The Nucleus of the Solitary Tract is Necessary for Apnea-Induced Respiratory Plasticity

by

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Abstract

The respiratory system is attentive, adaptive, learns and has memory. The respiratory system remembers repeated respiratory challenges to fine tune its motor activity by modulating neuronal synaptic strength. This phenomenon, respiratory long term facilitation (LTF), functions to strengthen the ability of respiratory motor neurons to enhance contraction of breathing muscles. LTF could serve as a protective mechanism against obstructive sleep apnea, a disease characterized by the collapse of upper airways, by restoring upper airway patency. LTF can be induced through modulation of vagal afferent feedback via repeated apneas. Here, we used reverse microdialysis, electrophysiology, neuropharmacology, and histology to determine if the nucleus of the solitary tract (NTS), a brain region exclusively receiving vagal afferents, is the origin of the neural circuit responsible for apnea-induced plasticity. My work shows bilateral injection of 5% lidocaine into the NTS prevented LTF. We conclude the NTS is required for triggering apnea-induced LTF.
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# Abbreviations

<table>
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<tbody>
<tr>
<td>NTS</td>
<td>Nucleus of the Solitary Tract</td>
</tr>
<tr>
<td>LTF</td>
<td>Long Term Facilitation</td>
</tr>
<tr>
<td>LTP</td>
<td>Long Term Potentiation</td>
</tr>
<tr>
<td>sec</td>
<td>Seconds</td>
</tr>
<tr>
<td>min</td>
<td>Minutes</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>5HT</td>
<td>Serotonin receptor</td>
</tr>
<tr>
<td>SARS</td>
<td>Slowly adapting stretch receptor</td>
</tr>
<tr>
<td>RARS</td>
<td>Rapidly adapting stretch receptor</td>
</tr>
<tr>
<td>CNQX</td>
<td>6-cyano-7-nitroquinoxaline-2,3-dione</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-Methyl-D-aspartic acid</td>
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<tr>
<td>GABA</td>
<td>Gamma-Aminobutyric acid</td>
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Chapter 1: Introduction

One of the most important biorhythms is breathing. The importance of breathing can be seen in its relentless appearance in philosophy and religion before it was even physiologically defined. Remarkably, the initial respiratory movements begin very early in development. In fact, there are intermittent practice breathes that take place in utero (Feldman et al., 2003). Once born the process becomes continuous and rhythmic (Feldman et al., 2003).

At first breathing had a very simplistic definition, the act of inspiration and expiration of air. However, in the last 200 years physiologists have expanded the knowledge of the respiratory system. A landmark moment in respiratory research was when Jean Pierre Flourens demonstrated that the destruction of the medulla abolished breathing. This was influential in demonstrating the brain had a center designated for respiration. Knowledge was added to Flourens work when Marckwald in 1888 performed both brainstem and cranial nerve transections on respiration in the rabbit. These two landmark studies laid a foundation for the investigation of the neural mechanism governing respiration. To this day we believe that there are structures which govern the process of breathing and these structures function to produce both the frequency of breathing (rhythm) and strengthen inspiratory amplitude.

1.1 Rhythm Generation and Brainstem Nuclei

The neural circuitry that creates rhythmic respiratory movements has been under investigation for more than a century, and still there is controversy on how the network generating this rhythm is organized (Feldman and Del Negro, 2006). This section will provide a short summary on the involuntary control of rhythm generation.
A novel experiment known as the *en bloc* experiment was a preparation that dissected out the components of the respiratory system so activities from the cranial and spinal nerve could be recorded. This preparation demonstrated that the respiratory rhythm was not being generated by the respiratory muscles or the lungs (Suzue, 1984). Therefore, central control must have been dictating the generation of rhythm.

Researchers soon found that critical groups in the medulla and the pons play a role in the control of breathing. The ventral respiratory group and dorsal respiratory group controls the generation of breathing rhythm whereas the pontine respiratory group shapes the rhythm (Feldman and Del Negro, 2006). The ventral respiratory column contains the pre-Bötzing complex, the Bötzing complex, the retrotapazoid nucleus, rostral and caudal respiratory groups (Ezure, 1990, Rekling and Feldman, 1998, Duffin, 2004, Guyenet and Mulkey, 2010). These brain regions are all found to be involved in rhythm generation; however, defining which of these regions as the main generator of respiratory rhythm creates much controversy. Currently, there is a strong debate between the pre-Bötzing complex and the retrotapazoid nucleus, which may both function as rhythm generators (Feldman and Del Negro, 2006).

The pre-Bötzing complex contains neurons that have inherent ability to burst in a rhythmic fashion (Rekling and Feldman, 1998). This is known as pacemaker properties and it is this very property that generated the pacemaker model of rhythm generation. The pacemaker model of rhythm generation stipulates that the intrinsic membrane properties of either single or groups of pacemaker neurons found in the pre-Bötzing complex generate a constant monotonous rhythm that is then transmitted to other respiratory regions to control both the shape and timing of respiratory muscle activity (Feldman and Del Negro, 2006).

If the pacemaker hypothesis were true then removal of the pacemaker cells should cease respiratory rhythm. However, it has been demonstrated that bilateral destruction of the
prebotzinger complex neurons in the adult rat does not abolish the respiratory rhythm (Gray et al., 2001). This evidence, along with other studies led some researchers to suggest that there is more than one brain area that are coupled together to produce respiratory rhythm (Hilaire and Pasaro, 2003). The network model of respiratory rhythm was proposed and stipulates that there are interactions between inhibitory and excitatory neurons (Hilaire and Pasaro, 2003). These respiratory related neurons fire at different times of the respiratory cycle and are therefore categorized as inspiratory (augmenting, decrementing, early, pre-inspiratory, or late) and expiratory (augmenting, decrementing, early, late) or phase spanning (inspiratory -expiratory, expiratory-inspiratory) (Ezure, 1990, Duffin, 2004). Multiple network models have been proposed, however, in general they revolve round the reciprocal inhibition between the respiratory related neurons which generate respiratory rhythm (Ezure, 1990, Duffin, 2004).

1.2 Respiratory Motor Neurons Drive Respiratory Muscles

Breathing requires the proper timing and contraction of respiratory muscles. Motor neurons function to link the activity of respiratory muscles with central respiratory rhythm (Rekling et al., 2000). Since motor neurons are critical in triggering effective breathing it becomes important to understand what influences motor neuron physiology (Rekling et al., 2000). Motor neuron excitability, the increased potential to produce action potentials, is influenced through a complex set of synaptic interactions (e.g., neurotransmission, plasticity) (Funk et al., 2000). This thesis is interested in the enhancement of motor neuron output through plasticity (discussed in Section 1.3). The focus of this thesis will be on the hypoglossal motor neurons that drive the genioglossus, as well as the phrenic motor nucleus which functions to drive the diaphragm contraction (Lowe, 1980, Prakash et al., 2000).
The upper airway can be thought of as a collapsible tube where the lack of bony structures allows the pharyngeal muscles to modulate upper airway resistance (Remmers et al., 1978). Upper airway respiratory motor neuron control is important for upper airway patency (Remmers et al., 1978). Failure of the upper airway respiratory motor neurons can lead to a less rigid airway which collapses under the negative pressure produced by the diaphragm contraction (Remmers et al., 1978, Horner, 1996). This collapse is the underlying cause of obstructive sleep apnea (Horner, 1996). Therefore, understanding how the hypoglossal and phrenic motor neurons are modulated by plastic mechanisms could increase our knowledge of the biochemical processes mediating sleep apnea.

1.2.1 **Hypoglossal Motor Nucleus**

The hypoglossal motor nucleus is a bilateral structure that extends the length of the medulla and is close to the midline (Liu et al., 2003, Berger, 2011). Electrophysiological recordings of hypoglossal motor neurons show phases of inspiration, early expiration and late expiration (Peever et al., 2002). The respiratory drive onto hypoglossal motor neurons originates from premotor neurons located in the lateral tegmental field and from the interneurons residing in the hypoglossal motor nucleus (Peever et al., 2002). During inspiration hypoglossal motor neurons are depolarized and repolarized as the expiratory phase begins (Peever et al., 2002). The inspiratory drive is thought to be primarily driven by glutamate acting through the non-NMDA type AMPA receptor (Funk et al., 1993, Funk et al., 1997). It is not truly understood if inhibition of the hypoglossal motor neurons occurs during expiration, however, it has been suggested that these motor neurons are disfacilitated rather than actively inhibited during expiration (Woch and Kubin, 1995, Peever et al., 2002).
The activity of motor neurons can also be influenced through neuromodulators like noradrenaline and serotonin (Rekling et al., 2000, Funk et al., 2011). Neuromodulators do not directly open ion channels as in the case of neurotransmitters; rather, they act on intracellular cascades (Piascik and Perez, 2001, Funk et al., 2011). In the case of noradrenaline, the brainstem regions A5 and locus ceruleus provide noradrenergic release onto the hypoglossal motor neurons (Moore and Bloom, 1979, Aldes et al., 1992). The exact intracellular mechanisms involved after activation of the noradrenaline α1-adrenergic receptor is not fully known. However, there is evidence that suggests that α1-adrenergic receptor is coupled to a Gq-protein and once activation of the Gq-protein takes place intracellular cascades are triggered (Piascik and Perez, 2001). One intracellular cascade thought to be initiated by Gq-protein activation is phospholipase C which will induce hydrolysis of phosphatidyionsitol-4,5-bisphosphate (Funk et al., 2011). This process generates second messengers inositol-(1,4,5)-trisphosphate (IP3) and diacyl-glycerol (DAG) cellular pathways (Funk et al., 2011). Protein kinase C is then activated by the release of intracellular calcium, triggered by IP3, and by direct activation of DAG (Zhong and Minneman, 1999). Protein kinase C is then thought to potentiate the AMPA receptors mediating inspiratory drive (Neverova et al., 2007).

1.2.2 Phrenic Motor Nucleus

The phrenic motor nucleus is located in the ventromedial region of the ventral horn of the spinal cord which is C3-C5 in rats (Mantilla and Sieck, 2008, 2011). Phrenic premotor neurons have been located within the ventral respiratory group and function to transmit inspiratory drive to the respiratory motor neurons (Tian and Duffin, 1996). Premotor neurons located in the VRG are glutamatergic in nature and provide inspiratory drive on phrenic motor neurons through NMDA and non-NMDA receptors (Chitravanshi and Sapru, 1996). Expiratory neurons of the Botzinger complex function to inhibit the phrenic motor neurons during expiration (Merrill and
Fedorko, 1984). This inhibition during expiration is mediated by the activation of GABA_A receptors (Fedorko et al., 1987).

Neuromodulators (e.g. serotonin, noradrenaline) also act on phrenic motor neurons (Rekling et al., 2000). Their effects are seen through the activation of intracellular cascades which change the excitability of motor neurons (Funk et al., 2011). An example of neuromodulation of the phrenic motor neurons can be seen through serotonin (Mitchell et al., 2001a). Premotor neurons residing in the caudal raphe provide serotonergic input onto hypoglossal motor neurons (Dobbins and Feldman, 1994). Activation of these serotonergic receptors is thought to activate intracellular cascades (e.g. PKC) which trigger the upregulation of post-synaptic glutamatergic receptors as well as increase presynaptic release of glutamate onto phrenic motor neurons (Feldman et al., 2003, Baker-Herman et al., 2004). This is important because respiratory plasticity is thought to be mediated by neuromodulation of hypoglossal motor neurons (see section 1.3 and 1.3.3) (Feldman et al., 2003).

1.3 The Respiratory System Exhibits Plasticity

One method to increase motor neuron excitability and therefore respiratory drive is to enhance synaptic communication to respiratory motor neurons. The synaptic efficacy refers to the efficiency of neurotransmitter transmission between neurons and it is not fixed (Kuffler et al., 2001). In fact, the synaptic efficacy between neurons can undergo changes that result from patterns of repeated activity (Kuffler et al., 2001). A change in synaptic efficacy is known as synaptic plasticity and can be identified as short term, lasting seconds, or long term, lasting minutes to hours (Christoffel et al., 2011). Evidence for long term changes in mammalian synapses was first demonstrated in the hippocampus of anesthetized rabbits (Bliss and Lomo,
Increases in hippocampal field potentials resulted from brief, high frequency stimulation of the inputs to the dentate gyrus (Bliss and Lomo, 1973). Bliss and Lomo were not aware of the mechanism responsible for the long term changes in hippocampal field potentials; however they speculated that brief high frequency stimulation was increasing neurotransmitter release, increasing the number of terminals releasing neurotransmitters or modulating the sensitivity of postsynaptic junction (Bliss and Lomo, 1973). The mechanism of LTP was further investigated and in the late seventies and early eighties it was determined that NMDA receptors had a role in LTP (Collingridge et al., 1983). NMDA receptors are cation channels that have high calcium conductance; however, at a normal cellular resting membrane potential these channels are blocked by magnesium (Dingledine et al., 1999). During membrane depolarization the magnesium block is removed and calcium enters the cell (Dingledine et al., 1999). Evidence for the role of NMDA receptors was demonstrated by NMDA receptor antagonists. If NMDA receptor antagonists were applied before the brief high frequency stimulation LTP did not occur (Collingridge et al., 1983). A role for calcium in LTP was demonstrated when researchers showed that injections of calcium chelators (BAPTA or EGTA) into the postsynaptic cell prevented the induction of LTP (Lynch et al., 1983, Yeckel et al., 1999). Once calcium enters the postsynaptic neuron, it binds to calmodulin which then activates calcium/calmodulin-dependent protein kinase II (CaMKII) (Malenka et al., 1989). The autophosphorylation of CaMKII allows this kinase to continue functioning within the cell after intracellular calcium concentrations return to baseline values (Miller and Kennedy, 1986). CaMKII functions to increase the excitability of the postsynaptic neuron by transporting AMPA receptors to the cell membrane as well as increasing AMPA receptor channel conductance through phosphorylation (Malenka et al., 1989, Malenka and Nicoll, 1999). Therefore, the connection between neurons can be modified in their strength through repeated episodes of stimuli.
The respiratory system is misconceived to be rigid and non-malleable. This is not the case; in fact, the respiratory system is attentive and adaptive (Mitchell et al., 2001a). Thus, the respiratory system displays plasticity and it can be induced by different stimuli, in various animals and under several conditions (Millhorn et al., 1980a, Fuller et al., 2000, Mitchell et al., 2001a, Tadjalli et al., 2010). The focus of this thesis is on a form of respiratory plasticity known as long term facilitation (LTF).

Respiratory LTF is characterized by progressive increases in respiratory motor output that last from minutes to hours in response to repeated respiratory challenges (Millhorn et al., 1980a, Mitchell et al., 2001a, Tadjalli et al., 2010). Some examples of how LTF can be induced are through carotid sinus nerve stimulation, repeated episodes of hypoxia and repeated vagal afferent modulation. These examples are discussed in the following sections (1.3.1 - 1.3.4). Changes in the inspiratory discharge of the carotid sinus nerve were measured after the LTF induction protocol (Millhorn et al., 1980a). Although it has been shown that multiple respiratory muscles can exhibit LTF, the majority of LTF studies monitored respiratory output of the hypoglossal and phrenic motor pools or their associated muscles (the genioglossus and diaphragm) (Fuller et al., 2001, Baker-Herman et al., 2004, Tadjalli et al., 2010).

1.3.1 Carotid Sinus Nerve Induced Respiratory Plasticity

The initial experiments that elicited LTF of the phrenic nerve activity was produced by carotid sinus nerve stimulation in cats (Millhorn et al., 1980a). The protocol for stimulation was five episodes that lasted two minutes in length. The results consisted of long term increases in phrenic nerve activity. The cats were urethane-anesthetized, paralyzed, mechanically ventilated and bilaterally vagotomized. Mechanical ventilation of the animal controlled the arterial blood gases and tracheal pressure. This excluded partial pressures of gases or receptors in the trachea as
potential stimuli for generating the increases in phrenic nerve activity. The paralysis reduced the myoelectric activity and the animals were vagotomized to prevent reductions in phrenic nerve activity as well as to avoid the entrainment of the phrenic nerve discharge to the ventilator. Thus, this initial study demonstrated that carotid sinus nerve stimulation could elicit long term changes in respiratory output and that the respiratory system was indeed plastic. In subsequent studies, (Millhorn et al., 1980b) determined that transections of the spinal cord at C7-T1 did not prevent LTF; however, serotonin antagonists did.

The respiratory system could adapt and adjust its output after repeated stimulation of the carotid sinus nerve; however, the electrical stimulations used were far beyond the range of excitation a nerve would ever experience under a natural chemoafferent stimulus. Researchers aimed to find a more relevant stimulus that acted within the physiological bounds of the system, hence, hypoxia - a potent and natural stimulus.

1.3.2 Hypoxia-induced Respiratory Plasticity

Currently, the most popular method to initiate LTF is intermittent hypoxia. The traditional hypoxia-induced LTF protocol consisted of anesthetized, paralyzed and vagotomized rats being exposed to three five-minute episodes of hypoxia (Cao et al., 1992, Bach and Mitchell, 1996). Each of these episodes had a recovery period where the animal was able to breathe normal levels of oxygen. Very early on, researchers noticed that respiratory LTF, like other forms of plasticity, is pattern sensitive (Baker and Mitchell, 2000). In the case of hypoxia-induced LTF, hypoxia must come in the form of repetitive episodes rather than one extended episode. Researchers commonly use three 5-min hypoxic episodes that are each separated by five minutes of hyperoxia to generate LTF, see Fig 1-1 (Fuller et al., 2000). When rodents are anesthetized and ventilated the intensity of the hypoxia and duration of hypoxic exposure do not
significantly affect the magnitude of LTF (Fuller et al., 2000, Mahamed and Mitchell, 2008). However, it was demonstrated that the recovery periods between hypoxic bouts are critical in eliciting LTF of the phrenic nerve when animals are ventilated and paralyzed (Bach et al., 1999). A recovery period of five minutes will elicit LTF; whereas, a recovery period that is much longer in length (30 minutes) will not trigger respiratory plasticity (Bach et al., 1999).

Experimental paradigms that do not involve anesthesia and mechanical ventilation but rather heart-brainstem preparation or awake rats demonstrate different results in the variables that contribute to the magnitude of LTF expression (McGuire et al., 2002, Tadjalli et al., 2007). In awake rats both the intensity and frequency of hypoxia determine the outcome of LTF (McGuire et al., 2002). In fact, the optimal hypoxic level found to produce ventilatory LTF is inhalation of ~10% oxygen (McGuire et al., 2002). Inhaled oxygen above or below this level failed to produce LTF (McGuire et al., 2002). Also, the number of hypoxic episodes increase the duration, not magnitude, of LTF (McGuire et al., 2002). Using the in situ heart-brainstem preparation of neonatal rats, respiratory plasticity was demonstrated in the elevation of respiratory frequency, however, not in the inspiratory amplitude of the phrenic nerve (Tadjalli et al., 2007).

Respiratory plasticity has been demonstrated in several animal species. As stated earlier in the introduction, cats were the original model of the initial LTF studies (Millhorn et al., 1980b, a). Moreover, this phenomenon has also been described in ducks, mice, goats, dogs and rats (Cao et al., 1992, Hayashi et al., 1993, Bach and Mitchell, 1996, Turner and Mitchell, 1997, Mitchell et al., 2001b, Peng and Prabhakar, 2003, Sokolowska and Pokorski, 2006). In humans, LTF has not been found in the wake state unless they are in the presence of elevated carbon dioxide levels (Harris et al., 2006). The rodent is the most widely used model to elucidate the mechanisms for respiratory plasticity, however, genetic variations do exist between strains of rats and these
genetic variations do play a factor in the expression of LTF. For example, Harlan Sprague Dawley colony 236 did not express LTF of the hypoglossal nerve activity whereas K62 colony from Charles River did (Fuller et al., 2001). Thus, animal model and stimulus influence mechanisms mediating respiratory plasticity.
Figure 1-1. Electrophysiological recording of phrenic LTF. Repeated episodes of hypoxia elicit increases in phrenic nerve activity that last at least sixty minutes. The dotted line represents baseline. Adapted from Feldman et al. (2003).
1.3.3 The Mechanisms of Hypoxia-induced Respiratory Plasticity

Repeated episodes of hypoxia could not elicit LTF if animals were pretreated with methysergide, a serotonin receptor antagonist, or ketanserin, a selective serotonin 5HT$_2$ receptor antagonist (Bach and Mitchell, 1996). This study demonstrated that serotonin, and more specifically 5HT$_2$ receptor activation was important for the manifestation of LTF; however, it did not assess whether 5-HT receptor activation was necessary for LTF induction, maintenance or both (Bach and Mitchell, 1996). Fuller et al. (2001) demonstrated that if 5HT receptor activation was blocked using ketanserin after hypoxic episodes phrenic LTF was not expressed. Therefore, activation of spinal serotonin receptors triggers (but does not maintain) phrenic LTF (Fuller et al., 2001, Baker-Herman and Mitchell, 2002). Currently, researchers are investigating the role of downstream intracellular cascades, which are triggered by 5HT$_2$ receptor activation, on hypoxia-induced LTF.

Previous literature has shown that 5HT$_2$ receptors are Gq protein-coupled metabotropic receptors that activate downstream kinases like protein kinase C (PKC) (Baker-Herman and Mitchell, 2002, Feldman et al., 2003, Baker-Herman et al., 2004). The current hypothesis stipulates that activation of 5HT$_2$ receptors on phrenic motor neurons will activate PKC. PKC further initiates protein synthesis and neurotrophic cascades such as brain-derived neurotrophic factor (BDNF). It has been determined that BDNF is necessary for the expression of phrenic LTF (Baker-Herman and Mitchell, 2002, Baker-Herman et al., 2004). BDNF is postulated to induce phrenic LTF via TrkB receptor activation because inhibition of receptor tyrosine kinases prevents phrenic LTF (Baker-Herman et al., 2004). Moreover, BDNF’s activation of TrkB receptors is speculated to trigger LTF via the enhancement of presynaptic glutamate release, increases in glutamate expression on phrenic motor neurons, reduce presynaptic GABA transmission and/or influence membrane potential of phrenic motor neurons (Levine et al., 1998,
Figure 1-2: The mechanisms mediating hypoxia-induced LTF. Activation of 5HT$_{2A}$ receptors on phrenic motor neurons result in new protein synthesis and the activation of neurotrophic cascades leading to increases in phrenic motor neuron excitability. Adapted from Feldman et al. (2003).
1.3.4 Apnea-induced Respiratory Plasticity

The respiratory system is not only sensitive to chemical stimuli (i.e. hypoxia) but also sensitive to the mechanical feedback from the lungs (Bonham et al., 1993, Bonham et al., 2006). This critical feedback is lost during vagotomy and therefore, our laboratory investigated whether intermittent vagal modulation could stimulate respiratory LTF. There were three lines of evidence that suggested repeated mechanical feedback, carried by the vagus nerve, could indeed evoke LTF. The first line of evidence comes from the experiments on Aplysia by Kandel (2001) where intermittent stimulation of respiratory mechanoreceptors results in long term plasticity of the respiratory gill withdrawal reflex. Evidence pertaining to vagal stimulation and LTF came from work by Zhang et al. (2003) where episodic high intensity vagus nerve stimulation induced phrenic LTF. Finally, other types of plasticity, like hippocampal long-term potentiation in rats and the word-recognition memory in human patients are enhanced following stimulation of vagal afferents (Clark et al., 1999, Zuo et al., 2007).

Although Zhang et al. (2003) showed stimulation of the vagus could produce phrenic LTF; the high intensity stimulation was far above anything that would be experienced under normal physiological conditions. Tadjalli et al., (2010) developed a physiological relevant model to elucidate the mechanisms of LTF where the vagus nerves are left intact, the rats can breathe spontaneously and the modulation of the vagal afferents are within the natural physiological range.

Tadjalli et al. (2010) induced apneas by the complete occlusion of the upper airway and measured the long term effects on breathing. They found that during apneas vagus nerve activity decreased by roughly 95% and thus, vagal modulation was possible through the physiological stimulus of apneas. The next sets of experiments were designed to test the hypothesis that vagal modulation, via repeated apneas, could induce expression of LTF in the diaphragm and
genioglossus muscle. Ten repeated apneas, each separated by 1 minute, evoked LTF of inspiratory activity. LTF was expressed only in the genioglossus muscle and not the diaphragm muscle or respiratory frequency.

During obstructive apnea arterial oxygen saturation levels were found to decrease significantly from baseline; however, this decrease was transient and returned to baseline levels after the apnea was terminated. The apnea protocol induced both repeated modulations in vagal activity as well as repeated hypoxic episodes. Although these repeated apneas are brief in length, the system is still subjected to repeated episodes of hypoxia and repeated episodes of hypoxia have been shown to cause long term changes in respiratory motor output (Bach and Mitchell, 1996). To dissociate repeated vagal modulation from repeated hypoxia-induced changes in respiratory motor output, Tadjalli et al. (2010) performed the apnea protocol under vagotomy and vagal cooling. Removal of vagal feedback by vagotomy caused immediate increases in the baseline genioglossus amplitude; post apnea genioglossus activity was compared to the new baseline level and LTF was not present in diaphragm or genioglossus muscle activity. This demonstrated that apnea-induced plasticity was not triggered by repeated hypoxic episodes and that this phenomenon is vagal-dependent. The authors also wanted to show that LTF was not masked by the vagotomy-induced increase in baseline genioglossus activity. After vagotomy, genioglossus muscle activity could be increased by either hypercapnia (5% CO2) or during an apnea. Therefore, they asserted that increases in the genioglossus could still occur after vagotomy. To further demonstrate that it is vagal modulation and not hypoxia causing LTF of the genioglossus muscle, Tadjalli et al. (2010) reversibly inactivated the vagus nerve by cooling the nerves to about 3°C during the repeated apneas. This technique prevented the increases in baseline genioglossus activity that results from vagotomy alone. Long term changes in genioglossus and diaphragm activity were not found when the vagus nerves were cooled during
repeated apneas. These authors also investigated repeated vagal cooling in the absence of apneas. When the vagus nerves were cooled for one minute in duration six times LTF was expressed in the genioglossus muscle and not the diaphragm which suggested that modulations of vagal activity can produce long term changes in genioglossus motor output. Therefore, these three experiments demonstrated that apnea-induced LTF of genioglossus muscle is due to vagal modulation and not hypoxia.

After demonstrating that the expression of LTF was due to vagal modulation, the next step was to uncover the neurochemical mechanisms that trigger apnea-induced LTF. The neurochemical mechanism was investigated at the hypoglossal motor nuclei. Using reverse microdialysis of ketanserin, a 5HT2A receptor antagonist, onto hypoglossal motor neurons Tadjallli et al. (2010) investigated if 5HT2A receptor activation was necessary for the expression of LTF in genioglossus inspiratory activity. This specific receptor was chosen because Zhan et al. (2002) showed hypoglossal motor neurons express 5HT2A receptors and because it is thought that hypoxia induced LTF requires 5HT2A receptor activation (see Section 1.3.3) (Bach and Mitchell, 1996, Fuller et al., 2000). Although unexpected, 5HT2A receptor activation was not required for apnea-induced LTF (Tadjallli et al., 2010).

Hypoxia-induced LTF of hypoglossal nerve activity was prevented if α1-adrenergic receptors were blocked both in vivo and in vitro (Neverova et al., 2007). Therefore, Tadjallli et al. (2010) hypothesized that antagonizing the α1-adrenergic receptors of hypoglossal motor neurons by reverse microdialysis of terazosin, an α1-adrenergic antagonist, would prevent LTF. In fact, they demonstrated that LTF of genioglossus inspiratory activity required the activation of α1-adrenergic receptors. Due to these findings, Tadjallli et al. 2010 postulated that apnea-induced plasticity was mediated by an alternative mechanism than hypoxia-induced LTF. The evidence for this was that hypoxia-induced LTF was serotonergic in nature, whereas, apnea-induced LTF
was noradrenergic dependent and did not require serotonergic input at the hypoglossal motor pool.

To conclude, in contrast to hypoxia-induced LTF, apnea-induced LTF is expressed in the genioglossus muscle activity and not in the diaphragm muscle activity. Also, unlike hypoxia-induced LTF vagal modulation is sufficient for generating LTF, requires $\alpha_1$-adrenergic receptor activation, not 5HT receptor activation, and is induced by vagal feedback not hypoxia.

1.4 Vagal Afferents

There are different types of receptors located below the larynx within the airways. These airway receptors are identified into three categories: slowly adapting stretch receptors (SARS), rapidly adapting stretch receptors (RARS) and bronchopulmonary C fibers (Kubin et al., 2006). The receptors are categorized based on their activation to various stimuli. Inhaled irritants (i.e. cigarette smoke) activate RARS, whereas the SARS are activated by lung volume (mechanical). The bronchopulmonary C fibers encompass the receptors that are not distinctively activated by irritants or lung volume. The bronchopulmonary C fibers are stimulated by abnormal amounts of interstitial fluid within the lung and by temperature (Kubin et al., 2006).

The focus of this section is on the receptors which respond to lung inflation, since manipulations in lung volume lead to apnea-induced LTF. These receptors reside within the airway smooth muscle. The volume of the lung and the transmural pressure is constantly monitored by the SARS (Ezure, 1990, Ezure and Tanaka, 1996, Kubin et al., 2006). These receptors react to both dynamic and static changes of lung volume and function to link lung mechanics with the central respiratory system. The SARs communicate changes in lung volume by modulating their firing pattern with decreases in stretch receptor discharge during deflation and increases during inflation (Persson et al., 1981, Vetter et al., 1999, Liu et al., 2003).
Artificial stimulation of SARs results in a decrease in the respiratory frequency due to an abrupt termination of inspiration and an increase in expiratory time. This phenomenon is defined as the Hering-Breuer reflex and it functions to regulate the phase shift between inspiration and expiration (Cohen and Feldman, 1984, Bonham et al., 1993). The axons of the SARS traverse the vagus nerve and terminate onto a specific region of the brainstem known as the nucleus of the solitary tract (Kalia and Sullivan, 1982).

1.5 Nucleus of the Solitary Tract

A bilateral brain region known as the nucleus of the solitary tract (NTS) receives inputs from a variety of visceral receptors which include cardiovascular, respiratory, gustatory, hepatic and renal control systems (Kalia and Sullivan, 1982, Andresen and Kunze, 1994, Bonham, 1995). This region is thought to be composed of 42,000 cells receiving over an estimated one million synapses from other various brain regions (Palkovits, 1981). It functions to integrate this extensive array of visceral afferent termination and accommodates this vast amount of input by its large rostral-caudal axis (Andresen and Kunze, 1994, Kubin et al., 2006). The anatomical composition of the NTS is heterogeneous, however, there is a general organization for the termination of the visceral afferents (Ezure and Tanaka, 1996, Miyazaki et al., 1999, Kubin et al., 2006). For example the more rostral regions of the NTS are predominantly for gustatory afferent termination whereas the more caudal regions are reserved for a mix of cardiopulmonary afferent termination (Barraco et al., 1992).

Non-pulmonary afferents are beyond the scope of this thesis and therefore, this section will focus on the termination of pulmonary afferents. The rat NTS has been divided into several distinct subnuclei: commissural (SolC), dorsal (SolD), dorsolateral (SolDL), gelatinosus (SolG), interstitial (SolI), intermediate (SolIM), medial (SolM), ventral (SolV), and ventrolateral

Neurons in the NTS which are monosynaptically activated by SAR afferents are known as pump cells (Pcells) and their electrophysiological activity is identical to the discharges of SARs (Berger and Dick, 1987). Therefore, Pcells exhibit phasic activities that constantly report the fluctuating lung volumes (Kubin et al., 2006). Bonham et al., (1993) demonstrated that pressure-injections of the excitatory amino acid, DL-homocysteine, into the SolIM produced apneas analogous to the Hering-Breuer reflex. This suggested that the NTS plays a role in mediating the Hering-Breuer reflex (Bonham et al., 1993). Bonham et al. (1993) also showed that although Pcells respond to both lung inflation and stimulation by DL-homocysteine, application of kynuric acid, an antagonist of excitatory amino acid, would block the response of Pcells to either stimulus. Taken together, Bonham and colleagues provided evidence that the NMDA and non-NMDA receptors are present on Pcells and glutamate was a potential neurotransmitter from SAR afferents. Evidence for the glutamatergic transmission from SAR afferents to Pcells was further demonstrated by Miyazaki et al. (1999) by the reduction in Pcells activity after application of NMDA antagonists APV and MK-801.

Miyazaki et al. (1999) demonstrated Pcells are not just simple relays of SAR activity. They also demonstrated that central respiratory drive does not have any influence on Pcells in the cat, however, in the rat it was quite different (Miyazaki et al., 1998, Miyazaki et al., 1999). Using extracellular in vivo recordings, Miyazaki et al. (1998) showed that the rat receives input from the central respiratory generator. Later, Miyazaki et al. (1999) demonstrated that as the inspiratory period progresses and eventually shifts to expiration, so too does the input from the
central respiratory drive. At first glycinergic inhibition was found during the onset of inspiration which was followed by a glutamatergic excitation near the end of inspiration and during the onset of expiration. Finally, Miyazaki et al. (1999) also provided evidence that phasic input from the central respiratory drive onto the Pcells occurs at the exact moment inspiration switches to expiration and the constant tonic inhibitory drive to Pcells operate through a GABA\textsubscript{A} mediated mechanism. This collection of data led Miyazaki et al. (1999) to speculate that at the exact moment where inspiration transitions to expiration NMDA receptors on Pcells are activated by an unknown group of respiratory neurons. This activation causes the termination of inspiratory drive by activating the Hering-Breuer reflex pathway. Thus, the nucleus of the solitary tract is an important area of integration for pulmonary afferent information regarding mechanical feedback from the lungs.

1.6 GABA Receptors

Gamma-aminobutyric acid (GABA) is a major inhibitory neurotransmitter of the mammalian brain and it is synthesized by the decarboxylation of L-glutamic acid via glutamic acid decarboxylase (GAD) (Jin et al., 2003). This neurotransmitter is stored in presynaptic vesicles and released after neuronal depolarization (Jin et al., 2003, Mody and Pearce, 2004). Once it is released from the presynaptic bouton it exerts its effects on nearby post synaptic GABA receptors (Mody and Pearce, 2004). There are three classes of GABA receptors: GABA\textsubscript{A}, GABA\textsubscript{B} and GABA\textsubscript{C} (Bormann, 2000). They are categorized into two main types: ionotropic receptors and metabotropic receptors (Bormann, 2000).

GABA\textsubscript{A} channels are fast acting and consist of five subunits arranged around a chloride channel pore which designates them as members of the cys-loop super family of similar ligand-gated ion channels (Bormann, 2000, Mohler, 2006). The receptors’ properties (e.g. conductance,
agonist affinity) are dependent upon the composition of subunits which come in various combinations (known as isoforms). Binding of the ligand GABA or GABA analogues (e.g. muscimol, GABA\textsubscript{A} agonist) opens the chloride pore resulting in the influx of chloride ions into the neuron (Sieghart, 2006). This activation hyperpolarizes the neuron and decreases the likelihood of excitatory neurotransmitters causing depolarization, thereby minimizing the chance of the neuron of generating an action potential.

The slow acting GABA\textsubscript{B} receptors are G protein coupled and activate either G\textbeta\gamma or Ga/Gao subunits (Chalifoux and Carter, 2011). Activation of the G\textbeta\gamma causes the inhibition of calcium channels and activation of potassium channels whereas the activation of the Ga/Gao leads to the inhibition of adenylyl cyclase (Chalifoux and Carter, 2011). Due to this inhibition, the concentration of cyclic AMP is decreased and this reduces the activity of protein kinase A which ultimately prevents the activation of NMDA receptors (Chalifoux and Carter, 2011).

1.7 Hypothesis and Experimental Approach

Tadjalli et al. (2010) demonstrated that apnea-induced plasticity is mediated by vagal modulation and we also know that the NTS is the exclusive termination point of the vagal afferents from the lungs. The ultimate goal of our laboratory is to map the neural network responsible for apnea-induced respiratory plasticity. Therefore, we aimed to investigate the role of the NTS on long term changes in upper airway activity. We hypothesized that inhibition of the NTS region would abolish apnea-induced LTF because descending projections from vagal afferents are mediated through this brain region. Activation of GABA\textsubscript{A} receptors on NTS neurons has been shown to be inhibitory (Bennett et al., 1987, Wang et al., 2010); hence, we inhibited the NTS via the perfusion of muscimol, a GABA\textsubscript{A} agonist. If inhibition of the NTS during repeated apneas prevents apnea-induced LTF, then the central pathway mediating apnea-
induced plasticity originates in the NTS. This thesis uses a combination of neuropharmacological, electrophysiological, histological techniques to test this hypothesis and was investigated using the following objectives:

**Objective 1:** To confirm that repeated apneas induce LTF?

**Objective 2:** To determine if the neurons of the NTS can be pharmacologically inhibited?

**Objective 3:** To determine if unilateral inhibition of the NTS prevents LTF?

**Objective 4:** To determine if bilateral inhibition of the NTS prevents LTF?
Chapter 2 : Methods

2.1 Ethical Guidelines

The experimental procedures in this study were performed in accordance with both the Canadian Council on Animal Care and University of Toronto Animal Care Committee. A total of 35 male Sprague-Dawley rats, 9-10 weeks old, (mean weight: 433 ± 42 g). All animals were housed in the University of Toronto’s Department of Cell and Systems Biology Animal Care facility. Animals were then relocated to the laboratory twenty-four hours prior to the experiment. This allowed time to acclimate to the new environment and reduce the stress from being relocated from the housing facility. Animals were housed in clean, rectangular polysulphone cages (Nalgene Lab ware, Nalge Nunc International, Rochester, NY) that were lined with corncob chip bedding with environmental conditions maintained at 20 ± 1C and 60% humidity, with a 12 hour light – 12 hour dark cycle. Animals had ad libitum access to drinking water and rat chow (Lab Diet, PMI Nutrition International, St. Louis, MO).

2.2 Surgical Preparation

Rats were weighed and then placed into an induction chamber where a 1:1 gas mixture of oxygen:nitrogen was presented. Gas flow was regulated via a variable flow-meter system (Western Medica, West Lake, OH) to a flow rate of 1800 mL/min. Outflow of gas from the induction chamber was vented into an activated-carbon scavenging canister (A.M. Bickford INC, Wales Center, NY). Isoflurane (Baxter, Mississauga, ON) was introduced to the gas mixture using an isoflurane vaporizer (T3IS0, Benson Medical Industries, Markham, ON). Animals were continuously monitored until they were unconsciousness, which took approximately 4 minutes. The animal was then removed from the induction chamber and placed in a supine position on the operating platform with their snout in a custom-made, sealed, nose cone providing the supply of
3% isoflurane, 50% oxygen: 50% nitrogen gas mixture. A heating pad (TC-1000; CWE, INC, Andromore, PA) was used to maintain a rectal body temperature of 37-38°C. The top of the head, neck and abdomen were shaved in preparation for surgery. The rear foot-pinched withdrawal reflex tested effective depth of anesthesia. Once this reflex was eliminated a midline ventral incision was made at the neck. The trachea was exposed below the larynx by blunt dissection of the overlying smooth muscle. A transverse incision between the tracheal rings was performed. Immediately after the incision between the tracheal rings was made, a custom-made silicone T-tube cannula was inserted into the proximal end of the trachea and secured using 4-0 gauge silk suture (Deknatel, Mansfield, MA). The silicon tubing carrying the isoflurane-oxygen gas mixture was connected immediately and anesthesia was reduced to 2%. All skin incisions were loosely closed using 9mm wound clips (Becton Dickinson, Sparks, MD). The custom T-tube cannula was also connected to an end-tidal CO$_2$ analyzer (MicroCapster Endtidal CO$_2$ analyzer, Ardmore, PA). To prevent the accumulation of mucosal secretions that could potentially occlude the tracheal T-tube, a subcutaneous injection of atropine sulfate was administered at a dose of 0.4mg/kg.

With the anesthetized animal still in the supine position a jugular catheter was prepared to allow infusion of lactated Ringer’s solution throughout the surgical procedure and experiment. A two-centimeter ventral cervical skin incision was made to the right of the midline with the caudal terminus at the level of the clavicle. The underlying tissues (salivary and lymphatic) were separated via blunt dissection to present the jugular vein. The jugular vein was then mobilized by blunt dissection away from the surrounding connective tissue. A catheter composed of polyethylene tubing (PE50, 0.023” ID), was secured to a blunt 20 gauge needle that was connected to a stop cock and 5mL plastic syringe for delivery of the infusion solution at a rate of 1.5mL hr$^{-1}$ (MD-1020, BASi, West Lafayette, IN). The catheter was beveled to 45 degrees to
allow for a more manageable insertion into the jugular vein. The distal end of the vein was tied off using 4-0 gauge silk suture (Deknatel, Mansfield, MA) to prevent drainage of the return blood flow. At a 45 degree angle a small incision was made to allow access for the catheter. The catheter was then inserted into the vein through this incision and secured with 4-0 gauge suture at the proximal end. An additional knot of silk suture was used at the distal end to secure the distal portion of the catheter. The skin incisions were loosely closed using 9 mm wound clips.

Previous literature has demonstrated that repeated apneas generate long term increases in respiratory motor outflow of the genioglossus (Tadjalli et al., 2010). Therefore, we monitored and recorded the motor outflow of the genioglossus muscle by bilaterally inserting a pair of bipolar needle electrodes into the genioglossus muscle (F-E2, Grass Technologies, West Warwick, RI). The diaphragm EMG activity was also monitored and recorded by custom-made needle electrodes. The diaphragm electrode contained three barb tipped needles secured and evenly distributed in parallel via dental cement. Insulated stainless steel wires were soldered to the barbed needle tips. A two-centimeter midline incision was made on the abdomen and the diaphragm electrode was fastened to the diaphragmatic fascia. The midline abdomen incision was loosely closed using 9 mm wound clips.

The goal of my thesis was to neuropharmacologically manipulate the NTS and determine how this intervention affects apnea-induced LTF. In order to reach this area with a microdialysis probe and focally apply drugs, the stereotaxic method was used. Once the surgical procedure consisting of the intubation of T-tube, EMG electrodes and insertion of jugular catheter was complete, the animal was gently repositioned in the prone position on the operating platform within the stereotaxic frame. The animal was secured with blunt ear bars and a snout clamp (David Kopf Instruments, Chatsworth, CA). A stereotaxic head leveler was used to ensure
bregma and lambda were level (David Kof Instruments). To expose the skull bone, a midline incision from the frontal bone to the caudal base of the skull was made. Following the incision, a cotton swab that was submerged in 3% hydrogen peroxide was used to clean and pull away the overlying connective tissue. Any bleeding that did not cease after one minute of pressure was controlled by topical application of adrenaline, a blood vessel clotting agent. To increase the appearance of skull sutures and prevent bleeding from the porous skull, bone wax (Ethicon, Knochenwacks, St. Vith, Belgium) was applied to the skull surface. The intersection of the sagittal and coronal sutures is known as the bregma landmark. All unilateral experiments had burr holes drilled (TX Series, Foredom Electric Co., Bethel, CT) at 13.3 mm caudal from bregma and 1.2 mm to the right lateral from the midline. The microdialysis probes (CMA/11, Holliston, MA) used to perfuse candidate drugs into the NTS had a membrane length of 1mm and a diameter of 250um. Molecules greater than 6KDa could not transverse the semi-permeable membrane of the microdialysis probe. Microdialysis probes were lowered into the burr holes to a ventral position of 9mm from bregma (Paxions & Watson, 2004). The microdialysis probe was then connected to Teflon tubing (inner diameter of 0.10; Elcom, Japan) that was then connected to a 1mL gastight syringe (MD00500 Gastight Syringes, BASi, West Lafayette, IN) via a liquid switch (UniSwitch, BASi, West Lafayette, IN). The probe was continuously perfused with filtered (0.2 um Nylon, Fisher Scientific, Ottawa, ON) saline or candidate drugs via a syringe pump driver and controller (MD-1020 Bee Give Pump Controllers, BASi, West Lafayette, IN) at a rate of 2ul/min.

In the case of the bilateral experiments, burr holes were bilaterally drilled at 13.3 mm caudal from bregma and 1.2 mm from midline in both directions. In both bilateral experiments, microdialysis of muscimol and microinjection of lidocaine required probes to be custom fitted together and lowered 1.2 mm off the midline in both directions to a depth of 9 mm from bregma.
In bilateral muscimol experiments, the probe was continuously perfused with filtered (0.2 um Nylon, Fisher Scientific, Ottawa, ON) saline or candidate drugs via a syringe pump driver and controller (MD-1020 Bee Give Pump Controllers, BASi, West Lafayette, IN) at a rate of 2ul/min. The bilateral lidocaine perfusion experiments used modified microdialysis probes. The modifications consisted of sealing the outlet tube with solder and removal of the semi-permeable membrane. In these experiments, the pump driver was set to deliver 1ul of drug (via modified microdialysis probes) over one minute. Probe placement was verified by post-mortem histology.
Figure 2-1. Schematic representation of experimental setup. An axial section of the rat head highlighting the genioglossus muscle, a sample genioglossus electromyogram recording and localization of the microdialysis probe to the NTS.
2.3 Electrophysiological Recording

Genioglossus and diaphragm EMG signals were amplified between 500-2000Hz using a Super-Z High Impedance Head Stage and a BMA-400 AC/DC Bioamplifier (CWE Inc., Ardmore, PA). Signals were filtered with a bandpass between 100Hz and 3kHz. EMG signals were sampled at 666 Hz. End tidal CO₂ and temperature were sampled at 40Hz (Spike2 software, 1401 Interface; CED, Cambridge, UK) and stored on a computer for offline analysis.
Figure 2-2. An experimental sample of electrophysiological recordings. An example recording from the Spike2 data acquisition software. Recordings of the genioglossus muscle, diaphragm muscle, rectal temperature, end-tidal CO2. The integrated smoothed records of the genioglossus and diaphragm electromyograms are also shown. Signals are represented as arbitrary units and the time scale is in seconds.
2.4 Drugs

All drugs were dissolved in saline (0.9% NaCl) vehicle. Muscimol (5-Aminomethyl-3-hydroxyisoxazole, FW: 114.1g/mol; Sigma, St. Louis, MO), a potent GABA<sub>a</sub> receptor agonist, was prepared to final concentration of 1mM and aliquoted into 250uL amounts and stored in -20°C freezer. The solution was thawed and filtered an hour before each experiment. A 5% solution of lidocaine hydrochloride monohydrate (2-Diethylamino-N-(2,6-dimethylphenyl)acetamide hydrochloride monohydrate, FW: 288.81g/mol; Sigma, St. Louis, MO), a Na<sup>+</sup> channel blocker, was prepared fresh prior to each experiment. All drugs were vortexed and filtered before perfusion (0.2um Nylon, Fisher Scientific, Ottawa, ON).

2.5 Experimental Protocols

2.5.1 Objective 1. Do repeated apneas induce LTF?

I wanted to confirm previous data in the laboratory where repeated apneas were shown to produce long term increases in genioglossus inspiratory activity. Rats were exposed to ten 15-second obstructive apneas each separated in time by one minute. Obstructing the collapsible custom T-tube at the end of expiration induced apneas. These obstructions mimicked the apneas suffered by OSA patients (Sanders and Moore, 1983; Sanders et al., 1985). Confirmation of the obstruction was made by monitoring the CO<sub>2</sub> levels as well as the real-time recording from the Spike2 acquisition software. Once the repeated apneas were completed, respiratory activity was monitored for at least sixty minutes.

2.5.2 Objective 2. Can the neurons in the NTS be pharmacologically inhibited?

Neurons in the region of the NTS mediate the Hering-Breuer reflex and therefore, we hypothesized that inactivation of these cells should abolish or suppress the reflex. Once rats were
stable under anesthesia occlusion of the custom tracheal tube was performed at the peak of inspiration in order to maintain lung inflation. The occlusion resulted in an abrupt termination of inspiration and extended expiratory period, known as the Hering-Breuer reflex. Withdrawal of the occlusion occurred once the first inspiration took place. A probe was then lowered into the NTS. The Hering-Breuer reflex was then induced three times under saline perfusion, 0.5mM CNQX perfusion and 1mM muscimol perfusion. Twenty-minute washouts took place after each drug perfusion.

2.5.3 Objective 3. Does unilateral inhibition of the NTS prevent LTF?

In Aim 2, we showed that we could successfully inhibit the NTS with muscimol. Thus, the purpose of Aim 3 was to determine if a functional NTS is required for LTF. Therefore, we perfused 1mM muscimol for 15 minutes prior to and during repeated apneas (total perfusion time of 28 minutes) in rats. The apnea occlusions were confirmed in the same method as described in Aim 1. Once the apnea protocol was completed, saline perfusion was resumed and respiratory activity was monitored for at least sixty minutes.

We also performed control experiments to characterize the effect of muscimol perfusion alone on genioglossus inspiratory activity. Therefore, we perfused 1mM muscimol for 28 minutes without apneas. After muscimol perfusion, saline perfusion was resumed and respiratory activity was monitored for sixty minutes.

2.5.4 Objective 4. Does bilateral inhibition of the NTS prevent LTF?

Since unilateral inhibition of the NTS did not prevent LTF, we hypothesized that bilateral inactivation of the NTS neurons would abolish LTF. First, we used microdialysis probes to bilaterally perfuse 1mM muscimol 15 minutes prior to and during apneas. In this next set of experiments, we custom fitted two CMA microdialysis probe guides together so that both would
enter the brain 1.2mm off the midline on each side. In a second set of experiments, we further modified these probes for microinjection. We removed the semipermeable membrane on both probes and lowered them into the NTS. As we lowered the probes 5% lidocaine was perfused. Once the probes reached the target we injected 1uL of the 5% lidocaine solution over the course of one minute. One minute after microinjection we performed the apnea protocol and respiratory activity was monitored for at least sixty minutes.

2.6 Histology

At the conclusion of every experiment, rats were overdosed with isoflurane (5%) until ventilation and cardiac activity ceased. Rats were immediately decapitated. The brains were dissected out and placed into chilled 4% paraformaldehyde in 0.1M phosphate-buffered saline (PBS) at 4°C for 48 hours. Next, the brains were cryoprotected by submerging them into 30% sucrose in 0.1M PBS solution over several days until they sank to the bottom of the vials. Brains were then transferred and frozen onto a freezing-microtome (Leica Microsystems, Wetzlar, De) and cut into 40 μm coronal sections. Sections were classified and separated based on whether they contained the probe lesion and were stored in PBS at 4°C. These sections were later mounted on to electrically-charged slides (Fisher Scientific) and stained with Neutral Red (Sigma). The stained slides were visualized under an optical microscope (BX50W1 Olympus Microscope, Carsen Group Inc., Markham, ON). Images were photographed using a high-resolution digital camera (3.3 RTV Micro Publisher, QIMAGING, Burnaby, BC). The location of lesion tracts were plotted on standardized brain maps (Paxinos & Watson, 2004).

2.7 Data Analysis

Using the cursor region function in Spike2 software (CED, Cambridge, UK), the expiratory period of the Hering-Breuer reflex was calculated between the breath preceding the
Hering-Breuer reflex and the first breath when breathing resumed. Three Hering-Breuer reflexes were elicited under each condition (no probe in brain, 0.9% saline perfusion into the NTS, 0.5mM CNQX perfusion into the NTS and 1mM muscimol perfusion into the NTS) were averaged using Excel (Microsoft, Mountain View, CA). Peak integrated inspiratory EMG amplitudes, from the genioglossus and diaphragm, and respiratory frequency were quantified on a breath-by-breath basis in one minute intervals during all LTF experimental protocols. Inspiratory amplitude and respiratory frequency were expressed as a percentage change from baseline ± standard error of the mean. Baseline values for inspiratory amplitude and respiratory frequency were acquired one minute before experimental interventions (e.g. repeated apneas, drug perfusion). Data were quantified and expressed at baseline, 15, 30, 45 and 60 minutes after experimental interventions.

2.8 Statistical Analysis

The specific statistical tests used for each experiment are stated within the results section. Dunnett’s student analysis was used to determine statistical significance post-hoc whenever repeated measures (RM) one-way ANOVAs were performed. Statistical analysis were performed electronically using Prism5 (GraphPad, La Jolla, CA) and applied a critical two-tailed alpha value of p < 0.05. Data are presented as a mean ± standard error of the mean.
Chapter 3 : Results

3.1 Neuropharmacological manipulation of the NTS affects the Hering-Breuer reflex

We needed to address if it was possible to prevent vagal feedback from reaching the NTS. The Hering-Breuer reflex is a vagus-dependent reflex that causes a prolonged expiratory time when activated. Since the vagus nerves terminate exclusively in the NTS, inhibition of the NTS should prevent this reflex from occurring.

3.1.1 The Hering-Breuer reflex significantly prolongs expiratory duration

First we wanted to quantify the average length of the Hering-Breuer reflex. The reflex was triggered by maintaining lung inflation via occlusion of the custom tracheal tube at the peak of inspiration. This method has been shown to be effective in eliciting the Hering-Breuer reflex (Tadjalli et al., 2010). Inspiratory genioglossus activity was abruptly abolished upon activation of the Hering-Breuer reflex producing a mean expiratory duration of 5.10 ± 2.0 seconds, and this prolonged expiration was significantly longer than the average expiratory period 0.89 ± 0.16 seconds (n=7, paired t-test, p = 0.0011, Fig 3-1A). These results demonstrate that maintaining lung inflation by occlusion of our custom tracheal tube triggers the Hering-Breuer reflex in our rats (Fig 3-1B).
Figure 3-1. Characterization of the Hering-Breuer reflex. A: Mean expiratory duration (seconds) during average breath by breath expiration (n=7), and average expiratory duration after evoked Hering-Breuer reflex (n=7). B: Raw recording of end tidal CO$_2$, diaphragm inspiratory activity (EMGDIA), genioglossus inspiratory activity (EMGGG) displaying the basal inspiratory and expiratory durations. C: Raw recording of end tidal CO$_2$, diaphragm inspiratory activity (EMGDIA), genioglossus inspiratory activity (EMGGG) demonstrating the increase in expiratory duration due to activation of the Hering-Breuer Reflex. All values are means ± SEM; * denotes p < 0.05.
3.1.2 The Hering-Breuer Reflex is not affected by CNQX

After showing that we could effectively activate and quantify the Hering-Breuer reflex, we attempted to determine if inhibition of the neurons within the NTS region could prevent the Hering-Breuer reflex because previous literature suggests that it is mediated by the vagal afferents release of glutamate onto pump cells within the NTS (Bonham et al., 1993, Bonham, 1995, Miyazaki et al., 1999). Therefore, we attempted to prevent vagal afferent feedback from reaching the NTS region by antagonizing non-NMDA receptors using 0.5mM CNQX into the region of the NTS. Previous research has demonstrated that 0.5mM CNQX, a non-NMDA receptor antagonist, is an effective dose for the suppression of motor neuron activity in the trigeminal motor nuclei (Burgess et al., 2008). Although not expected, perfusion of 0.5mM CNQX into the NTS region prior to activating the Hering-Breuer reflex did not significantly reduce the expiratory time $5.14 \pm 2.23$ (n=7, paired t-test, $p = 0.453$, data not shown) compared to the expiratory time of the Hering-Breuer reflex activated under saline perfusion. Hence, the drug was deemed ineffective at preventing the NTS region from receiving vagal afferent feedback. Therefore we decided to prevent the Hering-Breuer reflex using an alternative approach.

3.1.3 The Hering-Breuer Reflex is attenuated by Muscimol

Previous literature has identified the presence of GABA<sub>A</sub> receptors on neurons within the NTS region (Bennett et al., 1987, Wang et al., 2010). We hypothesized that perfusion of 1mM muscimol, a GABA<sub>A</sub> receptor agonist, would inhibit neurons in the NTS region and thereby abolish the Hering-Breuer reflex. Muscimol perfusion significantly reduced the duration of the Hering-Breuer reflex from $5.4 \pm 1.7$ seconds under saline perfusion to $2.88 \pm 1.2$ seconds under muscimol (1mM) perfusion (n=7, paired t-test, $p = 0.0004$, Fig 3-2A). However, the Hering-Breuer reflex under muscimol perfusion was still significantly longer than the average expiratory
time (n=7, paired t-test, p = 0.003). Therefore, muscimol perfusion attenuates the reflex but does not abolish it. These results demonstrate that muscimol can affect the NTS neurons from receiving appropriate vagal-afferent feedback. Locations of the microdialysis probe were confirmed to be in the region of the NTS by post mortem histology (Fig 3-3).
Figure 3-2. Unilateral inhibition of the NTS by muscimol decreases the Hering-Breuer reflex. A: Mean expiratory duration (seconds) of the Hering-Breuer reflex during reverse microdialysis of saline and the mean expiratory duration (seconds) of the Hering-Breuer reflex during reverse microdialysis of muscimol into the NTS region (N=7). Inset: Mean expiratory duration of the Hering-Breuer reflex during reverse microdialysis of muscimol and the mean expiratory duration between breaths (N=7) B: Raw recording of end tidal CO$_2$, diaphragm inspiratory activity (EMGDIA), genioglossus inspiratory activity (EMGGG) during the Hering-Breuer reflex under perfusion of saline. C: Raw recording of end tidal CO$_2$, diaphragm inspiratory activity (EMGDIA), genioglossus inspiratory activity (EMGGG) during the Hering-Breuer reflex under perfusion of muscimol. All values are means ± SEM; * denotes p < 0.05.
Figure 3-3. Histology of unilateral microdialysis perfusion of Saline, CNQX, and Muscimol in the NTS during Hering-Breuer reflex. A: An example of unilateral microdialysis probe localized in the region of the NTS. B: Histological plots of probe locations. The nucleus of the solitary tract region is outlined in red. The circle represents the tip of the probe’s 1mm long semipermeable membrane.
3.2 Unilateral saline perfusion in the NTS does not prevent repeated apnea-induced LTF

Prior to determining if neuropharmacological manipulation of the NTS influences respiratory LTF, we wanted to confirm that repeated apneas results in LTF of genioglossus inspiratory activity as previously shown by Tadjalli et al. (2010) and if the insertion of probes into the NTS affects the generation of LTF.

We lowered a microdialysis probe into the NTS and perfused 0.9% saline via reverse-microdialysis throughout the experiment from baseline until 60 min after repeated apneas. Insertion of the probe had no effect on overall baseline activity. Repeated apneas (ten, 15 sec apneas each separated by 1 min) produced significant increases in inspiratory genioglossus activity at 15, 30 and 60 minutes after apnea-induction (n=7, RM ANOVA, Dunnett’s post hoc, p < 0.05 for 15, 30 and 60 minutes after baseline, Fig. 3-4). Fifteen minutes after apneas, inspiratory diaphragmatic activity was not significantly different from baseline; however, there was a significant reduction in inspiratory diaphragmatic activity at 30, 45 and 60 minutes after repeated apneas (n=7, RM ANOVA, Dunnett’s post hoc, p < 0.05 for 30, 45 and 60 minutes after baseline) (Fig. 3-4) compared to baseline. Respiratory frequency was also significantly decreased at all time points after apneas (n=7, RM ANOVA, Dunnett’s post hoc, p < 0.05 for all time points measured, Fig. 3-4). These data shows that insertion of a microdialysis probe and perfusion of saline into the NTS do not affect the ability of repeated apneas to trigger LTF of the genioglossus inspiratory activity. Probe tracts were confirmed in the region of the NTS by post mortem histology (Fig 3-5).
Figure 3-4. Unilateral saline perfusion in the NTS does not prevent repeated apnea-induced LTF.  

A: Genioglossus and diaphragm inspiratory activity in one rat with saline perfused in the NTS throughout the experiment.  

B: Genioglossus inspiratory activity (∫EMG<sub>GG</sub>) with saline perfused in the NTS throughout the 60 minute recording period (n=7). Note that the inspiratory genioglossus tone increased above baseline levels (dotted line) at 15, 30 and 60 minutes demonstrating the expression of LTF  

C: Integrated diaphragm motor activity ∫EMG<sub>DIA</sub> and respiratory frequency over 60 minute recording periods (n=7). Note that there were no long term increases in diaphragm or respiratory frequency. All values are means ± SEM; * denotes p < 0.05 compared to baseline.
Figure 3-5. Histology of unilateral microdialysis perfusion of Saline into the NTS region. A: An example of unilateral microdialysis probe localized in the region of the NTS. B: Histological plots of probe locations. The nucleus of the solitary tract region is outlined in red. The circle represents the tip of the probe’s 1mm long semipermeable membrane.
3.3 Unilateral inhibition of NTS neurons by GABA_A receptor agonist does not prevent apnea-induced LTF

The next set of experiments was designed to determine if unilateral inhibition of the NTS prevents apnea-induced LTF of the genioglossus muscle. We unilaterally perfused 1mM muscimol into the right NTS (before and during) repeated apneas. We found increases in genioglossus inspiratory activity at 15, 30, 45 and 60 minutes when compared to baseline (n=5, RM ANOVA, Dunnett’s post hoc, p < 0.05 for all time points measured, Fig. 3-6). Diaphragm motor activity did not differ from baseline at any time point compared to baseline (n=5, RM ANOVA, Dunnett’s post hoc, p > 0.05 at 15, 30, 45 and 60 minutes) (Fig. 3-6). Similar to the control experiments with reverse-microdialysis of 0.9% saline into the NTS region, respiratory frequency was significantly reduced at 15, 30, 45 and 60 minutes compared to baseline (n=5, RM ANOVA, Dunnett’s post hoc, p < 0.05 at all time points measured) (Fig. 3-6). Probe insertion did not affect basal respiratory activity. All probe tracts from this set of experiments were found in the region of the NTS by post mortem histology (Fig 3-7).
Figure 3-6. Unilateral inhibition of NTS neurons by GABA<sub>A</sub> receptor agonist does not prevent repeated apnea-induced LTF A: Genioglossus and diaphragm inspiratory activity in one rat with muscimol perfusion into the NTS before and during repeated apneas. B: Genioglossus inspiratory activity (∫EMG<sub>GG</sub>) with saline perfused in the NTS throughout the 60 minute recording period (n=5). Note that the inspiratory genioglossus tone increased above baseline levels (dotted line) at 15, 30, 45 and 60 minutes demonstrating the expression of LTF C: Integrated diaphragm motor activity ∫EMG<sub>DIA</sub> and respiratory frequency over 60 minute recording periods (n=5). Note that there were no long term increases in diaphragm or respiratory frequency. All values are means ± SEM; * denotes p < 0.05 compared to baseline.
Figure 3-7. Histology of unilateral microdialysis perfusion of muscimol into the NTS region. Histological plots of probe locations. The nucleus of the solitary tract region is outlined in red. The circle represents the tip of the probe’s 1mm long semipermeable membrane.
3.4 Unilateral GABA<sub>A</sub> receptor agonist perfusion does not produce greater LTF

We next wanted to determine if the LTF expressed was greater in the unilateral muscimol perfusion group than in the saline control group. When we compared the time points after repeated apneas in the saline perfusion group to the muscimol treatment group, we found no significant difference in the increased integrated genioglossus motor activity at 15, 30 and 60 minutes but there was a significant increase at 45 minutes (two-way RM ANOVA, Bonferroni post hoc, p < 0.05 at 45 minutes) (Fig 3-8).
Figure 3-8. Unilateral perfusion of GABA-A receptor agonist enhances LTF at 45 minutes. A comparison of integrated genioglossus motor activity (∫EMG_{GG}) over 60 minute recording periods between the muscimol and saline perfusion groups (n=13). All values are means ± SEM; * denotes p < 0.05 compared to baseline. † denotes p < 0.05 between saline and muscimol groups at 45 minutes.
3.5 Unilateral perfusion of GABAA receptor agonist does not trigger LTF

Since perfusion of muscimol (1mM) prior to and during apneas resulted in a greater LTF of inspiratory genioglossus activity than apneas under saline perfusion, we speculated that muscimol may be potentiating LTF. Therefore, in the absence of apneas we perfused 1mM muscimol, a GABA<sub>A</sub>, into the NTS and assessed its affect on genioglossus and diaphragmatic motor activity. There was no effect on basal respiratory activity following probe insertion. Muscimol (1mM) perfusion without repeated apneas did not cause an increase in genioglossus motor activity compared to baseline at 15, 30, 45 or 60 minutes (n=7, RM ANOVA, Dunnett’s post hoc, p > 0.05 at all time points measured) (Fig. 3-9). Diaphragm activity remained constant and similar to baseline values at 15, 30, 45 and 60 minutes (n=7, RM ANOVA, Dunnett’s post hoc, p > 0.05 at all time points measured, Fig. 3-9). Respiratory frequency was significantly decreased compared to baseline at 15, 30, 45 and 60 minutes after muscimol perfusion (n=7, RM ANOVA, Dunnett’s post hoc, p < 0.05 at all time points measured) (Fig. 3-9). Post mortem histology confirmed probe tracts in the region of the NTS by post mortem histology (Fig 3-10).
Figure 3-9. Unilateral perfusion of GABA<sub>A</sub> receptor agonist does not trigger LTF. A: Integrated GG motor activity over 60 minute recording periods (n=7). Note that muscimol perfusion without apneas does not induce LTF of the GG muscle B: Integrated diaphragmatic motor activity over 60 minute recording periods (n=7). C: Respiratory frequency over 60 minute recording periods (n=7). All values are means ± SEM; * denotes p < 0.05 compared to baseline.
Figure 3-10. Histology of unilateral microdialysis of muscimol into the NTS region. Histological plots of probe locations. The nucleus of the solitary tract region is outlined in red. The circle represents the tip of the probe’s 1mm long semipermeable membrane.
3.6 Bilateral inhibition of NTS neurons by GABA-A receptor agonist is lethal

Our hypothesis was that unilateral reverse microdialysis of 1mM muscimol would prevent or diminish apnea-induced LTF; however, this was not the case. The NTS is a bilateral structure that receives vagal afferents into both nuclei, hence, our hypothesis evolved to investigate if bilateral inhibition of the NTS would prevent apnea-induced LTF (Kalia and Sullivan, 1982). In order to test this hypothesis, we examined the effects of bilateral reverse microdialysis perfusion with 1mM muscimol. Bilateral perfusion of muscimol (1mM) (without apneas) in, adjacent to, or ventral to the NTS was found to be lethal (n = 7). All probes were located within or ventral to the region of the NTS using post mortem histology (Fig. 3-11).
Figure 3-11. Histology of bilateral microdialysis perfusion of muscimol. A: Histological plots of probe locations. The NTS region is outlined in red. Probes are matched by their colour. The circle represents the tip of the probe’s 1mm long semipermeable membrane.
3.7 Bilateral inhibition of NTS neurons by a voltage gated Na\(^+\) channels blocker prevent repeated apnea-induced LTF

Since direct inhibition of the NTS neurons with the GABA\(_A\) agonist, muscimol, led to complications with the animal, we attempted to use a more general neural approach to decrease vagal afferent input to neurons of the NTS. Therefore, we perfused a sodium channel blocker, lidocaine, to prevent the neurons from responding to the vagal afferent excitatory inputs (Salazar et al., 1995). Also, to ensure that a specific concentration and limited amount of lidocaine reached the NTS region we opted to use microinjection versus microdialysis. Probe insertion and microinjection of 5\% lidocaine into the NTS caused a brief and transient decrease in genioglossus activity (lasting an average of 380 ± 170 sec) that returned back to baseline. We found that bilateral microinjection of 5\% lidocaine, a voltage gated Na\(^+\) channel blocker, into the NTS prior to repeated apneas abolished LTF of the genioglossus muscle (Fig. 3-12). Genioglossus inspiratory activity was not significantly different compared to baseline at 15, 30, 45 and 60 minutes after repeated apneas (n=5, RM ANOVA, Dunnett’s post hoc, p > 0.05 at all time points measured, Fig 3-12A). Repeated apneas performed after microinjection of 5\% lidocaine did not have any effect on either respiratory frequency (n=5, RM ANOVA, Dunnett’s post hoc, p > 0.05 at all time points measured) (Fig. 3-12C) or diaphragm inspiratory activity (n=5, RM ANOVA, Dunnett’s post hoc, p > 0.05 at all time points measured) compared to baseline (Fig 3-12B). Thus, bilateral inhibition of NTS neurons prevents LTF of the genioglossus inspiratory activity. The probe tracts for these experiments were confirmed to be within the region of the NTS by post-mortem histology (Fig 3-13).
Figure 3-12. Bilateral inhibition of NTS neurons by 5% Lidocaine prevent repeated apnea-induced LTF. A: Integrated GG motor activity over 60 minute recording periods (n=5). There was no significant increases from baseline values at any time point measured within the 60 minute recording period. B: Integrated diaphragmatic motor activity over 60 minute recording periods (n=5). C: Respiratory frequency over 60 minute recording periods (n=5). All values are means ± SEM.
Figure 3-13. Histology showing bilateral microinjection of lidocaine. A: Histological plots of probe locations. The NTS region is outlined in red. The circle represents the tip of the probe.
3.8 Verification of probe location

Post-mortem histological analysis provided visual evidence that probe insertion tracts were within or adjacent to the NTS. We were not able to confirm the microdialysis tract for three of the thirty-five animals in these experiments.
Chapter 4 : Discussion

Previous literature suggests that apnea-induced respiratory plasticity is mediated by vagal afferent feedback. Therefore, we investigated the effect of inactivating the NTS; a brain region which is the first termination site of vagal afferents and exclusively receives all vagal afferent communication. First, we demonstrate that inhibition of the NTS modulates the vagus-dependent Hering-Breuer reflex. Second, we show that apnea-induced LTF is unaffected by unilateral inactivation of the NTS. Third, we show LTF is blocked by bilateral inactivation of the NTS. We conclude that the NTS plays a role in apnea-induced LTF.

4.1 Technical Considerations

The majority of the studies conducted in this thesis used reverse-microdialysis to perfuse the nucleus of the solitary tract. The actual concentration of drug delivered to the NTS was not measured. It is likely that the concentrations of drug transferred to the NTS were lower than the concentration inside the microdialysis probe. Alessandri et al. (1996) demonstrated only 11-25% of the dose of glutamate in the microdialysis probe was delivered. Therefore, we may have not antagonized all of the GABA_A receptors on NTS neurons.

Another potential concern of the microdialysis method is that the spread of drug was not measurable. It is possible that drug spread from the caudal axis to the rostral axis and affected the rostral portion of the NTS as well. The rostral portion of the nucleus receives gustatory afferents, although it is unlikely that this spread affected LTF expression, the possibility of drug effect on collateral connections between the caudal and rostral regions could have had an effect on LTF expression.
It is possible that muscimol perfusion of the NTS reached the hypoglossal nucleus due to its close proximity to the NTS. It has been shown that microdialysis of muscimol into the hypoglossal will decrease genioglossus amplitude (Liu et al., 2003). Although we still observe LTF after unilateral perfusion of muscimol, it is possible that muscimol drug leak to the hypoglossal limited the magnitude of the expression. It is possible that the apnea-induced LTF had such a strong drive to the hypoglossal motor neurons that it compensated for the drugs effect on the motor neuron population even if muscimol had reached the hypoglossal motor pool.

The NTS is a highly heterogeneous area (Barraco et al., 1992). The delivery of drug to inhibit this region was not specific to any type of neuron. Neurons with functions other than pulmonary activity and interneurons may have been affected within the NTS. This nucleus largely facilitates the integration of many viscera and its ability to sort and process this input may have been affected. This could have caused modulations in the overall neural networks responsible for many physiological processes (Bonham and McCrimmon, 1990, Barraco et al., 1992, Koga and Fukuda, 1992). This in turn could have affected expression of genioglossus muscle activity. The experiments of this thesis targeted the caudal most region of the NTS, which is a mixed composition of cardiorespiratory output (Barraco et al., 1992). It is most likely that neurons receiving baroreceptor input were also affected.

Furthermore, blood pressure was not monitored. The nucleus of the solitary tract is not only involved in the pulmonary system, but also the cardiovascular system (Barraco et al., 1992). Therefore, we cannot be sure if unilateral muscimol or bilateral lidocaine injections into the NTS caused changes in blood pressure that could have affected genioglossus inspiratory activity or disrupted LTF. The literature involving the potential effect on blood pressure after perfusing GABA derivatives into the NTS is inconsistent (Andresen and Kunze, 1994) It was reported that
muscimol injections into the NTS had no effect on blood pressure (DiMicco et al., 1979) whereas by (Bousquet et al., 1982) found increases in blood pressure following muscimol injections. Therefore, we cannot be absolutely sure what effect muscimol perfusion had on blood pressure.

Blood pressure is often measured in LTF studies; however, there is a relatively small amount of literature detailing the effects of blood pressure on LTF. Hypotension and hypertension may elicit different effects on respiratory plasticity. Neverova et al., (2007) investigated the role of noradrenergic receptors in hypoglossal nerve LTF by preventing endogenous noradrenaline from binding through the use of prazosin, a α1-adrenergic receptor antagonist. Unfortunately, the action of prazosin on peripheral vasculature promotes hypotension (Neverova et al., 2007). To control for this confounding factor, Neverova et al., 2007 investigated if hypotension alone would affect the manifestation of in vivo hypoxia-induced plasticity. Blood was removed from the animal to induce hypotension and prazosin was not administered. They found that episodic hypoxia under hypotension without prazosin administration still induced LTF whereas episodic hypoxia under prazosin and hypotension did not induce LTF. When hypotension was not induced by blood removal and prazosin was administered without the episodic hypoxia LTF was not produced. This led the authors to conclude that hypotension had no effect on respiratory plasticity.

We found that apnea-induced respiratory LTF was still present despite unilateral inactivation of the NTS via perfusion of muscimol, a GABA<sub>A</sub> agonist. These results were unexpected because we had found that unilateral muscimol perfusion into the NTS region attenuated the duration of the Hering-Breuer reflex. The NTS is a bilateral region and the vagus nerve terminates in both bilateral nuclei (Kalia and Sullivan, 1982). It is possible that the
contralateral nucleus, which was not receiving muscimol perfusion, was able to compensate for the inactivated region. Apneusis can only be produced when the ventro-lateral region of the NTS is inhibited bilaterally and studies following this work have often opted for bilateral microinjection of GABA derivatives or inhibitors based on this knowledge (Wasserman et al., 2000, Wasserman et al., 2002). Therefore, the work by Wasserman et al. 2000 and Wasserman et al. 2002 has led to the hypothesis that effects produced by bilateral microinjection into the NTS do not occur with unilateral microinjection of the same drug.

From our results, we hypothesized that the side contralateral to muscimol perfusion was compensating for the unilateral inactivation. We decided to bilaterally inhibit the NTS. However, we chose to use lidocaine in case GABA_A receptor activation was inducing changes in the activity pattern of the NTS which may lead to overall changes in the network mediating apnea-induced plasticity. Lidocaine’s mechanism of inactivation differs from muscimol.

Muscimol binds to a GABA_A receptor where it induces calcium efflux which leads to hyperpolarization of the membrane and in doing so, the neuron activity is reduced and more difficult to excite via endogenous release of excitatory neurotransmitters (Wasserman et al., 2002). Lidocaine works by inhibiting neuronal activity through inhibition of sodium channels (Khodorova et al., 2001). One ion that action potentials require is sodium and therefore, lidocaine blocks the ability of the neuron to generate an action potential and prevents the flow of communication between neurons (Khodorova et al., 2001).

Bilateral perfusion of lidocaine prevented apnea-induced respiratory plasticity. Neurons of the NTS were unable to respond to the communication about mechanical feedback from the vagal afferents. Vagal afferent feedback has previously been reported to be required for apnea-induced LTF (Tadjalli et al., 2010). The purpose of our experiments was to determine if
inactivation of this region abolished or reduced the expression of LTF. Therefore, despite some technical considerations, our findings support our hypothesis that this region which receives vagal afferent feedback exclusively, is important in triggering vagal-mediated apnea-induced plasticity.

4.2 Peripheral feedback is required in apnea-induced LTF but not hypoxia-induced LTF

It was hypothesized that repeated episodes of hypoxia causes repeated activation of chemoafferent neurons (Bach and Mitchell, 1996). This repeated action of chemoafferent neurons was then thought to elicit LTF through central mechanisms (Bach and Mitchell, 1996). To test the involvement of the carotid bodies in hypoxia induced LTF through carotid denervation, rats had their carotid sinus nerves sectioned bilaterally previous to hypoxic intervention (Bavis and Mitchell, 2003). These animals still expressed LTF, but it was attenuated in comparison to animals that had intact carotid sinus nerves (Bavis and Mitchell, 2003). This suggested that hypoxia triggers hypoxia-induced LTF through a central mechanism and does not require peripheral feedback from the carotid bodies (Bavis and Mitchell, 2003). However, our lab has previously shown that peripheral feedback via the vagus nerve is required for apnea-induced LTF (Tadjalli et al., 2010). Also, here we show that inactivating NTS from receiving this repeated peripheral feedback input abolishes LTF expression. Therefore, the two induction methods of LTF differ. Hypoxia-induced LTF does not require peripheral feedback, whereas, apnea-induced LTF requires it. This does not suggest that apnea-induced LTF is not also mediated by a central mechanism, but rather that there is a peripheral component required to trigger LTF.
4.3 Neural pathways of apnea induced plasticity

Apnea-induced LTF has been shown to be vagal-mediated (Tadjalli et al, 2010). Our results support this claim since the NTS receives vagal afferents exclusively, and we show that inactivation of this nucleus abolishes vagal mediated apnea induced LTF.

Tadjalli et al. (2010) demonstrated that noradrenergic input but not serotonin to the hypoglossal motor nucleus was required for apnea-induced LTF. Not only do all of the vagal afferents terminate onto the NTS, but this nucleus has efferent projections, both direct and indirect, to multiple noradrenergic regions. The more direct monosynaptic efferents of the NTS terminate on the Peri-Locus Coeruleus and the A5 (Cedarbaum and Aghajanian, 1978, Byrum and Guyenet, 1987, Van Bockstaele et al., 1999). Moreover, the NTS does have indirect connections to brainstem noradrenergic regions: locus coeruleus, A5, and A7 (Ennis and Aston-Jones, 1989, Van Bockstaele et al., 1989, Iwasaki et al., 1999). Therefore, we hypothesize that the NTS is acting through the paragigantocellularis, which could then proceed to excite the LC (Ennis and Aston-Jones, 1988). This increase in excitation of the LC could potentially lead to increases in noradrenergic transmission onto hypoglossal motor neurons which would increase motor activity of the genioglossus muscle. This increase in genioglossus activity would increase upper airway patency (Fig. 4-1).

Direct projections from the NTS to the hypoglossal have been found (Borke et al., 1983, Amri and Car, 1988). The specifics on whether these connections are excitatory or inhibitory is unknown, however, it is possible that the NTS mediates stimulatory input directly onto the hypoglossal motor nucleus and increasing genioglossus inspiratory effort.
Figure 4-1. Neural substrates involved in apnea-induced respiratory plasticity. Schematic representing the hypothesized neural substrates mediating apnea induced plasticity. Mechanical feedback from the lungs are delivered to the nucleus of the solitary tract (NTS) via the vagus nerve. Widespread projections (both monosynaptic and disynaptic) from the NTS to other noradrenergic regions such as the A5, A6 (locus coeruleus), A7 occur. There is also evidence to suggest a synaptic connection between the NTS and hypoglossal motor nucleus. We speculate that apnea-induced LTF results in an increase of noradrenergic release onto the hypoglossal motor neurons which increases the respiratory effort of the genioglossus muscle.
4.4 Clinical Relevance

Obstructive sleep apnea is a debilitating sleep disorder that causes numerous health complications (Horner, 1996). Intermittent airway occlusions are a feature of OSA, and it is possible that the body is compensating physiologically through LTF to promote stability of breathing when the system is challenged by respiratory challenges (Mahamed and Mitchell, 2007). Stability is promoted by LTF by increasing the upper airway patency which then leads to the prevention of decreased airflow associated with sleep apnea (Mahamed and Mitchell, 2007). Preventing the reductions in airflow resistance will reduce the incidence of apneas (Remmers et al., 1978).

Currently, there are no effective pharmacological FDA approved drugs to treat obstructive sleep apnea (Smith and Quinnell, 2004). Surgery to correct or modify the structure of the upper airways does not boast an effective success rate (Pang, 2005). Moreover, the time associated with the process of surgery, recovery and the low success rate of surgery may lead patients to be apprehensive about the surgical process (Pang, 2005, Won et al., 2008). The most effective and common form of treatment is from a device known as the CPAP (continuous positive airway pressure) (Loube, 1999). Unfortunately, due to the low compliance with this device individuals often fail to see the needed results (Sullivan et al., 1981, Loube, 1999). Therefore, pharmacological drugs would have potential advantages in both the ease of administration and compliance amongst patients.

Understanding the mechanisms that underlie the phenomenon of LTF will contribute to the development of novel pharmaceuticals. However, before pharmaceuticals can be developed the neural substrates mediating LTF must be known. Understanding the key regions of the brain involved in the manifestation of increasing upper airway stability is critical. Here we show that
the NTS tract is involved in apnea-induced respiratory plasticity. We plan to continue solving the pathways and neural substrates of this phenomenon. Once we understand the critical processes mediating apnea induced plasticity we can start to develop drugs which stimulate or enhance the pathways during sleep as to prevent decreases in both upper airway patency and respiratory motor output.

4.5 Future Directions

The purpose of this thesis was to investigate the role of the nucleus of the solitary tract on vagal mediated apnea-induced respiratory plasticity. We found that bilateral but not unilateral, inactivation of the nucleus prevented apnea-induced respiratory plasticity. There are many open avenues of study that could proceed from this direction.

This thesis investigated if the NTS had any role in mediating apnea-induced plasticity. We did not attempt to identify if the region had a role in the induction or maintenance of apnea-induced plasticity. Future studies could be constructed to evaluate if it has a specific role, or if it is important in both the induction and maintenance.

Although it is possible to dissect the intracellular intricacies of the NTS that are responsible for apnea induced plasticity, a more logical step would be to investigate noradrenergic brain regions receiving NTS efferents such as the A5, A6 (locus coeruleus), A7, and the subcoeruleus (Cedarbaum and Aghajanian, 1978, Byrum and Guyenet, 1987, Ennis and Aston-Jones, 1989, Van Bockstaele et al., 1999). The NTS makes monosynaptic connections with some of these regions (eg. A5 and A7) but also synapse onto the noradrenergic regions indirectly. This would include the nucleus prepositus hypoglossi and the paragigantocellularis nucleus (Ennis and Aston-Jones, 1989, Van Bockstaele et al., 1989). We know that the regions that the NTS projects to (both directly and indirectly) synapse onto the hypoglossal motor
neurons, and therefore, it is of interest to identify the function of these regions in apnea-induced LTF so we can determine the circuits that both underlie and trigger apnea-induced LTF. The ultimate goal of dissecting the neuronal mechanism that mediates this form of respiratory plasticity is to improve breathing and lung ventilation through pharmacological approaches.

Here we used reverse microdialysis, electrophysiology, neuropharmacology and histology to determine if the NTS is the origin of the neural circuit responsible for apnea induced plasticity. These same methods could be used to inactivate other noradrenergic regions and assess their roles in apnea-induced plasticity. A more interesting set of approaches would include novel techniques like optogenetics and pharmacogenetic manipulation (e.g. DREADD technology) to tease apart the neural networks. These advanced techniques allow for the gain or loss of specific cell types within a neuronal population (e.g. noradrenergic neurons of the A5). These techniques could help further identify regions of the neural network and their biochemical mechanisms mediating apnea-induced respiratory plasticity.

The anesthesia model used in our experimental paradigm allows for the control of motor expression, which could otherwise be altered during different conscious states or specific behaviours. The anesthetized preparation is a reduced approach that allowed analysis of the apnea-induced plasticity and the role of the NTS in mediating it. However, there are still caveats to this approach. Anesthetics alter the modulation of neural transmission that can affect the basal motor neuron excitability. Anesthesia can cause enhanced GABAergic transmission, reduced glutamatergic activity and fluctuations in the magnitude of conductance in the potassium channel of motor neurons (Franks and Lieb, 1994, Winegar and Yost, 1998, Peters et al., 2008). Neuronal slices containing the NTS were used to investigate the effects of isoflurane on second order NTS neurons. Isoflurane was found to enhance the inhibitory transmission through GABA<sub>A</sub> receptors.
and decreases in excitatory transmission (via glutamate) by reducing the ability of the neurons to propagate action potentials (Peters et al., 2008). Nevertheless, we were still able to evoke respiratory plasticity through repeated apneas as well as localize the origin of the neural network mediating this process.

Optogenetics is a powerful tool that allows for the modulation of specific neurons via specific wavelengths of light after the transfection of a light sensitive receptor. It allows for the gain-or-loss of function of specifically targeted neuron phenotypes using recombinase driver mouse lines (Deisseroth, 2011). After identification of regions of interest in mediating apnea-induced plasticity, we could use this technology to activate these regions during different conscious states, thereby moving our anesthetized experimental paradigm into freely behaving animals. This would provide a more relevant physiological condition and it will allow us to evaluate if LTF can be observed and beneficial during sleep.

One other final future direction could be the quantification of noradrenergic release at the hypoglossal motor nuclei. A microdialysis probe or voltammetry properly targeted to the hypoglossal motor nuclei would allow the sampling and quantification of noradrenaline in a time dependent manner. It would be interesting to know what happens to noradrenaline release onto the hypoglossal motor neurons before, during, and after apneas. Also, it would be interesting to then compare these levels to the levels of noradrenaline found during behavioral states such as wakefulness and sleep. If there is a difference between wakefulness and sleep investigations should be undertaken to examine if LTF during sleep will increase noradrenergic concentrations back to wakefulness levels.

Our laboratory’s ultimate goal is to understand vagal-mediated apnea-induced plasticity and apply what we learn from this phenomenon into practical use. We believe that future
experiments will further our knowledge of respiratory plasticity. Furthering our knowledge of this phenomenon could allow for the development of pharmacological approaches which contribute to both evoking and enhancing respiratory LTF as well as provide stable healthy breathing in individuals who struggle with obstructive sleep apnea.
References


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