EMG_{dia} activity during control (C) and occluded (O) breathing. $intEMG_{dia}$/effort is shown during C from the 3 s prior to occlusion, and the first 3-4 s of O on the first and last day of ITTO (A), and the difference between O and C on the last day of ITTO conditioning was significant (*p=0.03). Normalized values (O/C) for $intEMG_{dia}$/effort on the first and last day of ITTO conditioning were significantly different (B, *p=0.05).

CHAPTER 4
STRESS AND ANXIETY RESPONSES TO REPEATED TRACHEAL OCCLUSIONS

Introduction

An animal's acute response to stress is characterized by activation of neural pathways that lead to arousal in preparation for handling the threat posed by the stressor. Arousal may include increases in heart rate, respiratory rate, blood pressure, and heightened startle responses. The stress-related neural pathways converge upon the paraventricular nucleus (PVN) of the hypothalamus (Herman *et al.*, 2003; Herman *et al.*, 2008; Flak *et al.*, 2009). The median parvocellular part (PaMP) is the neurosecretory division of the hypothalamus responsible for producing corticotropin releasing hormone (CRH) (Vale *et al.*, 1981), released in response to stress (Herman *et al.*, 2003; Herman *et al.*, 2008; Flak *et al.*, 2009). CRH is released in the anterior pituitary and promotes the secretion of adrenocorticotropic hormone (ACTH) (Vale *et al.*, 1981; Antoni, 1986) which circulates to the adrenal glands where glucocorticoids like corticosterone (Cort; cortisol in humans) are released by the adrenal cortex. This pathway is known as the hypothalamic-pituitary-adrenocortical (HPA) axis. Cort, via its many neurophysiologic actions, plays a role in normal neuroendocrine function and assists an animal in adapting to stressors (Sapolsky *et al.*, 2000). Thus, measuring blood plasma Cort levels is one way to determine the extent of HPA axis activation. Measuring PVN CRH or blood CRH is not linearly correlated with chronic stress because repeated exposure to the stress leads to a blunted CRH response (Harbuz *et al.*, 1992). Additionally, the acute HPA response to the presentation of a stimulus used in a chronic stress paradigm shows habituation (Girotti *et al.*, 2006; Grissom *et al.*, 2007), while basal HPA activation remains elevated (Fernandes *et al.*, 2002; Armario, 2006; Filipovic *et al.*, 2010).

The physiological response to stressful stimuli varies depending upon a number of factors such as duration, predictability, and controllability of the stimulus (Levine, 2000; Herman *et al.*, 2003; Arnhold *et al.*, 2007; Grissom *et al.*, 2007). Stress-inducing stimuli can be categorized as internal (visceral) physical, external physical, or psychological (Li *et al.*, 1996; Levine, 2000; Herman *et al.*, 2003), and stimuli can fall under more than one category. Respiratory afferents activated by resistive loads to breathing project to subcortical neural areas, discriminative (Davenport *et al.*, 1985; Davenport *et al.*, 1991; Davenport *et al.*, 1993; Davenport & Hutchison, 2002), and affective sensory regions (von Leupoldt & Dahme, 2005b; Schon *et al.*, 2008) that may contribute to both internal physical and psychological stress. Patients with respiratory obstructive diseases such as chronic obstructive pulmonary disease (COPD), the fourth leading cause of death in the United States (Heron & Tejada-Vera, 2009), experience psychological stress and often have affective disorders such as anxiety and panic (Brenes, 2003; Wagena *et al.*, 2005). Additionally, individuals diagnosed with affective disorders are more likely to have higher salivary Cort levels upon waking (Vreeburg *et al.*, 2010). It remains unclear, however, what effect repeated, stressful respiratory stimuli may have on HPA activation in healthy conscious animals, and whether the stimuli are sufficient to cause affective responses.

Our laboratory has developed a model of unavoidable, intrinsic, transient tracheal occlusions (ITTO) in conscious rats. A tracheal occlusion is, functionally, an infinite resistive load to breathing, and our laboratory has shown that intense resistive loads are stressful stimuli (Alexander-Miller & Davenport, 2010). Measuring resting plasma Cort allows for the approximation of stress-related HPA activation. With repeated or chronic

stress, an animal's adrenal glands become enlarged due to sustained increased Cort production (Harbuz *et al.*, 1992; Fernandes *et al.*, 2002; Marquez *et al.*, 2004). Comparing Cort levels and adrenal gland weight in animals after 10 days of ITTO conditioning to values obtained from animals not receiving occlusions allowed us to approximate the extent of stress caused by ITTO. In addition, the Elevated Plus Maze (EPM) has been used as a behavioral test for anxiety in rodents (Pellow *et al.*, 1985). All animals completed an EPM test prior to ITTO and before their last day of ITTO to determine changes in state anxiety caused by conditioning. It was hypothesized that after 10 days of uncued ITTO, an unavoidable stress, rats would have elevated basal plasma Cort, increased adrenal weights, and increased anxiety-like behavior measured via the EPM.

Methods

Animals

These experiments were performed on 25 male Sprague-Dawley rats (374.0 ± 55.0 g). The animals were housed two to a cage in the University of Florida Animal Care Facility where they were exposed to a 12 h light/12 h dark cycle. The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the University of Florida.

Surgical Procedures

Animals were initially anesthetized using isoflurane gas (2-5% in O_2) administered in a whole-body gas chamber. Anesthetic depth was verified by the absence of a withdrawal reflex from a rear paw pinch. Buprenorphine (0.01-0.05 mg/kg body weight) and carprofen (5mg/kg body weight) were administered preoperatively via subcutaneous injection. Incision sites were shaved and sterilized with povidine-iodine

topical antiseptic solution. Body temperature was maintained at 38°C by a heating pad, and anesthesia was maintained by isoflurane gas via a nose cone.

The trachea was exposed by a ventral neck incision. The trachea was freed from surrounding tissue and a saline-filled inflatable cuff (Fine Science Tools) was sutured around the trachea, two cartilage rings caudal to the larynx. The actuator tube of the cuff was plugged and routed subcutaneously to the dorsal surface of the animal, externalized via an incision between the scapulae. The tissue exposed in the ventral incision was pulled over the cuff and the skin was sutured closed. The skin at the dorsal incision was sutured closed and the tube was secured in place by tying the ends of the suture around a bead on the tube. Rats were then administered warm normal saline (0.01-0.02 ml/g body weight) and isoflurane anesthesia was gradually reduced. The animal was placed in a recovery cage on a heating pad and was returned to the Animal Care Facilities once fully mobile. Postoperative analgesia was provided for two to three days using buprenorphine (0.01-0.05 mg/kg body weight) and carprofen (5mg/kg body weight) given every 24 hours. Animals were allowed a full week of recovery before experiments began.

Protocol

The animals were retrieved from the Animal Care Facility on the morning of Experiment Day 1 and were taken to the laboratory. Each animal was placed on the EPM in a sound-proof room. The animal remained on the EPM for 5 minutes where it was allowed to explore the two open arms and two closed arms freely and undisturbed. The animal's movement was tracked with a video recording device and details of its movement were analyzed with the EPM software. At the end of the 5 minutes the animal was removed from the EPM and the EPM was cleaned with alcohol wipes. After

all animals completed the maze trial they were brought to the testing laboratory and placed in one of two recording chambers placed side by side and separated by a visual barrier. During this acclimatization period lasting 15 minutes, the externalized actuator tube of the tracheal cuff from each animal was connected to a saline-filled syringe outside the chamber but no pressure was applied to the syringe. At the end of the 15 min protocol animals were returned to their home cages and the chambers were cleaned with alcohol wipes. Once all animals had completed the acclimatization protocol they were returned to the Animal Care Facility.

The animals were retrieved from the Animal Care Facility on the morning of Experiment Day 2 and were brought to the testing laboratory. They were divided into two groups: experimental (n=13) and control (n=12). Tails were marked with colors to identify group and unique animal number. One experimental and one control animal were placed individually in the two recording chambers and the actuator tube was connected to a saline-filled syringe. The experimental animal was allowed to rest undisturbed in the chamber for 2.5 minutes, followed by a series of cuff inflations. The syringe and cuff were pressure calibrated so a known amount of fluid movement in the syringe would result in pressure required to fully compress and occlude the trachea. Removing the pressure allowed for full recovery of the trachea with no interference to breathing. The cuff of the experimental animal was inflated 3-6 seconds with at least 15 seconds separating the occlusions for ~35 trials in a 15 minute period. The ITTO trials were applied in a random time pattern. After the final ITTO trial, the experimental animal was allowed to rest undisturbed in the recording chamber for 2.5 minutes. The control animal remained undisturbed for the duration of the 20 minute protocol. At the end of

the 20 minutes, both animals were removed from the chambers and returned to their cages. The recording chambers were cleaned with alcohol wipes between animals. The next control and experimental animals were placed in the recording chambers and the 20 minute protocol was repeated. This was continued for all animals. All animals were returned to the Animal Care Facility at the end of Experiment Day 2. This procedure was repeated daily for Experiment Days 3-11. Before the protocol on Experiment Day 11, all animals completed the EPM test for the second time.

The animals were brought to the laboratory on Experiment Day 12 and they were allowed to remain undisturbed for 2 hours. Each animal was removed from its cage in a random order and placed in a chamber with isoflurane gas (5% in O_2). It was removed from the chamber in an anesthetized state, within 1.5 min, and sacrificed. Adrenal glands were removed and weighed. Trunk blood was collected. Clotted blood was centrifuged at 4°C for 15 minutes, and plasma was extracted and stored at -80°C. Plasma Cort (ng/ml) was measured using a radioimmunoassay (RIA) kit (rat Cort 125I, MP Biomedicals).

Analyses

Adrenal gland weight was normalized by dividing adrenal weight by the animal's body weight on the day the adrenals were removed, and these values were compared between groups using a t-test (significance at $p<0.05$). Blood Cort levels were also compared between groups using a t-test (p-value <0.05). Percent time spent in the open, closed and center regions of the EPM during the test were obtained for each animal on the first and ninth day of ITTO conditioning. Percent times were analyzed (Pellow *et al.*, 1985; Rodgers & Dalvi, 1997; Korte & De Boer, 2003) for each maze region using a one-way repeated measures analysis of variance (RMANOVA),

comparing group (experimental vs. control) and day (pre- vs. post-ITTO conditioning). Differences were significant when $p<0.05$, and post-hoc comparisons were performed using Tukey's HSD with overall significance of $p<0.05$. All data are reported as means ± SEM.

Results

Figure 4-1 shows averages for basal plasma Cort levels in the control (2.9 ± 1.2 µg/dl) and experimental (5.2 ± 1.5 µg/dl) groups after 10 days of ITTO ($p=0.02$). The difference in group averages for adrenal/body weight is shown in Figure 4-2 (control: 0.14 ± 0.009; experimental: 0.17 ± 0.006; $p=0.03$). The group averages for the percent time spent on the open arms, closed arms, and center of the EPM during the 5 minute protocol are shown for values obtained before (Pre-Tx) and after (Post-Tx) ITTO conditioning in Figure 4-3. There were no group differences for parameters measured Pre-Tx or Post-Tx, and there were no Pre-Post differences for parameters measured in the control group; however, the percent time spent on the open arms of the maze Post-Tx was significantly less than during Pre-Tx for the experimental group ($p=0.05$). There were no Pre-Post differences in the experimental group for time spent on the closed arms or on the center of the EPM.

Discussion

Ten days of uncued, uncontrollable ITTO elicited elevated Cort levels and increased adrenal weight to body weight ratios in conscious rats. Increased anxiety, measured by the decreased time in the open arms of the EPM, in the experimental group was also observed after 10 days of ITTO. The results of this study suggest that repeated exposure to tracheal occlusions in conscious animals can cause elevated resting stress and anxiety levels in animals after only 10 days of conditioning. These

results support the link between intense respiratory stimuli, stress, and anxiety reported with COPD.

We assessed resting blood Cort in the morning when the levels were lowest (Windle *et al.*, 1998), and obtained adrenal to body weight ratios to corroborate the Cort measures. The combined results support the hypothesis that repeated bouts of ITTO augment basal HPA activation. This is similar to stress responses elicited by a variety of stimuli (Fernandes *et al.*, 2002; Armario, 2006; Filipovic *et al.*, 2010). Our study indicated that ITTO is a respiratory stimulus sufficient to evoke the same kind of stress responses seen with exposure to other stressful stimuli. It is clear that the stress response pattern to ITTO including adrenal hypertrophy and increased basal Cort is similar to that of other unavoidable intense stressors such as restraint and shock (Raone *et al.*, 2007), chronic variable stress (Herman *et al.*, 1995), and intermittent restraint (Fernandes *et al.*, 2002), suggesting that repeated respiratory obstructions qualify as stress-evoking stimuli. These responses are mediated by both physical and psychological neural pathways, further suggesting that consciousness plays an important role in the stress response to respiratory stimuli.

Ten days of ITTO in conscious rats led to increased anxiety. It is known that uncontrollable stress causes blood Cort to increase (Levine, 2000), and sustained HPA activation is linked with affective disorders such as anxiety and depression (Roy-Byrne *et al.*, 1986; Kling *et al.*, 1991; Stenzel-Poore *et al.*, 1994; Maier & Watkins, 2005). Anxiety and depression share similar behavioral characterizations such as disrupted sleep patterns, enhanced fear responses, fatigue, cognitive deficits, and weight loss due to decreased appetite (Stenzel-Poore *et al.*, 1994; Frazer & Morilak, 2005). In humans,

anxiety and depression often have overlapping symptoms according to DSM-IV (APA, 1994). Furthermore, depression is accompanied by hypercortisolism in some individuals (Roy-Byrne *et al.*, 1986; Vreeburg *et al.*, 2010), and anxiogenic behavior is enhanced by CRH overproduction (Stenzel-Poore *et al.*, 1994) and correlated with elevated plasma Cort levels in animals (Pellow *et al.*, 1985). Although we did not test our animals for depression after 10 days of ITTO, it is possible that they develop anxiety and depression behaviors since both are linked with HPA dysregulation and share similar behavioral characteristics.

The PVN projects to the dorsal raphe (DR) (Geerling *et al.*, 2010), and the caudal DR is excited by CRH (Lowry *et al.*, 2000; Hammack *et al.*, 2002). Serotonin (5-HT) is released by the DR and is subject to CRH input (Valentino *et al.*, 2009); 5-HT plays a role in stress-induced CRH and ACTH secretion (Jorgensen, 2007). 5-HT and its receptors have important implications in the psychological and behavioral responses to uncontrollable stressors (Maier, 1984; Graeff, 1994; Graeff *et al.*, 1996; Maier & Watkins, 2005; Jorgensen, 2007; Valentino *et al.*, 2009). According to Graeff and colleagues (Graeff *et al.*, 1996), stress activates the DR which releases 5-HT into the periaqueductal gray (PAG) and amygdala, causing immediate escape behaviors that ultimately become anxiety behaviors through conditioning or learning. When a stressor is unavoidable, 5-HT released in the hippocampus helps the animal adapt to the stress. If this pathway becomes dysfunctional, perhaps resulting from repeated activation with chronic stress, depression behaviors emerge (Graeff *et al.*, 1996). Serotonergic pathways stimulated by stress likely play a role in the ITTO conditioning responses seen in our animals, both with HPA axis and anxiety behaviors.

During the 10 days of ITTO conditioning, we observed the animals decreasing their exploratory behavior and withdrawal fear responses to ITTO, and developing immobility throughout the 20 minute ITTO trial. These responses are consistent with the animals developing depression behaviors. However, we did not test for depression and future studies need to include measures of depression. In another study done by our laboratory we have found alterations in 5-HT receptor gene expression in thalamic neural areas after one exposure to ITTO (Bernhardt *et al.*, 2008) and after 10 days of ITTO (Bernhardt *et al.*, 2010). It appears that serotonergic signaling is modulated in response to ITTO conditioning, likely from activation of stress responses (Maier, 1984; Graeff, 1994; Graeff *et al.*, 1996; Maier & Watkins, 2005; Jorgensen, 2007; Valentino *et al.*, 2009). Changes in serotonergic and stress pathways may suggest that ITTO can modulate behavioral load compensation, control of respiration (Hodges *et al.*, 2009) and respiratory neural plasticity (Feldman *et al.*, 2003; Doi & Ramirez, 2008). The PVN also projects to the brainstem respiratory network, including the nucleus ambiguus (nA) and the nucleus of the solitary tract (nTS) (Geerling *et al.*, 2010), and the nTS has both direct and indirect projections to the PVN (Hermes *et al.*, 2006). This loop may be useful in modulating respiratory motor output during situations of both respiratory and non-respiratory related stress. The afferents activated by ITTO likely have direct projections to the PVN that influence the internal physical stress response, while activated limbic regions may mediate the psychological stress response that includes the HPA axis (Jankord & Herman, 2008).

It is significant that conscious animals were used in these experiments because consciousness introduces a behavioral component to the stress response and is

important in the cognitive processing of sensations associated with ITTO. Tracheal occlusions are infinite resistive loads, and breathing through resistive loads causes individuals to experience sensations of discomfort, known as dyspnea (von Leupoldt & Dahme, 2005b; O'Donnell *et al.*, 2007; Schon *et al.*, 2008; von Leupoldt *et al.*, 2008). Dyspnea is a common symptom of respiratory obstructive diseases (von Leupoldt & Dahme, 2005a; O'Donnell *et al.*, 2007), and includes both a discriminative and affective component (von Leupoldt & Dahme, 2005b). The discriminative component is relayed to the somatosensory brain network, which is processed in a similar fashion in both humans and animals (Davenport *et al.*, 1985; Davenport *et al.*, 1991; Davenport *et al.*, 1993; Davenport & Hutchison, 2002). The affective component is relayed to parts of the limbic neural network and shares similarities with the affective component of pain via processing in the insular and anterior cingulate cortices (Aleksandrov *et al.*, 2000; von Leupoldt & Dahme, 2005a; Schon *et al.*, 2008). Thus, although dyspnea cannot be assessed in animals as it is in humans, the neural correlates that mediate dyspnea are similar. It is therefore plausible that respiratory obstructions in animals cause intense sensations of discomfort, leading to an experience of negative affect. Over time, this negative affect may result in disorders such as anxiety or depression, which are both highly prevalent in patients with COPD who also experience dyspnea (Karajgi *et al.*, 1990; Brenes, 2003; Wagena *et al.*, 2005). Dyspnea is a highly aversive sensation and individuals modify their behavior in order to adapt or avoid experiencing the sensation. Patients diagnosed with respiratory obstructive diseases often lead sedentary lifestyles (ZuWallack, 2007; Bourbeau, 2009) and sedentary behavior can be a symptom of

depression (Roshanaei-Moghaddam *et al.*, 2009). Determining if our animals experience symptoms of depression as a result of ITTO is an important future study.

We hypothesized that repeated ITTO would change basal stress levels in animals. We measured blood Cort the morning after the last exposure to ITTO. Habituation of the acute HPA response to a homotypic stressor and sensitization to new stressors after a chronic stress paradigm has been reported (Fernandes *et al.*, 2002; Grissom *et al.*, 2007). It is possible that animals in the current study could express a blunted acute Cort and PVN CRH response to ITTO after multiple days of conditioning, but these responses would likely be increased if the animals experienced a novel stress such as shock or restraint stress. These results suggest increased stress reactivity in individuals who experience airway occlusions or intermittent, unpredictable increases in airway resistance on a regular basis.

In general, one trial on the EPM is accepted as an indicator of anxiety levels resulting from treatment differences. Behavior during a second exposure to the EPM is thought by some to originate from other factors such as the animal's fear of heights, and is resistant to anxiolytics during a short but not long test duration (File, 1993; File *et al.*, 1993). However, according to Treit and colleagues (Treit *et al.*, 1993), open arm avoidance during the EPM test was not related to a fear of heights. This group also found that animals do not habituate to repeated exposures to the EPM, even after forced exploration of the open arms. In the present study, a two-exposure protocol was valid if used in conjunction with our appropriate controls. Hence, the decreased open arm time after ITTO conditioning in the experimental animals supports our hypothesis that repeated exposure (10 days) to unexpected, unavoidable, uncontrollable ITTO

produces state changes in conscious animals characterized by increased anxiety and stress.

ITTO performed once a day for 10 days lead to elevated basal HPA activation, anxiety, and potentially depression behavior. Intense, unavoidable respiratory stimuli like tracheal occlusions likely produce intense physical discomfort and severe psychological stress due to the life-threatening quality of ITTO, which is relevant for individuals who experience respiratory stress on a regular basis, such as those with COPD and asthma. Chronic experience with one type of stress can make an individual more reactive to new types of stress, a dangerous fact when patients with asthma and COPD have other physiological and psychological symptoms that could be worsened by airway obstruction-dependent HPA activation. Determining the links between respiration, sensations, stress, and psychological state is extremely important work that needs to be continued.

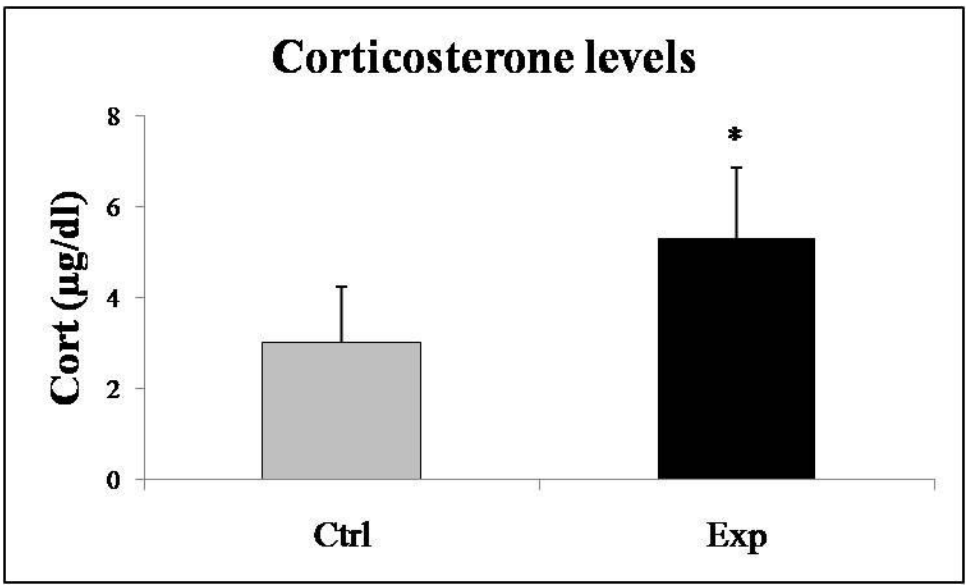

Figure 4-1. Basal plasma corticosterone levels (µg/dL) after 10 days of ITTO training (Exp) or handling without tracheal occlusions (Ctrl). The difference in group averages was significant (*p=0.02).

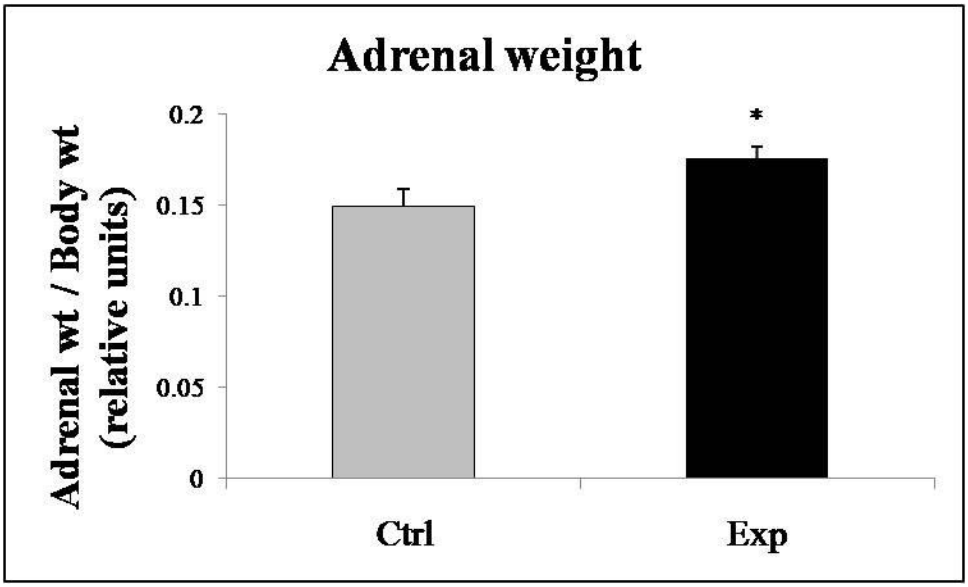

Figure 4-2. Adrenal weight normalized to animal body weights after 10 days of ITTO (Exp) or handling without tracheal occlusions (Ctrl). The difference in group averages was significant (*p=0.03).

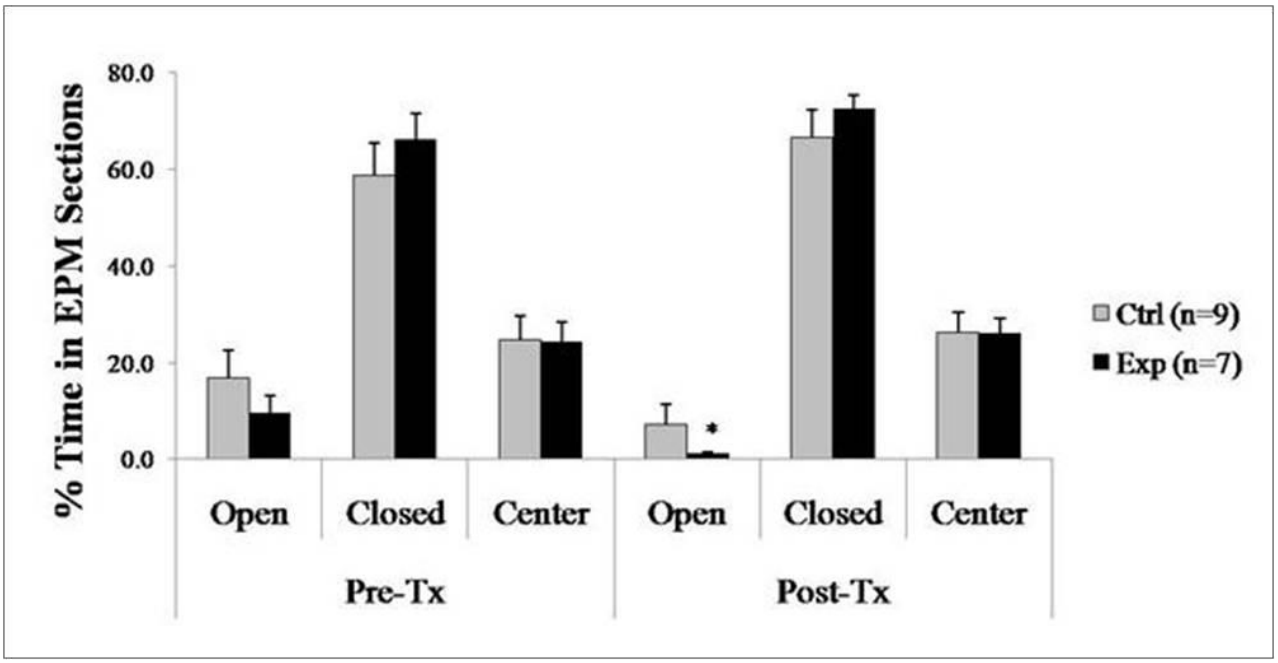

Figure 4-3. Percent time spent in each section of the EPM before (Pre-Tx) and after (Post-Tx) 10 days of ITTO (Exp) or handling without tracheal occlusions (Ctrl). Time spent on the open arms of the EPM Post-Tx was significantly different from time spent on the open arms of the EPM Pre-Tx in Exp animals (*p=0.05).

CHAPTER 5
NEURAL PLASTICITY IN RESPONSE TO REPEATED TRACHEAL OCCLUSIONS

Introduction

The complexity of the respiratory system enables it to respond to a variety of mechanical, metabolic, and voluntary stimuli, allowing for the continuation of ventilation through changes in breathing pattern. Early respiratory studies focused on defining normal respiratory patterns and control mechanisms and were primarily conducted in anesthetized animals where reflexes and metabolic needs drive the system. For example, mechanically challenging the respiratory system using resistive loads to breathing causes respiratory load compensation, characterized by a decrease in loaded phase volume and increase in the phase duration, and the unloaded phase of the breath remains unchanged (Zechman *et al.*, 1976). This response is a vagal-dependent reflex (Clark & von Euler, 1972; Zechman *et al.*, 1976). Others have described further components of the load compensation reflex in anesthetized animals including abdominal muscle activity (Koehler & Bishop, 1979), pulmonary stretch receptor (PSR) discharge (Davenport *et al.*, 1981a; Davenport *et al.*, 1984; Davenport & Wozniak, 1986), influence of neural activation (Zhang *et al.*, 2009), and the importance of upper airway afferents during loading (Webb *et al.*, 1994, 1996).

Consciousness and behavior play a major role in modulating breathing, however, and voluntary control of breathing is capable of overriding the expected responses to metabolic needs. Studies in conscious animals are less numerous, but they add a vital component to the understanding of respiratory load compensation. Respiratory loads elicit respiratory sensations that are detected in conscious animals presented with inspiratory resistive loads (Davenport *et al.*, 1991). Respiratory loads also elicit cortical

evoked potentials in conscious lambs (Davenport & Hutchison, 2002), suggesting the load compensation response in conscious states may include suprapontine cognitive behavioral components. The pattern of behavioral load compensation in conscious animals is variable (Hutt *et al.*, 1991; Forster *et al.*, 1994; Watts *et al.*, 1997) and differs from the pattern seen in anesthetized animals. Also unlike anesthetized animals, conscious animals do not require pulmonary or diaphragm afferents to respond to resistive loading (Forster *et al.*, 1994), and respiratory muscle activity is not suppressed by serotonergic inhibition in conscious animals (Sood *et al.*, 2006). The neural mechanisms responsible for these differences are unclear.

Most of what is known about the neural control of breathing has been determined from acute studies in anesthetized animals using immunohistochemical or electrophysiological methods. In anesthetized rats, electrical stimulation of the phrenic nerve causes neural activation, indicated by c-Fos immunohistochemistry, in the nucleus of the solitary tract (nTS), rostral ventral respiratory group (rVRG), and ventrolateral medullary reticular formation (Malakhova & Davenport, 2001). These nuclei contribute to vagal efferents and are termination sites of vagal afferents, which transduce information to and from the airways and lungs (Kalia & Mesulam, 1980b, a; Kalia, 1981b, a). In a related study, c-Fos was found in the periaqueductal gray (PAG) in response to superior laryngeal nerve (SLN) stimulation (Ambalavanar *et al.*, 1999). Afferents from tracheal receptors travel in the SLN and recurrent laryngeal nerve (RLN) (Traxel *et al.*, 1976; Sant'Ambrogio *et al.*, 1977; Lee *et al.*, 1992), respond to changes in pressure (Traxel *et al.*, 1976; Sant'Ambrogio & Mortola, 1977; Citterio *et al.*, 1985) and stretch (Davenport *et al.*, 1981c), and have cortical projections via various relay nuclei

(O'Brien *et al.*, 1971; Fukuyama *et al.*, 1993). The PAG is a region involved in defensive behaviors (Brandao *et al.*, 1994; Vianna *et al.*, 2001). Electrical stimulation and chemical disinhibition of the dorsal PAG (dPAG) in anesthetized animals activates respiration (Hayward *et al.*, 2003; Zhang *et al.*, 2005), and respiratory activation is greater with caudal compared to rostral dPAG stimulation (Zhang *et al.*, 2007). Furthermore, chemical activation of the dPAG modulates the brainstem volume-timing reflex in response to inspiratory and expiratory occlusions in anesthetized rats (Zhang *et al.*, 2009). The lateral parabrachial nucleus (lPBN) has been shown to mediate the respiratory responses evoked by the dPAG (Hayward *et al.*, 2004), and is a potential site during voluntary breath holding where suprapontine efferents are integrated, resulting in a cohesive response that exerts inhibitory control over brainstem respiratory nuclei (McKay *et al.*, 2008). Although brainstem and suprapontine nuclei important in mediating acute respiratory responses in anesthetized animals would be expected to play a role in mediating respiratory responses in conscious animals, their exact role is unknown. It is additionally unknown whether neural activity in these nuclei may increase or decrease in response to repeated respiratory stimuli.

Our laboratory has developed a method to study the load compensation reflex in conscious rats via intrinsic, transient and reversible tracheal occlusions (ITTO). ITTO is performed by inflating a rubber cuff around the trachea with enough pressure to reversibly close the lumen of the trachea. Tracheal squeeze with ITTO is expected to activate lung, airway, and muscle afferents that project to central neural structures and elicit load compensation. In addition, because a conscious animal's response to respiratory stimuli can be voluntarily modulated, higher brain centers were expected to

increase activity as a result of repeated ITTO. The somatosensory cortex, an area responsible for processing discriminative components of stimuli, has increased activity in response to phrenic (Davenport *et al.*, 1985; Davenport & Vovk, 2009) and intercostal (Davenport *et al.*, 1993) nerve stimulation, and inspiratory occlusion in conscious lambs (Davenport & Hutchison, 2002). The ventroposterior (VP) thalamic complex is also an important area in discriminatory processing, regulating activation in the somatosensory cortex (Rausell *et al.*, 1992). Phrenic nerve-evoked cortical activation in the cat is relayed through the VP (Yates *et al.*, 1994; Zhang & Davenport, 2003), and the VP responds to somatosensory stimuli depending upon body region (Bushnell *et al.*, 1993). It was expected that the VP would be involved in encoding the discriminatory components of ITTO.

The anterior insular cortex (AI) is part of an animal's limbic system and is known to be involved in affective sensory processing (Davenport & Vovk, 2009). The AI activates respiration upon stimulation (Aleksandrov *et al.*, 2000), and becomes active when conscious humans breathe through large resistive loads (von Leupoldt & Dahme, 2005a; von Leupoldt *et al.*, 2008). The symptom of dyspnea, or difficult and uncomfortable breathing, is often associated with respiratory obstructive diseases and is accompanied by activation in the AI (Banzett *et al.*, 2000; Evans *et al.*, 2002; von Leupoldt & Dahme, 2005a; von Leupoldt *et al.*, 2008) and amygdala (Evans *et al.*, 2002; von Leupoldt *et al.*, 2008). The amygdala is also involved in affective sensory processing and is vital in fear conditioning (LeDoux *et al.*, 1988; Beck & Fibiger, 1995; Campeau & Davis, 1995; Day *et al.*, 2008), stress responses (Bremner *et al.*, 1996; Chowdhury *et al.*, 2000), and is activated by phrenic nerve stimulation (Malakhova &

Davenport, 2001). The affective or emotional component evoked by ITTO should result from activation in the AI and amygdala.

To determine the neural substrates that may mediate behavioral and reflex load compensation, we stained neural tissue for cytochrome oxidase (CO), an enzyme involved in oxidative metabolism in the electron transport chain. CO is used to indicate changes in brain steady state activity levels in response to stimuli (Wong-Riley, 1979; Wong-Riley, 1989; Gonzalez-Lima & Garrosa, 1991; Hevner *et al.*, 1995), and is therefore best used in studies of prolonged rather than acute duration. CO can reveal adaptation within brain nuclei, and neural adaptation has been documented in response to inspiratory resistive loading in humans (Gozal *et al.*, 1995). CO staining would help determine increases or decreases in neural activity in response to repeated ITTO, significantly advancing our understanding of the neural areas mediating behavioral load compensation. We designed a 10 day ITTO protocol in conscious rats that would elicit and behaviorally condition the animal to respiratory load compensation. It was hypothesized that 10 days of ITTO would induce state changes in respiratory brainstem nuclei, areas involved in stress responses, and suprapontine nuclei involved in discriminative and affective respiratory information processing.

Methods

Animals

These experiments were performed on 10 male Sprague-Dawley rats (363.2 ± 38.3 g). The animals were housed two to a cage in the University of Florida animal care facility where they were exposed to a 12 h light/12 h dark cycle. The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the University of Florida.

Surgical Procedures

Animals were initially anesthetized using isoflurane gas (2-5% in O_2) administered in a whole-body gas chamber. Anesthetic depth was verified by the absence of a withdrawal reflex from a rear paw pinch. Buprenorphine (0.01-0.05 mg/kg body weight) and carprofen (5mg/kg body weight) were administered preoperatively via subcutaneous injection. Incision sites were shaved and sterilized with povidine-iodine topical antiseptic solution. Body temperature was maintained at 38°C by a heating pad, and anesthesia was maintained by isoflurane gas via a nose cone.

The animal was placed in a supine position and the trachea exposed by a ventral neck incision. The trachea was freed from surrounding tissue and a saline-filled inflatable cuff (Fine Science Tools) was sutured around the trachea, two cartilage rings caudal to the larynx. The actuator tube of the cuff was plugged with a blunt needle and routed subcutaneously dorsally and externalized through an incision between the scapulae. The tissue exposed in the ventral incision was pulled over the cuff and the skin was sutured closed. The skin at the dorsal incision was sutured closed and the tube was secured in place by tying the ends of the suture around a bead on the tube. Rats were then administered warm normal saline (0.01-0.02 ml/g body weight) and isoflurane anesthesia was gradually reduced. The animal was placed in a recovery cage on a heating pad and returned to the Animal Care Facilities once fully mobile. Postoperative analgesia was provided for two to three days using buprenorphine (0.01-0.05 mg/kg body weight) and carprofen (5mg/kg body weight) given every 24 hours. Animals were allowed a full week of recovery before experiments began.

Protocol

The animals were retrieved from the Animal Care Facility on the morning of Experiment Day 1 and were brought to the testing laboratory and placed in one of two recording chambers side by side and separated by a visual barrier for an acclimatization period of 15 minutes. During this time the externalized actuator tube of the tracheal cuff from each animal was connected to a saline-filled syringe outside the chamber but no pressure was applied to the syringe. Chambers were cleaned with alcohol wipes between each animal. Animals were then returned to the Animal Care Facility.

Training

The animals were retrieved from the Animal Care Facility on the morning of Experiment Day 2 and were brought to the laboratory. They were randomly divided into two groups: Experimental (n=5) and Control (n=5). One experimental and one control animal were placed individually in the two recording chambers and the actuator tube was connected to a saline-filled syringe. The experimental animal was allowed to rest undisturbed in the chamber for 2.5 minutes, followed by a series of cuff inflations. The syringe and cuff were pressure calibrated to a known amount of fluid movement in the syringe that would result in the pressure required to fully compress and occlude the trachea. Removing the pressure allowed for full recovery of the trachea with no interference to breathing. The 10 minute protocol consisted of twenty 10 second inflations. The trials were applied in a random time pattern. After the last trial, the experimental animal was allowed to rest undisturbed in the recording chamber for 2.5 minutes. The control animal remained undisturbed for the duration of the 15 minute protocol. At the end of the 15 minutes, both animals were removed from the chambers and returned to their cages. The recording chambers were cleaned with alcohol wipes

between animals. The next control and experimental animals were placed in the recording chambers and the 15 minute protocol was repeated. This was continued for all 5 pairs of animals. This protocol was repeated once daily for Experiment Days 2-11.

Experiment Day 12 began by retrieving the animals from the Animal Care Facility and bringing them to the testing laboratory where they were allowed to remain undisturbed for 2 hours. Each animal was removed from its cage in a random order and placed in a chamber with isoflurane gas (5% in O_2). It was removed from the chamber in an anesthetized state and brains were removed, blocked into three pieces via coronal cuts, and placed in 4% paraformaldehyde.

Cytochrome oxidase (CO)

The blocked brains remained in the paraformaldehyde solution for 3 days, and then were transferred into a 30% sucrose solution for 2 days. Brains were cut with a microtome and the tissue was placed one slice per well in 24-well plates. Each well was filled with phosphate buffer saline, pH 7.4 (PBS), and the tissue remained in the PBS for 5 days. One full series (column) of tissue per animal brain was used for the staining protocol, adapted from (Wong-Riley, 1979). Briefly, the CO solution was made [600 ml PBS, 60 g sucrose, 300 mg cytochrome C (Sigma, C2506), 200 mg catalase (Sigma, C9322) and 150 mg diaminobenzidine (Sigma, D5905)] and applied to the tissue. All plates were put on a shaker and covered to prevent light exposure. They remained on the shaker overnight, were removed 17 hours later and transferred into PBS. The following day, tissue was mounted on glass microscope slides, dehydrated with ethanol, cleaned with xylene, and coverslipped with Eukitt.

Analyses

Imaging

Slides of brain tissue were viewed using light microscopy (Zeiss Axioplan 2), images were captured using a computer software system (ImagePro Plus), converted to 8-bit mono images, and stored. During each capture session the image of a blank slide was also captured using the same settings. A standard optical density calibration (arbitrary units) was created by setting black levels to zero and using the image of the blank slide as the incident light reference. This corrects for differences in background light levels between capture sessions where different settings may have been applied. The standard calibration was applied to all relevant images before any measurements were made. Three line readings were taken from within a nucleus, defined by stereotaxic coordinates (Paxinos & Watson, 1997), for at least one slide per nucleus per animal (Hevner *et al.*, 1995). This results in at least three readings per nucleus per animal that were averaged. The intensity of staining in each nucleus was determined for each animal. Those values were normalized to the average intensity of staining in an area of white matter (optic tract) for that animal to control for variability between staining batches.

Statistics

The normalized values were combined according to group and were used to conduct a t-test for each nucleus analyzed. Statistical significance was determined at p-values < 0.05. The group means ± SEM are reported.

Results

Brainstem

In the brainstem, the primary respiratory neural areas included the VRG and nTS. The level of CO staining measured in the rostral nTS in experimental animals was significantly greater than in controls (Figure 5-1; p=0.01). There were no significant differences in staining intensities between groups in the cnTS, VRG, AP, lPBN, or locus coeruleus (LC).

Midbrain

Staining intensities in the PAG and the dorsal raphe (DR) (Table 5-2) were greater in the experimental group compared to control, and differences between group averages were statistically significant for all areas except the rostral dPAG (p=0.06). The most significant difference between group averages in all nuclei was seen in the caudal ventral PAG (vPAG) (Figure 5-2; p=0.004).

Higher Brain Centers, Discriminative & Affective

The amount of CO staining in the experimental group was significantly greater compared to control in the ventroposteromedial thalamic nucleus (VPM), and the agranular insular cortex (AI) (Figures 5-3, 5-4; p=0.02). There were no significant differences in average CO staining between groups in the arcuate (Arc), PaMP, centromedial thalamus (CM), ventroposterolateral thalamus (VPL), cingulated cortex (Cg1), or central nucleus of the amygdala (CeA).

Discussion

The load compensation response in conscious animals arises as a result of activated reflexive respiratory pathways and the modulation of these pathways by conscious input. Ten days of repeated ITTO in conscious rats caused significant state

changes in the rostral nTS, PAG, DR, VPM and AI. This is the first evidence of neural compensation in response to respiratory load conditioning in conscious animals. Other techniques of conscious respiratory loading include tracheal banding (Greenberg *et al.*, 1995; Rao *et al.*, 1997), restricting airflow to a head chamber (Farre *et al.*, 2007), and applying loads via a tracheostomy (Davenport *et al.*, 1991; Osborne & Road, 1995; Davenport & Hutchison, 2002) or facemask (Davenport & Hutchison, 2002). Tracheal banding is a sustained increase in airway resistance that leads to compensation from both mechanical and chemical activation. Restricting airflow via a head chamber, tracheostomy, and facemask are extrinsic airway resistances and are often implemented acutely. The model used by our laboratory is unique in that it allows for intrinsic, transient, tracheal occlusions in conscious animals with a return to normal airway resistance between cuff inflations.

Increases in neural metabolic activity after 10 days of ITTO were seen in the experimental group compared to control in the rostral nTS but not the brainstem respiratory nuclei such as the VRG or caudal nTS. The caudal nTS is the primary nucleus targeted by cardiorespiratory afferents and is involved in respiratory control (Kubin *et al.*, 2006), whereas the rostral nTS is involved in gustatory sensory processing. In other studies in our laboratory (unpublished results), we have shown that ITTO elicits transient changes in blood pressure that temporally correspond with each occlusion. This is likely due in part to sympathetic activation and in part to the hemodynamic response to such large negative intrathoracic pressures. During each occlusion, afferents activated in the lung, airway, trachea, and blood vessels ascend to the caudal nTS. In addition, it is possible that afferents from chemoreceptors might also

ascend to the nTS if the 10 second tracheal occlusions are long enough to cause a slight degree of hypoxia by the end of the occlusion. Farre and colleagues (Farre *et al.*, 2007) found a 10 s obstruction reduced the SpO_2 to 83% in rats, and results from Yasuma et al. (Yasuma *et al.*, 1991) showed increased arousal in dogs at higher SaO_2 levels when resistive loads were also applied. The mechanical stimulation may enhance chemical stimulation, which would be relayed to the nTS by carotid body afferents traveling in the glossopharyngeal nerve. With ITTO, the caudal nTS should receive great amounts of intermittent, convergent mechanical and potential chemical input. This input was not sufficient to induce permanent modulatory changes in activity level within the caudal nTS after 10 days of ITTO, however. The observation of increased basal activity in the rostral nTS due to ITTO conditioning could be related to the proximity of the cuff to the pharynx and oral cavity, in which are taste receptors sending sensory information to the rostral nTS. Rostral nTS activation could also be related to the fact that cranial nerves relaying gustatory information to the rostral nTS are also involved in mediating visceral sensations, some potentially related to cuff inflation, and that this overlap resulted in the increase in CO activity seen in these results.

Plasticity is known as a “persistent change in the neural control system based on prior experience” (Mitchell & Johnson, 2003). Neural modulation in the respiratory circuit is known to occur in response to respiratory stimuli (Baker *et al.*, 2001; Mitchell & Johnson, 2003), and also occurs in other brain structures (Huang & Kandel, 1994; Feldman *et al.*, 1999). The nTS is an area well-known for its plastic abilities (Chen *et al.*, 2001; Kline, 2008), and it is possible that the rostral nTS alters basal neural activity as an adaptation to the intermittent convergent input caused by repeated ITTO

conditioning. This could indicate that the nTS changes its state for conditioned load compensation to subsequent airway obstruction challenges, allowing for adaptation of physiological responses to the severe respiratory challenge posed by repeated ITTO. McKay and colleagues (McKay *et al.*, 2003) suggest that the brainstem respiratory network, including the nTS, may be an important site for the voluntary control of breathing in humans. Thus, the nTS may also be an important site for the voluntary modulation of breathing in animals, with the rostral division undergoing plastic changes as a result of ITTO conditioning and potentially generating a new response pattern for the animal.

Veening and colleagues (Veening *et al.*, 2009) found increased c-Fos expression in the AP as a result of an anxiety-producing paradigm, and the AP projects to the nTS (Bonham & Hasser, 1993). No group differences were seen in the present study in the AP as a result of ITTO, indicating it did not undergo changes in baseline activity; however, that does not rule out a potential role for the AP in the acute response to ITTO. The AP projects to the lPBN (Herbert *et al.*, 1990), and neurons in the nTS activated by vagal afferents send projections to pontine respiratory groups, including to the lPBN (Herbert *et al.*, 1990; Ezure *et al.*, 1998). The lPBN is involved in PAG-activated cardiovascular (Hayward *et al.*, 2004; Hayward, 2007) and respiratory responses (Hayward *et al.*, 2004), and the PAG has projections to other areas like the medulla that also mediate these responses (Cameron *et al.*, 1995; Farkas *et al.*, 1998). The lack of group differences in CO staining seen in the lPBN suggests that there was no change in steady state activity after 10 days of ITTO, but this does not mean the lPBN was not activated during ITTO or that this activation could not lead to steady state

changes in interconnected nuclei. The lPBN is not a brain region where a change in basal activity is often seen, but the lPBN is known to play a role in plasticity in other neural areas (Lopez de Armentia & Sah, 2007). Surprisingly, no change in baseline activity was seen in the LC in response to 10 days of ITTO. The LC is involved in the noradrenergic response to stressful stimuli (Bremner *et al.*, 1996; Van Bockstaele *et al.*, 2001), and our laboratory has shown that ITTO is a stress-evoking stimulus (unpublished results). The LC receives efferent projections from the CeA, nTS, and the PAG (Van Bockstaele *et al.*, 2001). There can be opposing actions on the LC from these nuclei and others in response to different types of stressors (Van Bockstaele *et al.*, 2001); excitatory and inhibitory inputs to this area may prevent an alteration in steady state activity. Alternatively, a change in baseline neural activity in the LC may not be a necessary response to repeated ITTO. A study looking at the fMRI response to inspiratory loading in humans showed that the level of activation in regions corresponding with the LC decreased upon the second presentation of the load (Gozal *et al.*, 1995). Perhaps the LC initially responds to ITTO by increasing activity but does not remain active if the stimulus is not present, having no effect on baseline activity levels.

The midbrain PAG was the brain region with the largest differences in staining between groups. The PAG is involved in defensive behaviors (Bandler & Carrive, 1988; Brandao *et al.*, 1994; Vianna *et al.*, 2001), pain modulation (Mayer *et al.*, 1971; Behbehani, 1995), respiratory activation (Hayward *et al.*, 2003; Zhang *et al.*, 2005), and modulation of the load compensation response (Zhang *et al.*, 2009). Neural activation was observed in the PAG in response to SLN stimulation (Ambalavanar *et al.*, 1999),

which is one group of tracheal afferents likely stimulated by cuff inflation. After 10 days of ITTO we noticed the greatest differences in the caudal compared to the rostral PAG. Interestingly, the results of (Zhang *et al.*, 2007) showed greater respiratory activation in response to caudal vs. rostral dPAG stimulation. The caudal PAG has descending input to the nTS and the ventrolateral PAG innervates the VRG and the paraventricular hypothalamus (PVN) (Farkas *et al.*, 1998). The dPAG showed significantly increased steady state excitation in animals exposed to 10 days of ITTO, while increases in the vPAG approached significance. The response of the PAG may have contributed to the significant changes also seen in the rostral nTS. The dPAG and vPAG have been implicated in panic and anxiety, respectively (Carrive, 1993; Bandler & Shipley, 1994; Brandao *et al.*, 1994; Vianna *et al.*, 2001; Cunha *et al.*, 2010), and the vPAG appears to be involved in conditioned fear responses (Vianna *et al.*, 2001). Repeated ITTO may initially produce panicogenic responses that ultimately become anxiogenic as a result of contextual fear during ITTO conditioning in our rats.

The raphe nucleus is a main source of serotonin (5-HT) in the brain. The DR is the subdivision with the greatest serotonergic input to other nuclei, and the amygdala receives most of its 5-HT from the DR (Azmitia & Segal, 1978; Li *et al.*, 1990). After 10 days of ITTO the DR had elevated steady state activity, suggesting a potential role in the adaptive response to repeated severe respiratory stimuli. 5-HT has been implicated in respiratory (Lindsay & Feldman, 1993; Bianchi *et al.*, 1995; Pena & Ramirez, 2002; Hodges *et al.*, 2009) and cardiovascular control (Merahi *et al.*, 1992; Dergacheva *et al.*, 2009), and is also involved in respiratory plasticity (Baker *et al.*, 2001; Fuller *et al.*, 2001; Bocchiaro & Feldman, 2004; Doi & Ramirez, 2008). The DR and serotonin also

play a role in stress responses and mental disorders (Maier, 1984; Maier & Watkins, 2005; Jorgensen, 2007; Mizoguchi *et al.*, 2008). Many individuals with respiratory obstructive diseases have anxiety, and 5-HT is often pharmacologically manipulated to treat anxiety in these patients (Brenes, 2003). Also, learned helplessness caused by uncontrollable stressors can lead to sensitization of serotonergic neurons in the DR, which respond to future stressors with exaggerated 5-HT release that subsequently leads to behavioral changes (Maier & Watkins, 2005). ITTO is an unexpected, uncontrollable, life-threatening stressor, and the increase in steady state DR activity may be an indicator that the serotonergic system is sensitized as a result of our 10 day protocol. This has implications for individuals who experience transient uncontrollable respiratory stress, especially in the form of obstructive events occurring with asthma and chronic obstructive pulmonary disease (COPD). These individuals may be sensitized to exaggerated stress responses to other stimuli as well, creating an unhealthy physiological and psychological state.

Serotonergic influence is dependent upon the nucleus and the receptor(s) activated. It is generally considered that 5-HT1A and 5-HT1B receptor activation is inhibitory (Barnes & Sharp, 1999), and both are present in the CeA (Saha *et al.*, 2010). The amygdala shares a critical link with the PAG in the fear circuitry of the brain and is also involved in aversive responses (Brandao *et al.*, 1994; Davis *et al.*, 1994; LeDoux, 2000; Martinez *et al.*, 2006). The CeA is the site of many convergent inputs, including nociceptive afferents from the PBN and spinal cord (LeDoux, 2000). The basolateral and lateral amygdala act as the regulators of information received from aversive stimuli reaching the CeA, the motor output subnucleus of the amygdala (Campeau & Davis,

1995). Our laboratory has shown (Shahan *et al.*, 2008) that electrical stimulation of the CeA leads to an increase in respiratory rate in anesthetized rats, potentially through output to the PAG. We expected to see down regulation of CeA activity as a result of ITTO conditioning, potentially via DR activity, assuming increased DR activation after ITTO would result in greater 5-HT release in the CeA; however, there was no observable change in CeA activity. The CeA has inhibitory projections to the PAG and nTS via gamma aminobutyric acid (GABA) (Davis *et al.*, 1994; Saha *et al.*, 2010). Down-regulation of CeA GABA-ergic innervation could disinhibit the PAG (Saha, 2005; Oka *et al.*, 2008). Thus, if baseline activity in the CeA decreased, activation of the PAG and nTS could increase through disinhibition, potentiating the fear and anxiety component of behavioral respiratory load compensation observed after repeated airway obstruction experiences such as ITTO. This might allow the animal to modulate its physiological responses to repeated respiratory mechanical stressors or fearful stimuli via CeA plasticity. The lack of staining differences in the CeA suggests there are other neural areas playing a larger role in the modulation of responses.

The PVN is another nucleus known to respond to stressful stimuli. The PaMP is the neurosecretory division of the PVN responsible for producing corticotropin releasing hormone (CRH) (Vale *et al.*, 1981), and it projects to a number of brain regions including the DR, PAG, PBN, nTS, and VRG (Dampney, 1994; Geerling *et al.*, 2010). Because ITTO is a stressful stimulus (unpublished results), the PaMP was expected to have increased basal activity after 10 days of ITTO, however no significant differences between groups were found. According to Arnhold and colleagues (Arnhold *et al.*, 2007), the PaMP response to acute stress may be different from repeated stress. They

found decreased c-Fos expression in CRH-positive parvocellular neurons after repeated episodes of restriction-induced drinking, a stress-evoking stimulus. They suggested that the inhibition could be a result of conditioning. The lack of increased or decreased activity in the PaMP after ITTO conditioning may be due to the duration of the protocol. The 10 days of conditioning may be long enough that an increase in activity is no longer observed, but short enough that a persistent downregulation of activity has not yet occurred.

Subcortical neural networks are essential for maintaining normal cardiorespiratory function, generating reflexes, and adapting to recurring or prolonged stimuli. Some components of these networks can be voluntarily modulated in conscious animals via cortical input. An awake animal has two defined systems involved in sensory processing. The discriminative system encodes stimulus details such as intensity, timing, and location of the stimulus, whereas the affective system integrates the qualitative emotional aspects associated with the stimulus. The discriminative pathway includes a relay through the thalamus to the somatosensory cortex, and the affective pathway involves structures such as the amygdala, anterior cingulate (Cg1), and AI (von Leupoldt & Dahme, 2005b, a; Schon *et al.*, 2008; von Leupoldt *et al.*, 2008; Davenport & Vovk, 2009). Both these pathways are involved in respiratory sensations (von Leupoldt & Dahme, 2005b, a; Schon *et al.*, 2008; von Leupoldt *et al.*, 2008; Davenport & Vovk, 2009). With ITTO we have found long-term increases in activity in the VPM thalamus and AI. The VPM is an area that responds to facial stimuli. Prior experiments have found activation in the VPL in response to phrenic nerve stimulation (Yates *et al.*, 1994; Zhang & Davenport, 2003). It is possible that tracheal afferents activated upon cuff

inflation send projections beyond the brainstem to the VPM via polysnaptic pathways (Ambalavanar *et al.*, 1999). In addition, Cechetto and Saper (Cechetto & Saper, 1987) found that the VPM projects visceral sensory information to the AI, some of which includes cardiopulmonary afferent information. The present study shows changes in the activity state of the discriminatory cortical pathway but the afferents activated by ITTO mediating these changes remain unknown.

The affective AI had significantly increased CO staining in the experimental animals compared to controls. The AI has an excitatory projection to the PAG (Behbehani *et al.*, 1993), responds to respiration, arterial chemoreceptors, and cardiovascular baroreceptors (Cechetto & Saper, 1987), and is active during voluntary breath-holding maneuvers (McKay *et al.*, 2008). Aleksandrov and colleagues (Aleksandrov *et al.*, 2000) found a respiratory related area in the insular cortex that produced either excitatory or inhibitory respiratory responses when stimulated, depending upon whether the region was posterior or anterior, respectively. Of note, the AI also plays a vital role in the neural processing of dyspnea, or the sensation of difficult and uncomfortable breathing (von Leupoldt & Dahme, 2005a; von Leupoldt *et al.*, 2008; Davenport & Vovk, 2009). Because loaded breathing has been shown to produce dyspnea (Schon *et al.*, 2008), it is likely that the AI is activated by the sensations associated with ITTO and may play a significant role in modulating the behavioral load compensation responses to repeated ITTO. This modulatory function might arise from plasticity within the region, suggested by the increase in basal activity after 10 days of ITTO.

In order to understand the neural mechanisms involved in load compensation responses to respiratory stimuli in human subjects, non-invasive methods such as fMRI and cortical evoked potentials (CEP) are most often used (Gozal *et al.*, 1995; Davenport *et al.*, 2006; Chan & Davenport, 2008; von Leupoldt *et al.*, 2008; Chan & Davenport, 2009). Both methods provide information regarding the brain's immediate response to a stimulus. fMRI produces information about both inhibition and activation, and CEP indicates temporal activity patterns primarily in the cortex. Both methods have been used in animals. Although they produce large quantities of data, they offer little information about specific neural networks and plasticity. Because of the ability to conduct invasive experiments in animals, researchers can also use histochemistry to determine specific neural changes in response to stimuli after the stimulus has been delivered. A common method is staining neural tissue for c-Fos, which only depicts areas of activation, not inhibition. Furthermore, c-Fos cannot differentiate between afferent or efferent activation, and many control groups are needed. CO is another histomchemical method, but unlike with c-Fos it can show both excitatory and inhibitory state changes in brain activity but does not provide information on acute neural activity. One potential difficulty with using CO lies in the fact that brain nuclei have inherent differences in metabolic rates. Certain areas are always less active than others and will have less intense CO staining. Determining group differences in metabolic activity for these nuclei can be difficult when the changes are small. Similar to the observations of Hevner and colleagues (Hevner *et al.*, 1995), the greatest staining was observed in special sensory and motor nuclei such as the dorsal and ventral respiratory groups, while the lowest staining intensities were seen in limbic nuclei like the amygdala, which

is known for less CO reactivity. The between-group differences in steady state activity in limbic nuclei resulting from ITTO may actually be more robust than is indicated by CO staining, especially if the change is a net decrease in activity level as was seen in the amygdala in this study.

This study shows state changes in specific brain nuclei elicited by conscious load compensation conditioning to respiratory mechanical stimuli. Significant findings include increases in basal activity of the nTS, PAG, DR, VPM, and AI. This list includes important respiratory nuclei, nuclei involved in an animal's stress response, and nuclei mediating discriminative and affective sensory processing. These results suggest a modulation of sensory brain regions, which is especially relevant to pulmonary diseases characterized by transient, unexpected, inescapable, uncontrolled airway obstruction such as asthma, COPD, and obstructive sleep apnea. Patients with these pulmonary diseases have a high incidence of stress and affective disorders. Repeated obstructions to breathing during sleep have resulted in impaired detection of R loads in conscious humans (McNicholas *et al.*, 1984), suggesting that neural structures involved in the respiratory sensory processing in these patients may have been altered. The present study reports state changes in brain pathways that are consistent with neural plasticity related to affective disorders in pulmonary obstructive diseases. Characterizing the load compensation response in conscious animals, how this response is altered after repeated experience, and what neural structures mediate these responses is essential to our understanding of respiratory diseases and rehabilitation.

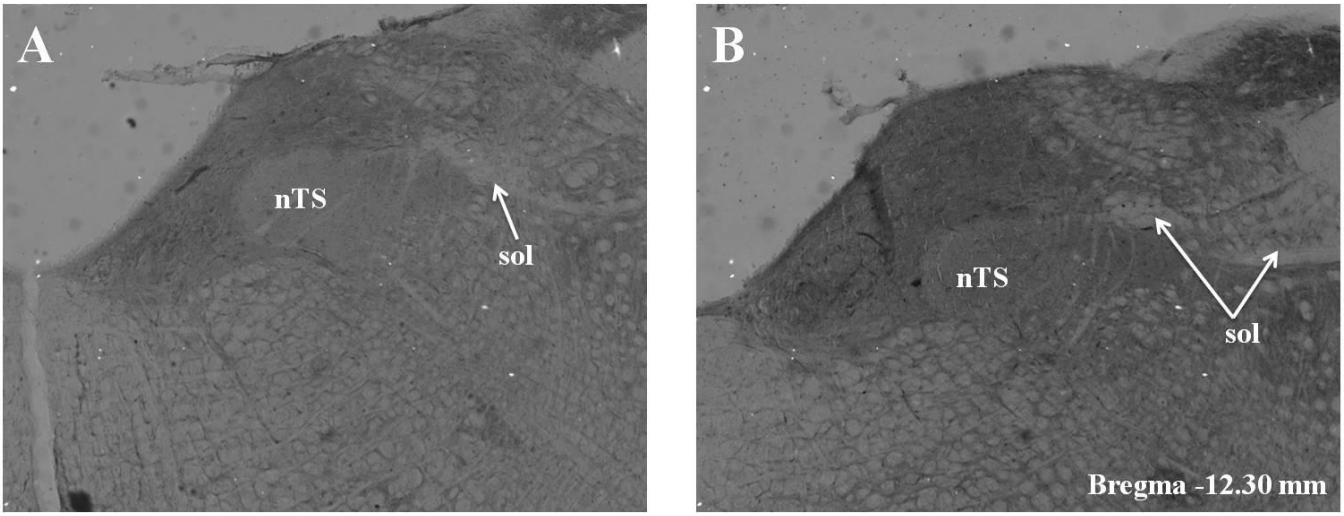

Figure 5-1. CO staining in the rostral nTS after A) 10 days of handling and no tracheal occlusions, and B) 10 days of ITTO. Differences in staining averages between groups were significant (p=0.01).

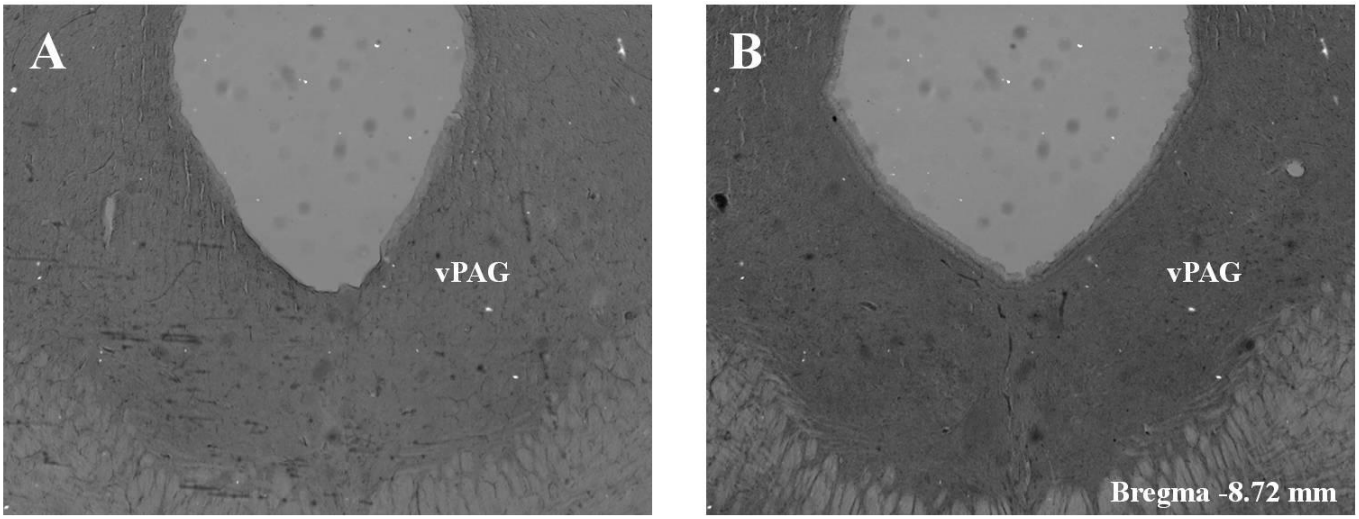

Figure 5-2. CO staining in the caudal vPAG after A) 10 days of handling and no tracheal occlusions, and B) 10 days of ITTO. Differences in staining averages between groups were significant (p=0.004).

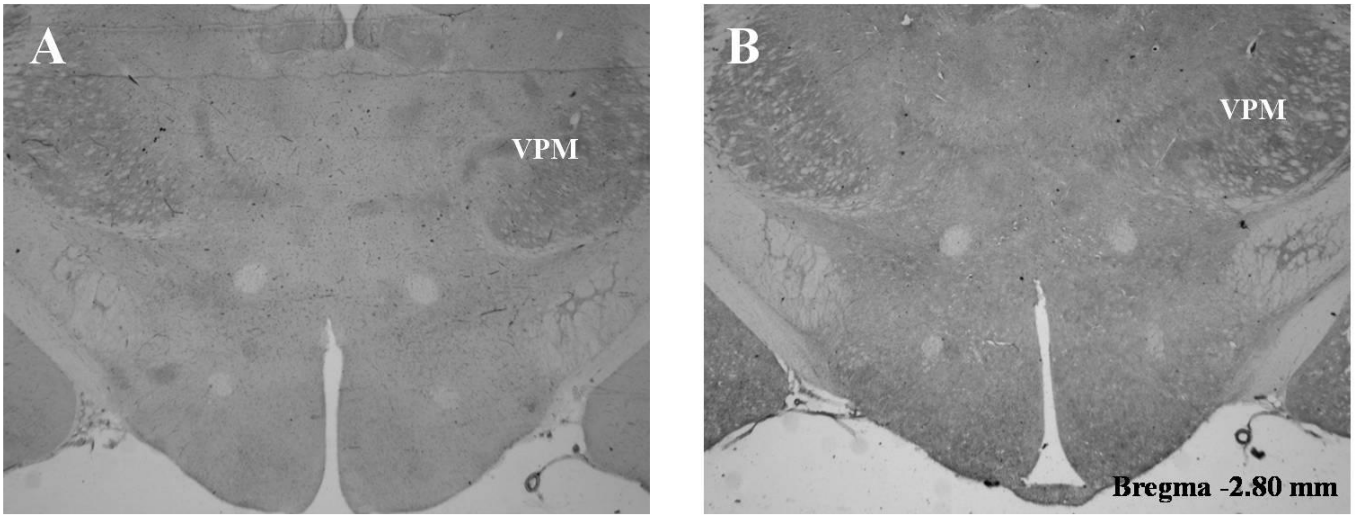

Figure 5-3. CO staining in the VPM thalamus after A) 10 days of handling and no tracheal occlusions, and B) 10 days of ITTO. Differences in staining averages between groups were significant (p=0.02).

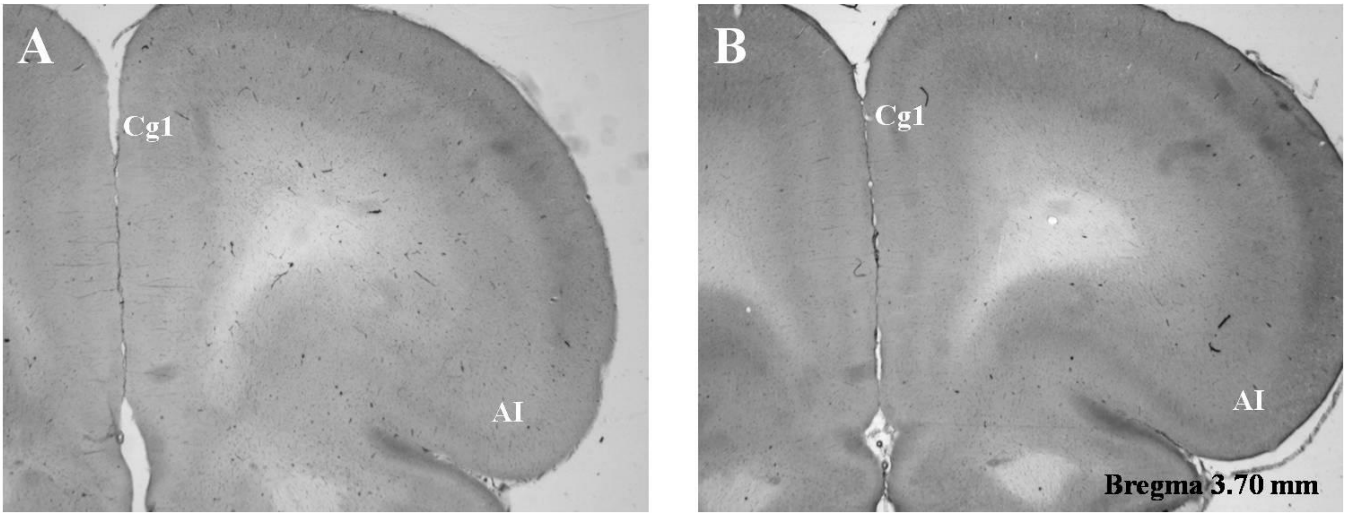

Figure 5-4. CO staining in the AI and Cg1 after A) 10 days of handling and no tracheal occlusions, and B) 10 days of ITTO. Differences in staining averages between groups were significant in the AI only (p=0.02).

Table 5-1. Brainstem CO staining after 10 days of ITTO (Exp) or handling without tracheal occlusions (Ctrl).

Nucleus	Exp	Ctrl	p-value
caudal nTS	2.66 (.13)	2.46 (.10)	0.31
rostral nTS	2.87 (.19)	2.12 (.14)	0.01
cVRG	3.04 (.17)	2.86 (.10)	0.40
rVRG	3.08 (.16)	2.43 (.16)	0.02
AP	2.76 (.20)	2.85 (.30)	0.82
lPBN	2.07 (.10)	1.93 (.20)	0.57
LC	2.25 (.09)	1.85 (.17)	0.08

Reported values are staining intensities in each nucleus normalized to unstained white matter from the same animal and staining batch.

Table 5-2. Midbrain CO staining after 10 days of ITTO (Exp) or handling without tracheal occlusions (Ctrl).

Nucleus	Obs	Ctrl	p-value
caudal dPAG	2.11 (.10)	1.74 (.08)	0.01
caudal vPAG	2.25 (.07)	1.90 (.04)	0.004
rostral dPAG	1.98 (.08)	1.70 (.10)	0.06
DR	2.11 (.06)	1.80 (.11)	0.05

Reported values are staining intensities in each nucleus normalized to unstained white matter from the same animal and staining batch.

Table 5-3. Higher brain centers, discriminative, and affective CO staining after 10 days of ITTO (Exp) or handling without tracheal occlusions (Ctrl).

Nucleus	Obs	Ctrl	p-value
Arc	2.22 (.14)	2.16 (.19)	0.79
PaMP	2.09 (.11)	2.12 (.27)	0.93
CM	1.74 (.08)	1.47 (.12)	0.09
VPL	2.45 (.13)	2.27 (.13)	0.37
VPM	2.48 (.08)	2.11 (.10)	0.02
AI	2.69 (.06)	2.43 (.06)	0.02
Cg1	2.60 (.13)	2.32 (.12)	0.19
CeA	1.34 (.03)	1.45 (.12)	0.39

Reported values are staining intensities in each nucleus normalized to unstained white matter from the same animal and staining batch.

CHAPTER 6
GENERAL DISCUSSION

Acute, Anesthetized Respiratory Load Compensation

Our method of intrinsic, transient, tracheal obstructions for 2-3 breaths in anesthetized rats elicited respiratory load compensation, including a prolongation of Ti and relatively longer Te, increased EMG_{dia} activity, and more negative inspiratory P_{es}. End-expiratory P_{es} did not change with obstruction or recovery in any animals, indicating that ITTO does not change lung compliance. The changes in breath timing and respiratory motor responses resulted from the load compensation response of the respiratory neural control system to breathing efforts against a closed airway, and tracheal squeeze alone was not sufficient to evoke responses. These responses were mediated by respiratory afferents, including vagal PSR's responding to transpulmonary pressure (P_{tp}), which is influenced by lung volume (Davenport *et al.*, 1981b). The elevated P_{es} and EMG_{dia} during recovery breaths are consistent with other studies (Forster *et al.*, 1994; Watts *et al.*, 1997), and it appears that whether respiratory motor responses return to baseline during recovery depends upon the severity and properties of the load (i.e. duration, magnitude, and whether it's applied to inspiration, expiration, or both). Breath timing parameters are often reported to return to baseline during recovery (Hutt *et al.*, 1991; Forster *et al.*, 1994). The augmentation of parameters during recovery breaths after ITTO is likely a result of the respiratory system acting to restore ventilation after prolonged perturbation.

Respiratory load compensation in the anesthetized animal is mediated by reflexive neural mechanisms and is dependent upon lung, airway and muscle afferent activation, including PSR's (Zechman *et al.*, 1976; Davenport *et al.*, 1981a; Davenport *et al.*, 1984;

Webb *et al.*, 1994, 1996). ITTO in anesthetized rats induced neural activation in respiratory brainstem nuclei, and nuclei involved in cardiovascular, stress, and affective responses, indicating that nuclei within these pathways may participate in the breath timing and motor load compensation responses to ITTO. Our neural pathway model is represented in Figure 6-1. The nTS is the primary target of lung (Kalia & Mesulam, 1980a, b; Kalia, 1981a) and upper airway afferents (Sant'Ambrogio *et al.*, 1977; Kalia, 1981b; Lee *et al.*, 1992). The nA is activated by the nTS (Loewy & Burton, 1978), phrenic, vagus, carotid sinus, and superior laryngeal nerve stimulation, bronchoconstriction, and inspiratory occlusions (Davenport & Vovk, 2009), and also participates in load compensation responses. The increased FLI in medullary respiratory nuclei results from tracheal afferents stimulated by the squeeze of the cuff, pulmonary afferents activated by lung inflation, and efferents mediating changes in respiratory pattern. ITTO also evoked significant neural activation in the dlPBN, which has been shown to respond to occlusions (Davenport & Vovk, 2009) and neurons from the nTS (Loewy & Burton, 1978; Ezure *et al.*, 1998). The lPBN plays a role in the descending pathway from the PAG involved in cardiovascular (Hayward *et al.*, 2004; Hayward, 2007) and respiratory (Hayward *et al.*, 2004) control, and the PAG was also activated by ITTO with the greatest response seen in the caudal region. The PAG is an important nucleus involved in defensive behaviors (Bandler & Carrive, 1988; Brandao *et al.*, 1994; Vianna *et al.*, 2001), pain modulation (Mayer *et al.*, 1971; Behbehani, 1995), panic and anxiety, (Carrive, 1993; Bandler & Shipley, 1994; Brandao *et al.*, 1994; Vianna *et al.*, 2001; Cunha *et al.*, 2010), conditioned fear (Vianna *et al.*, 2001), and respiratory responses (Hayward *et al.*, 2003; Zhang *et al.*, 2005, 2007, 2009). The FLI

seen in the lPBN after ITTO could be a response to afferent projections to that nucleus, but it could also be a result of projections from the lPBN to other neural areas such as the limbic system (Saper & Loewy, 1980). The FLI seen in the PAG after ITTO could be respiratory-specific and/or related to stress and fear pathways. The potential contribution of other sensory afferents to respiratory volume-timing, motor, and neural responses to ITTO cannot be ruled out. It is clear that reflexive respiratory load compensation has a neural component that includes both sensory- and motor-activated neurons involved in respiratory, cardiovascular, stress, and affective pathways (Figure 6-1).

Conditioned, Conscious Respiratory Load Compensation

The results of the first study in the anesthetized animal provide evidence that our model of ITTO is effective for eliciting respiratory load compensation and elucidating the neural component of that compensation. Behavioral load compensation is fundamental to adaptation to respiratory loads in conscious animals (i.e. escape is a critical compensation behavior in obstructive pulmonary diseases). It is known that changes in respiratory behavior can be elicited through conditioning in conscious humans (Gallego & Perruchet, 1991) and animals (Nsegbe *et al.*, 1997; Nsegbe *et al.*, 1998; Nsegbe *et al.*, 1999; Durand *et al.*, 2003). Stimuli such as hypoxia and hypercapnia can be paired with a discrete cue, like a tone or odor, or be implemented in an consistent context. Conditioned responses were variable, depending upon the stimuli used, species, and age of the animal. However, the results of those studies indicate that stimuli can change respiratory behavior, which, over time, may become a persistent response as a result of learning (conditioning).Thus, we developed a conscious rodent model of ITTO using the same surgical procedures as in the first study in order to characterize the conscious

response to tracheal occlusions and understand how the behavioral control of breathing modulates reflexive responses, especially after conditioning. The results of that study showed that the first day response to ITTO consisted of a prolongation of Te with no significant changes in Ti, Ttot or diaphragmatic activation. After 10 days of conditioning, conscious rats lengthened Te and increased diaphragmatic activation during occluded (O) compared to control (C) breaths, which were not accompanied by increases in Ti. There were no significant changes in total breath timing parameters from the first day of ITTO to the last day; however, there was a significant increase in the O/C ratio of diaphragmatic activity per breath. This suggests that after 10 days of ITTO, conscious animals preferentially change respiratory drive rather than timing in response to the occlusions, and that the volume-timing reflex seen in anesthetized animals is modulated in conscious rats.

The prolongation of Te during O was hypothesized to be a result of behavioral breath holding rather than lung hyperinflation since we attempted to occlude animals at FRC and only evaluate breaths where that was accomplished. The discomfort caused by trying to breathe through a large resistive load (Simon *et al.*, 1989; Simon *et al.*, 1990; ATS, 1999) is aversive (von Leupoldt & Dahme, 2005b; von Leupoldt *et al.*, 2008) and linked with the fear that arises in response to the threat of suffocation (Lang *et al.*, 2010) or asphyxiation (Campbell, 2007). The prolongation of Te observed during tracheal occlusions was likely the attempt of the animals to hold their breath and avoid the sensations rather than breathe through them. There was an increase in Te on both the first and last day of ITTO training, however those values were not different from one another suggesting that even with conditioning the animals will continue to breath-hold

and avoid the aversive sensations associated with ITTO. Aversive sensations associated with respiratory stimuli may not habituate, which is beneficial when considering the survival of the animal. We observed no other changes in breath timing between O and C or between the first and last day of ITTO indicating that the primary respiratory load compensation response in conscious animals does not include modulation of breath timing, which is consistent with respiratory studies in humans (Clark & von Euler, 1972; Axen *et al.*, 1983).

Increased respiratory muscle activation appears to be a more consistently reported response in both the conscious and anesthetized human and animal (Axen *et al.*, 1983; Lopata *et al.*, 1983; Hutt *et al.*, 1991; Frazier *et al.*, 1993; Xu *et al.*, 1993a; Xu *et al.*, 1993b; Osborne & Road, 1995; Zhang *et al.*, 2009). The diaphragmatic activation per breath in conscious rats was increased during O compared to C in this study, and the O/C ratio was greater on the last compared to the first day of ITTO. This was a result of increased EMG_{dia} amplitude (i.e. muscle fiber recruitment) during Ti, indicative of stronger inspiratory efforts. These results suggest the enhanced diaphragm motor response was a result of conditioning, attributed to behavioral sensitization. ITTO produces stress responses in conscious rats (unpublished results), and others have reported fear potentiation in response to uncontrollable stressors (Korte *et al.*, 1999). Additionally, fear-potentiated behaviors are observed as a result of contextual affective conditioning (Risbrough *et al.*, 2009) and the increased O/C diaphragm response seen on the last day of ITTO compared to the first day may also due to a heightened affective state from contextual conditioning. These potentiated behaviors were only apparent during stimulus presentation and not during C breathing, consistent with habituation of

resting behaviors during context exposure (Beck & Fibiger, 1995). These results suggest that there is a respiratory load compensation response to tracheal occlusions in conscious rats that is different from the anesthetized reflexive response and from other patterns reported in conscious humans and animals exposed to respiratory loads. This response includes breath holding and increased inspiratory effort potentially due to a sensitized behavioral affective response. Thus, the volume-timing reflex may be modulated in conscious animals by behaviorally mediated increased respiratory drive as the preferential load compensatory mechanism.

Stress and Affective Responses to ITTO Conditioning in Conscious Animals

ITTO causes neural activation of nuclei involved in stress and affective pathways in anesthetized animals and the pattern of the respiratory load compensation response in conscious animals is influenced by behavioral control. To determine whether stress and/or aversive mechanisms play a role in the behavioral modulation of respiratory load compensation we conditioned conscious animals to 10 days of ITTO. Load compensation responses were determined by physiological assays for stress and a behavioral measure of anxiety. We found that 10 days of unexpected, unavoidable, uncontrollable ITTO elevated basal Cort levels, increased adrenal weight, and heightened anxiety in conscious rats. These results suggest that repeated exposure to tracheal occlusions in conscious animals can cause elevated resting stress and anxiety levels in animals after only 10 days of ITTO conditioning.

The augmentation of basal HPA activity is similar to responses evoked by other repeated stressors (Fernandes *et al.*, 2002; Armario, 2006; Filipovic *et al.*, 2010). Our study indicated that ITTO is a respiratory stimulus sufficient to evoke the same kind of stress responses seen with exposure to other stressful stimuli such as restraint and

shock (Raone *et al.*, 2007), chronic variable stress (Herman *et al.*, 1995), and intermittent restraint (Fernandes *et al.*, 2002). Sustained HPA activation is linked with affective disorders such as anxiety and depression (Roy-Byrne *et al.*, 1986; Kling *et al.*, 1991; Stenzel-Poore *et al.*, 1994; Maier & Watkins, 2005), and 10 days of ITTO increased anxiety in our rats. We did not test for depression in our animals, but because anxiety and depression have many similarities (APA, 1994; Stenzel-Poore *et al.*, 1994; Frazer & Morilak, 2005), especially related to HPA activity (Pellow *et al.*, 1985; Roy-Byrne *et al.*, 1986; Stenzel-Poore *et al.*, 1994; Vreeburg *et al.*, 2010), it is possible that our animals developed depression behaviors. These responses are mediated by both physical and psychological neural pathways, further suggesting that consciousness plays an important role in the response to respiratory load stimuli.

It was hypothesized that the stress-anxiety response observed after 10 days of ITTO is mediated in part by the PVN and CRH release, as well as the DR and 5-HT release. These nuclei and neurotransmitters modulate the activity of one another (Lowry *et al.*, 2000; Hammack *et al.*, 2002; Jorgensen, 2007; Valentino *et al.*, 2009; Geerling *et al.*, 2010) and 5-HT and its receptors have important implications in the psychological and behavioral responses to uncontrollable stressors (Maier, 1984; Graeff, 1994; Graeff *et al.*, 1996; Maier & Watkins, 2005; Jorgensen, 2007; Valentino *et al.*, 2009), including anxiety and depression behaviors arising from PAG and limbic activation (Graeff *et al.*, 1996). We have shown alterations in 5-HT receptor gene expression in thalamic neural areas after one exposure to ITTO (Bernhardt *et al.*, 2008) and after 10 days of ITTO (Bernhardt *et al.*, 2010). Alterations in the serotonergic and stress pathways suggests that tracheal occlusions can cause changes in the neural modulators mediating

behavioral load compensation, the control of respiration (Hodges *et al.*, 2009) and respiratory neural plasticity (Feldman *et al.*, 2003; Doi & Ramirez, 2008). Furthermore, the PVN has reciprocal projections with the brainstem respiratory network (Hermes *et al.*, 2006; Geerling *et al.*, 2010) which may modulate respiratory motor output during situations of both respiratory and non-respiratory related stress.

Because these experiments were performed in conscious animals, we were expecting a substantial portion of the animal's responses would be a result of the sensations associated with large resistive loads and tracheal occlusions since these sensations, collectively referred to as dyspnea, are highly aversive (von Leupoldt & Dahme, 2005b; O'Donnell *et al.*, 2007; Schon *et al.*, 2008; von Leupoldt *et al.*, 2008). Dypsnea includes both discriminative and affective components (von Leupoldt & Dahme, 2005b) that are relayed to the somatosensory (Davenport *et al.*, 1985; Davenport *et al.*, 1991; Davenport *et al.*, 1993; Davenport & Hutchison, 2002) and limbic neural networks (Aleksandrov *et al.*, 2000; von Leupoldt & Dahme, 2005a; Schon *et al.*, 2008). Dyspnea cannot be assessed in animals but the neural correlates that mediate dyspnea are similar, and it is plausible that respiratory obstructions in animals cause similar intense and aversive sensations, leading to an experience of negative affect. Negative affect, if experienced repeatedly may result in psychological disorders such as anxiety or depression, which are both highly prevalent in patients with COPD who also experience dyspnea (Karajgi *et al.*, 1990; Brenes, 2003; Wagena *et al.*, 2005). Indeed, we observed anxiety responses in our animals after 10 days of ITTO. Furthermore, individuals modify their behavior in order to adapt, avoid or escape from experiencing the sensation of dyspnea. It was hypothesized that the prolongation of Te in response to

the occlusions in the previous study was a behavioral breath holding technique used to avoid the sensations associated with breathing through a respiratory load. Intense, unavoidable respiratory stimuli like tracheal occlusions likely produce intense physical discomfort and stress due to the life-threatening quality of ITTO, which is highly relevant to individuals who experience these types of respiratory stressors on a regular basis, such as those with COPD and asthma. This is also important because chronic experience with one type of stress can make an individual more reactive to new types of stress, which would be detrimental in patients with asthma and COPD who often have other physiological and psychological symptoms that could be worsened by airway obstruction-dependent HPA activation. Determining the neural mechanisms behind these responses in future studies is extremely important.

Neural Plasticity Responses to ITTO Conditioning in Conscious Animals

This project investigated the neural structures and mechanisms mediating the behavioral, stress, and anxiety responses observed in animals after 10 days of ITTO. We hypothesized that respiratory brainstem nuclei, areas involved in stress responses, and regions participating in discriminative and affective sensory processing would show modulation in response to repeated ITTO. Significant state changes in the medullary dorsal respiratory group, PAG, DR, discriminatory VPM and the affective AI were found, indicated by alterations in CO staining. This was the first evidence of neural state modulation in response to respiratory load conditioning in conscious animals. In this study we used 10 s tracheal occlusions rather than the 3-6 s used in the other studies to ensure a consistent pattern of neural activation. It is possible that chemoreceptor afferents would have increased activation if the 10 s occlusions were long enough to cause a slight hypercapnic or hypoxic response. However, Farre and colleagues (Farre

et al., 2007) found that a 5 second obstruction reduced the SpO_2 to 85% in rats, and that a 10 second obstruction only reduced the SpO_2 to 83%. These values are both greater than the 67-80% SaO_2 that would result from the oxygen partial pressures of 35-45 mmHg often used in intermittent hypoxia studies (Zabka *et al.*, 2001; Lee & Fuller, 2010). Chemoreceptor afferents project to the nTS, which is also the primary target of cardiorespiratory afferents in the lung, airway, trachea, and blood vessels. The pattern of stimulation in the nTS from convergent input during ITTO was sufficient to induce plasticity after 10 days, but the relationship between mechanical and chemical stimulation mediating this plasticity are unknown. The plasticity in the nTS could indicate that the nTS changes its state for conditioned load compensation to subsequent airway obstruction challenges, allowing for adaptation of physiological responses to the severe respiratory challenge posed by repeated ITTO. The results of McKay et al. (McKay *et al.*, 2003) suggest that the brainstem respiratory network, including nTS, may be an important site for the voluntary control of breathing in humans. Thus, the nTS may also be an important site for the behavioral modulation of breathing in animals, and may undergo plastic changes as a result of ITTO conditioning, potentially generating a new respiratory load compensation pattern for the animal.

Similar to the results of the acute ITTO protocol in anesthetized animals, differences in neural activation between control and experimental groups were observed in the midbrain dorsal and ventral PAG. This indicates that the PAG plays an important role in the respiratory load compensation response in conscious animals and mediates the behavioral, stress, and anxiety responses observed after ITTO conditioning. The PAG participates in defensive behaviors (Bandler & Carrive, 1988; Brandao *et al.*, 1994;

Vianna *et al.*, 2001), pain modulation (Mayer *et al.*, 1971; Behbehani, 1995), respiratory activation (Hayward *et al.*, 2003; Zhang *et al.*, 2005), modulation of the load compensation response (Zhang *et al.*, 2009), stress pathways (Farkas *et al.*, 1998), and fear responses (Carrive, 1993; Bandler & Shipley, 1994; Brandao *et al.*, 1994; Vianna *et al.*, 2001; Cunha *et al.*, 2010). Interestingly, we also found a significant increase in DR activity after ITTO conditioning. This result supports our hypothesis that the DR and 5-HT are important in mediating the stress and anxiety behaviors we observed, and indicates a possible role in the adaptive response to repeated severe respiratory load stimuli. The plasticity within the DR may have implications for plasticity in other pathways and responses in which 5-HT has a modulatory function (Maier, 1984; Merahi *et al.*, 1992; Lindsay & Feldman, 1993; Bianchi *et al.*, 1995; Baker *et al.*, 2001; Fuller *et al.*, 2001; Pena & Ramirez, 2002; Bocchiaro & Feldman, 2004; Maier & Watkins, 2005; Jorgensen, 2007; Doi & Ramirez, 2008; Mizoguchi *et al.*, 2008; Dergacheva *et al.*, 2009; Hodges *et al.*, 2009).

After ITTO conditioning we observed non-significant decreases in neural activity within the CeA and PaMP. The CeA is part of the limbic system in the brain and is essential for fear conditioning and aversive responses (Brandao *et al.*, 1994; Davis *et al.*, 1994; LeDoux, 2000; Martinez *et al.*, 2006). Our laboratory has shown (Shahan *et al.*, 2008) that electrical stimulation of the CeA leads to an increase in respiratory rate in anesthetized rats, possibly via output to the PAG. CeA activity decreased in response to repeated ITTO that may, in part, be due to the increase in DR activity after ITTO. The CeA has inhibitory projections to the PAG and nTS (Davis *et al.*, 1994; Saha *et al.*, 2010), so a potential down-regulation of CeA GABAergic innervation is likely to disinhibit

the PAG (Saha, 2005; Oka *et al.*, 2008). Thus, a decrease in CeA activity could lead to activation of the PAG and nTS through disinhibition. This suggests that the animal modulates its physiological responses to repeated respiratory mechanical stressors via CeA plasticity. A change in basal activity within the CeA may influence the behavioral sensitization of the inspiratory efforts during occlusions observed on the 10th day of ITTO. The PaMP also responds to stressful stimuli by producing CRH (Vale *et al.*, 1981). Because we have shown that ITTO evokes stress responses, the PaMP was initially expected to increase activity in response to 10 days of ITTO. However, the PaMP showed a non-significant decrease in steady state activity. Although circulating CORT is known to be elevated as a result of chronic stress, basal PVN activity is blunted as a result of conditioning (Girotti *et al.*, 2006; Arnhold *et al.*, 2007). The CeA and PaMP downregulation support our hypothesis that these nuclei mediate fear-potentiated behavioral sensitization of inspiratory effort, breath holding, stress, and anxiety responses to ITTO.

The discriminative pathway includes a relay through the thalamus to the somatosensory cortex, and the affective pathway involves structures such as the amygdala, Cg1, and AI (von Leupoldt & Dahme, 2005b, a; Schon *et al.*, 2008; von Leupoldt *et al.*, 2008; Davenport & Vovk, 2009). These pathways are involved in respiratory sensations (von Leupoldt & Dahme, 2005b, a; Schon *et al.*, 2008; von Leupoldt *et al.*, 2008; Davenport & Vovk, 2009), and have shown plasticity in response to 10 days of ITTO. Specifically, significant increases were found in steady state activity in the VPM thalamus and AI. It is possible that tracheal afferents stimulated during occlusion project to the VPM via polysnaptic pathways (Ambalavanar *et al.*, 1999). In

addition, (Cechetto & Saper, 1987) found that the VPM projects visceral sensory information to the insular cortex, some of which includes cardiopulmonary afferent information. The AI has an excitatory projection to the PAG (Behbehani *et al.*, 1993), responds to respiration, arterial chemoreceptors, and cardiovascular baroreceptors (Cechetto & Saper, 1987), and is active during voluntary breath-holding maneuvers (McKay *et al.*, 2008), a behavior our animals appeared to use in response to ITTO. The AI is known to play a vital role in the neural processing of dyspnea (von Leupoldt & Dahme, 2005a; von Leupoldt *et al.*, 2008; Davenport & Vovk, 2009) and we have previously established that loaded breathing produces dyspnea (Schon *et al.*, 2008). Thus, affective neural processing likely takes place in the AI during ITTO and results in significant modulation of the behavioral load compensation responses to repeated ITTO. This modulatory function might arise from plasticity within the region, suggested by the increase in steady state activity after ITTO conditioning.

Conclusion

Collectively, the results of these studies show that tracheal occlusions elicit neural and behavioral load compensation responses in conscious animals, supporting the link between intense respiratory stimuli, stress, and affective disorders. This has important implications for individuals with respiratory obstructive diseases such as COPD, asthma, and obstructive sleep apnea, who experience repeated life-threatening, intermittent, unpredictable increases in airway mechanical load. The increase in airway load leads to sensory activation and load compensation behaviors. Sensory activation in response to the airway obstructions can result in intense physical discomfort, or dyspnea (von Leupoldt & Dahme, 2005a; O'Donnell *et al.*, 2007) and compensation behaviors often include leading a sedentary lifestyle (ZuWallack, 2007; Bourbeau,

2009). It is well established that emotions and respiration are tightly linked (Ley, 1999), and individuals with respiratory obstructive diseases are frequently diagnosed with affective disorders such as anxiety and depression. Furthermore, sedentary behavior can be a symptom of depression (Roshanaei-Moghaddam *et al.*, 2009). The severe chronic stress experienced by these individuals could potentially make them more reactive to new types of stress (Fernandes *et al.*, 2002), which is particularly problematic since patients with asthma and COPD often have physiological and psychological symptoms that may be worsened by airway obstruction-dependent HPA activation. In addition, uncontrollable stressors have been shown to sensitize serotonergic neurons in the DR, which respond to future stressors with exaggerated 5-HT release that may cause behavioral changes such as learned helplessness (Maier & Watkins, 2005). We observed an increase in steady state activity in the DR so it is likely that this pathway was activated by repeated ITTO. Additionally, the activities of sensory brain regions were modified by 10 days of ITTO, similar to repeated obstructions to breathing during sleep that resulted in impaired detection of R loads in conscious humans (McNicholas *et al.*, 1984). The results of McNicholas et al. suggest that neural structures involved in the respiratory sensory processing in OSA patients may have been altered. In conclusion, characterizing the acute and chronic respiratory load compensation responses in conscious animals, determining how these responses are altered after repeated experience, and elucidating the neural structures involved in generating these responses is essential to our understanding of respiratory obstructive diseases and rehabilitation.

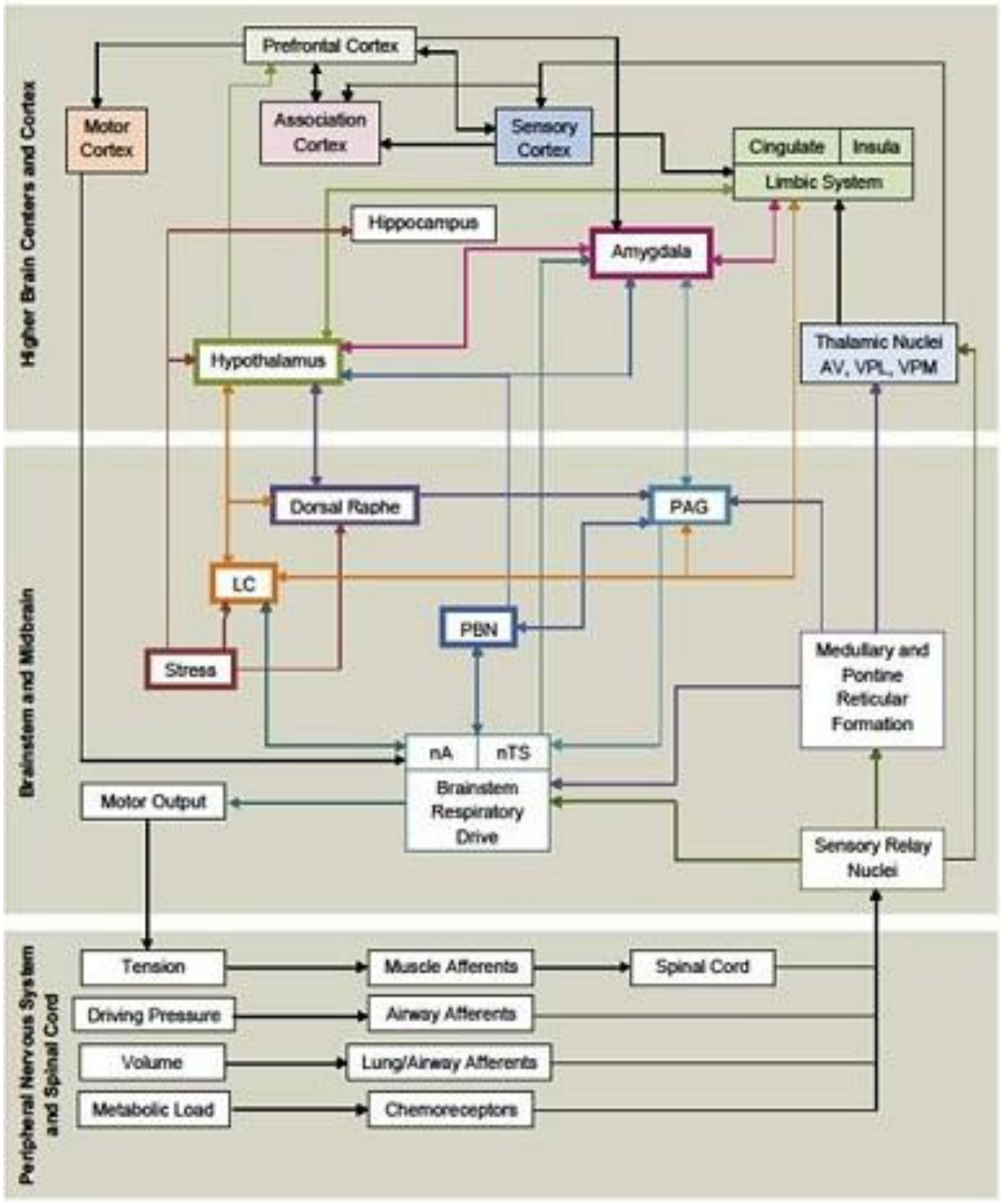

Figure 6-1. Representation of central nervous system nuclei and pathways involved in respiratory control.

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BIOGRAPHICAL SKETCH

Kathryn Mackenzie Pate was born in Sebastian, Florida in 1985. She grew up with her parents, Stephen and Constance, and older brothers, Robert, Matthew, and Michael. She graduated from the University of Florida with a Bachelor of Science in Zoology in May 2006 and began her Ph.D. career at the University of Florida's College of Veterinary Medicine in August 2006. Kate spent four years under the guidance of Dr. Paul Davenport in the Department of Physiological Sciences, and completed her Ph.D. in August, 2010. Upon receiving her Ph.D., she began her post-doctoral training at National Jewish Health respiratory hospital in Denver, Colorado to investigate the role of redox imbalance in disease. Her new mentor is Dr. James Crapo.