HYPOCRETIN NEURONS ENDOGENOUSLY REGULATE SOMATIC MOTONEURON EXCITABILITY

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Cell & Systems Biology
University of Toronto

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ABSTRACT

The role of hypocretin neurons in modulating somatic motoneuron excitability and hence muscle tone is poorly understood. We investigated whether hypocretin neurons influence the hypoglossal and trigeminal motor pools that innervate the genioglossus and masseter muscles respectively, both of which function to maintain upper airway patency. We hypothesized hypocretin neurons facilitate motor outflow at the motor pools. We pharmacologically manipulated hypocretin neuron activity in anaesthetized mice to determine their role in somatic motoneuron excitability. We also antagonized hypocretin receptors in the hypoglossal motor pool to determine the pathway through which hypocretin neurons influence motoneuron excitability. We demonstrated that hypocretin neurons potently excite somatic motoneurons and hence facilitate genioglossus and masseter muscle tone. Furthermore, we demonstrated that an endogenous hypocretinergic drive on somatic motoneurons facilitated muscle tone under anaesthesia. These studies demonstrate that hypocretin is an excitatory neuromodulator of muscle tone and contributes to the excitatory regulation of somatic motoneurons.
Acknowledgements

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<tr>
<td>µL</td>
<td>Microliter</td>
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<tr>
<td>µM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>aa</td>
<td>Amino acids</td>
</tr>
<tr>
<td>ABC</td>
<td>Avidin biotinylated enzyme complex</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-Amino-3-hydroxy-5-methylisoxazole-4-propionic acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AP</td>
<td>Action potential</td>
</tr>
<tr>
<td>AU</td>
<td>Arbitrary units</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>DAB</td>
<td>3,3'-Diaminobenzidine</td>
</tr>
<tr>
<td>EMG</td>
<td>Electromyogram</td>
</tr>
<tr>
<td>EPSP</td>
<td>Excitatory postsynaptic potential</td>
</tr>
<tr>
<td>FW</td>
<td>Formula Weight</td>
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<tr>
<td>g</td>
<td>Grams</td>
</tr>
<tr>
<td>GG</td>
<td>Genioglossus</td>
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<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
</tr>
<tr>
<td>Hcrt</td>
<td>Hypocretin</td>
</tr>
<tr>
<td>Hcrtr</td>
<td>Hypocretin receptor</td>
</tr>
<tr>
<td>hr</td>
<td>Hour</td>
</tr>
<tr>
<td>Hz</td>
<td>Hertz</td>
</tr>
<tr>
<td>IPSP</td>
<td>Inhibitory postsynaptic potential</td>
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<tr>
<td>kHz</td>
<td>Kilohertz</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>KO</td>
<td>Knock out</td>
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<tr>
<td>M</td>
<td>Molar</td>
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<tr>
<td>MCH</td>
<td>Melanin concentrating hormone</td>
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<tr>
<td>min</td>
<td>Minute</td>
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<tr>
<td>mL</td>
<td>Milliliter</td>
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<td>mm</td>
<td>Millimeter</td>
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<tr>
<td>mM</td>
<td>Millimolar</td>
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<tr>
<td>ms</td>
<td>Millisecond</td>
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<tr>
<td>Na</td>
<td>Sodium</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartic acid</td>
</tr>
<tr>
<td>NREM</td>
<td>Non-rapid eye movement</td>
</tr>
<tr>
<td>O</td>
<td>Oxygen</td>
</tr>
<tr>
<td>OSA</td>
<td>Obstructive sleep apnea</td>
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<tr>
<td>P</td>
<td>Probability</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>REM</td>
<td>Rapid eye movement</td>
</tr>
<tr>
<td>RLS</td>
<td>Restless leg syndrome</td>
</tr>
<tr>
<td>s</td>
<td>Second</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
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<tr>
<td>vGluT</td>
<td>Vesicular glutamate transporter</td>
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<td>WT</td>
<td>Wild type</td>
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CHAPTER 1:

INTRODUCTION
Chapter 1: INTRODUCTION

1.1. Overview

One of the main characteristics of the sleep-wake cycle is the stereotypical pattern of muscle tone across different behavioural states. This pattern of muscle tone was first characterized in 1962 by Michel Jouvet who observed that muscle tone is maximal during alert waking, reduced in quiet waking, further reduced during non-rapid eye movement (NREM) sleep, and potently suppressed in rapid eye movement (REM) sleep (Jouvet 1967). The complex interactions between the central nervous system and the muscular system that are responsible for coupling muscle tone to behavioural state rely fundamentally on the actions of neurotransmitters (Rekling, Funk et al. 2000). Several studies have been undertaken to elucidate the neurochemical mechanisms controlling muscle tone across the sleep-wake cycle; from these works, numerous neurotransmitter systems have been implicated in the control of movement and natural behaviour. Determining the mechanisms responsible for the pattern of muscle tone across the sleep-wake cycle is not only important in understanding the basic neural control of muscles, but also a dysregulation of muscle activity is implicated in numerous sleep disorders associated with abnormal motor control and behavioural state, making an understanding of these neurotransmitter systems imperative for the development of effective therapies.

One of these notable sleep disorders is obstructive sleep apnea (OSA); the most prevalent sleep disorder in North America, muscles of the upper airway become overly relaxed during sleep, which narrows the airway leading to airway collapse and cessation of breathing (Horner 1996; Jordan and White 2008). REM sleep behaviour disorder, another disorder of motor dysregulation, is characterized by the absence of REM sleep
muscle atonia, resulting in overt, often injurious movements usually associated with dream enactment (Boeve, Silber et al. 2007). Narcolepsy is a neurological disorder characterized by excessive daytime sleepiness, fragmented sleep patterns, and cataplexy—the sudden loss of muscle tone during periods of wakefulness (Mitler, Boysen et al. 1974). Additional examples of sleep disorders with motor dysfunctions include restless leg syndrome, periodic limb movements disorder, and bruxism (Montplaisir 2004). To date, pharmacological therapies to treat these sleep disorders of motor dysregulation are lacking; a better understanding of the neurochemical mechanisms underlying normal motor control is needed for the development of effective treatments for these debilitating disorders.

Our laboratory investigates the neural mechanisms that control muscle tone during sleep and wakefulness. We have examined the role of a number of neurotransmitter systems in the control of muscle tone across the different behavioural states (Brooks and Peever 2008; Burgess, Lai et al. 2008; Schwarz, Yee et al. 2008). Of interest to the thesis is the previously published study examining the endogenous role of glutamate in the control of the stereotypical pattern of muscle tone across the sleep-wake cycle (Burgess, Lai et al. 2008). Glutamate is the most abundant excitatory neurotransmitter in the mammalian central nervous system (CNS) (Nakanishi 1992). Glutamate is also the primary transmitter responsible for controlling motoneuron excitability and for mediating motor behaviours (Rekling, Funk et al. 2000). Antagonism of glutamatergic activity during wakefulness in both the trigeminal and hypoglossal motor pools dramatically reduces masseter and genioglossus (GG) muscle tone, respectively (Burgess, Lai et al. 2008; Steenland, Liu et al. 2008). This reduction in
activity, however, does not reach the suppression of motor tone seen in REM sleep; therefore, additional excitatory neuromodulators must also directly contribute to the control of muscle tone. Accordingly, this study aimed to determine the role of hypocretin (Hcrt) in modulating muscle tone.

A dysregulation of Hcrt neurotransmission is implicated in a number of sleep-related and motor related disorders that involve abnormal muscle activity. Hcrt neurons project to motoneurons, which express Hcrt receptors (Hcrtr) (Peyron, Tighe et al. 1998; Cutler, Morris et al. 1999; Fung, Yamuy et al. 2001; Hervieu, Cluderay et al. 2001; Hwang, Chen et al. 2001). However, the direct effect of Hcrt neurons on motoneuron excitability and ensuing muscle tone has not been determined. Thus, we aimed to elucidate whether changes in Hcrt activity would affect motoneuron activity and thus, muscle tone. For this purpose, we employed an anaesthetised mouse model that allowed for the pharmacological manipulation of Hcrt activity. We used the hypoglossal and trigeminal motor systems because it enabled us to measure the direct effect of manipulations of Hcrt activity on cranial motoneurons and muscle tone.

The following introductory chapter will provide a basic background of information relevant to this study. It includes a brief overview of the Hcrt system and existing evidence that supports the role of Hcrt in modulating motoneuron excitability. Furthermore, information about the motor systems pertaining to this study will be provided.
1.2. The hypocretin system

1.2.1. Hypocretin

Our knowledge of the Hcrt system was initiated in 1998 when it was discovered by two independent groups using two different methods. In January of 1998, the group of Sutcliffe et al. published a study describing a rat hypothalamic mRNA species coding for a precursor peptide 130 amino acids (aa) in length, which generated two separate peptides 33aa and 28aa in length. They named these peptides the Hcrt1s on the basis of their location and production in the hypothalamus, and the sequence similarities to the secretin family of peptides. The precursor peptide is the prepro-hypocretin and its two final peptide products are Hcrt-1 and Hcrt-2. Simultaneously, a group led by Sakurai isolated two hypothalamic peptides that activated G-coupled receptors. On the basis of peptide sequences, Sakurai et al. cloned cDNA for a precursor peptide (preprorexin: 130aa). The peptides were named orexins because they increased food intake in non-fasted rats when injected into the lateral ventricle. It soon became apparent that both groups were describing an identical group of neuropeptides and therefore, the 33aa long peptide is known as both Hcrt-1 and orexin-A, while the 28aa long peptide is referred to as both Hcrt-2 and orexin-B (de Lecea, Kilduff et al. 1998; Sakurai, Amemiya et al. 1998). While only a few thousand Hcrt cells are found solely within the perifornical region of the lateral hypothalamus, they project to virtually the entire CNS. (Peyron, Tighe et al. 1998; Nambu, Sakurai et al. 1999) (Figure 1-1). Cleavage of one prepro-hypocretin peptide and further modification leads to the production of one molecule each of Hcrt-1 and Hcrt-2.
Figure 1-1: Schematic drawing of sagittal section through the rodent brain summarizing the organization of the hypocretin neuronal system. Hypocretin neurons are found only in the lateral hypothalamic area and project to the entire central nervous system. Abbreviations: 3V, third ventricle; TMN, tuberomammillary nucleus; LC, locus coeruleus; MoV, trigeminal nucleus; 12N, hypoglossal nucleus. Figure from Sakurai 2005. Reprinted with permission from Elsevier.
Hcrt is an excitatory neuropeptide whose actions are mediated through two different receptors. These receptors, called Hcrt receptor-1 (Hcrtr-1) and Hcrt receptor-2 (Hcrtr-2), are a class of G-protein coupled receptors. The receptors differ in their specificity for the Hcrt peptides: Hcrtr-1 is selective for Hcrt-1 only, whereas Hcrtr-2 is non-selective and is receptive to both peptides (Cluderay, Harrison et al. 2002). A common electrophysiological response of neurons to Hcrt stimulation appears to be an increase in the spontaneous action potential frequency, as shown by studies recording extracellular neuronal activity (van den Pol, Gao et al. 1998); increased action potential frequency has been measured in the hypothalamus, brain stem, spinal cord, and also in peripheral neurons. (Bourgin, Huitron-Resendiz et al. 2000; Hwang, Chen et al. 2001; Burlet, Tyler et al. 2002).

1.2.2. Functions of the hypocretin system

Over a decade of investigation has implicated the Hcrt system in a number of different functions such as eating, sleep/wakefulness, and neuroendocrine function (Sakurai, Amemiya et al. 1998; van den Pol, Gao et al. 1998; Kukkonen, Holmqvist et al. 2002; B.E. 2005; Sakurai 2007). In the original paper describing Hcrt, it was shown that intracerebroventricular injection of Hcrt induced feeding in non-fasted rats (Sakurai, Amemiya et al. 1998; B.E. 2005). Blockade of Hcrtrs through intraperitoneal injections does in fact cause a decrease in baseline feeding and normal weight gain (Haynes, Jackson et al. 2000). The degree to which Hcrt can regulate long-term feeding is, however, subject to debate with some data indicating that Hcrt only plays an acute role in food-intake that is balanced within 24-hrs of injections (Willie, Chemelli et al. 2001).
Any long term effects on feeding could be a by-product of the important role Hcrt plays in the regulation of sleep/wakefulness.

Release of the Hcrt peptides is maximal during waking periods and minimal during sleep (Fujiki, Yoshida et al. 2001; Lee, Hassani et al. 2005; Mileykovskiy, Kiyashchenko et al. 2005). Hcrt neurons are wake promoting and when Hcrt is injected intracerebroventricularly into rodents, it causes a remarkable increase in wakefulness duration at the expense of NREM and REM sleep (Hagan, Leslie et al. 1999). In rats, c-Fos expression of Hcrt neurons is increased during the dark, active period, and Hcrt levels in cerebrospinal fluid also peak during the dark, active period and decrease during the light, rest period (Yoshida, Fujiki et al. 2001). These observations suggest that Hcrt neurons are active during the wake period promoting and maintaining wakefulness, and are inactive during most of the sleep period.

Hcrt neurons innervate and excite different wake promoting monoaminergic neurons in the brainstem that fire tonically during waking, less during NREM sleep, and cease firing completely during REM sleep (Kayama and Koyama 2003). There is a synchrony between monoaminergic activity and behavioural state with the two parameters being positively correlated. Of interest is the profile of monoaminergic activity when comparing it to muscle activity across different behavioural states: during wakefulness when muscle activity is highest, monoaminergic activity is positively correlated and the correlation holds during NREM sleep when muscle activity is suppressed. REM sleep is unique in that muscles express atonia with occasional bursts in activity; these phasic events of activity, however, are not correlated with any increases in monoaminergic activity indicating the possibility of an additional source of motor control.
(Nicholson, Belyavin et al. 1989; Kayama and Koyama 2003). Hcrt neurons are a plausible candidate for this additional source of motor control as they fire only during the phasic twitches seen in REM sleep and may promote muscle activity through a direct pathway that is independent of the monoaminergic systems (Kiyashchenko, Mileykovskiy et al. 2002; Kayama and Koyama 2003).

1.2.3. Hypocretin and motor control

The hypothalamus has long been known to be involved in the regulation of a number of physiological functions. In fact, areas in the hypothalamus exert an influence over most, if not all, systems in the body. Neurons of the hypothalamus are situated in an ideal position to exert an influence throughout the different systems comprising the CNS, and Hcrt producing cells are no different as evident by their widespread projections. As early as 1958, studies attributed a respiratory function to neurons located in the vicinity of the perifornical region of the lateral hypothalamus (Redgate and Gellhorn 1958). Electrical stimulation of regions that are now known to house the Hcrt cells in the CNS caused an increase in the tidal volume of anaesthetized cats. Interestingly, the stimulation of this general region in the human during surgery has also been reported to have respiratory effects (Segundo, Arana et al. 1955). Despite the link to motor function, few studies were conducted to ascertain the role of the hypothalamus in the motor system.

The discovery of Hcrt shifted focus back onto the role of the perifornical region of the hypothalamus in physiological function. Perhaps the most convincing evidence for a link between the Hcrt system and the motor system stems from its endogenous activity pattern, as well as the disorders caused by a lack of Hcrt activity. As mentioned
previously, Hcrt neurons fire in a strikingly coordinated manner that shadows the activity of an animal; in freely moving cats, the concentrations of the neuropeptide, as measured through microdialysis in different regions of the brain, display a positive correlation with the level of muscle tone across the sleep-wake cycle, being highest during active waking, reduced in quite waking, further reduced in NREM sleep and only present during the phasic twitches of REM sleep (Kiyashchenko, Mileykovskiy et al. 2002). These studies concluded that Hcrt neurons discharge most when postural muscle tone is highest and decrease discharge in the absence of movement.

In both the trigeminal and hypoglossal motor nuclei, Hcrt immunoreactive varicose fibers were observed in close proximity to motoneurons (Fung, Yamuy et al. 2001). This finding suggests that Hcrt neurons exert global action on different groups of somatic motoneurons. Furthermore, trace studies performed to determine the source of Hcrt innervation onto hypoglossal motoneurons, confirmed that direct, monosynaptic innervations exist from the Hcrt field onto the cranial motoneurons (Fung, Yamuy et al. 2001).

In decerebrate cats, the application of Hcrt into the trigeminal motor nucleus increases masseter muscle tone, and Hcrt application into the hypoglossal motor nucleus increases GG muscle activity (Fung, Yamuy et al. 2001; Peever, Lai et al. 2003). Hcrt is capable of exciting motoneurons and thereby, it elicits increases in muscle activity. Whether the Hcrt system plays an endogenous role in maintaining the excitation of motoneurons has never been addressed and is the subject of this thesis.

The loss of Hcrt function provides an interesting perspective into the important endogenous role this ligand plays in muscle tone. A malfunction in Hcrt activity, whether
it is the ligand, receptors or loss of the neurons themselves, leads to dysregulation of physiological activity: narcolepsy is a disorder that can be characterized as an abnormal intrusion of REM sleep features, most importantly muscle atonia, into the waking behaviour of a subject (Mitler, Boysen et al. 1974; Chemelli, Willie et al. 1999; Kilduff and Peyron 2000; Nishino, Okura et al. 2000; Thannickal, Moore et al. 2000; Nishino 2007; Overeem, Black et al. 2008). Furthermore, patients who suffer from narcolepsy have a significantly higher frequency of developing OSA (Dahm, Wessendorf et al. 2003).

1.2.4. Modulation of hypocretin neuron activity

Neurons that release Hcrt are present solely within the perifornical region of the lateral hypothalamus. The physiological characteristics of these neurons are of great interest and their modulation is important for studies attempting to orchestrate the output of this diffuse hypothalamic system. Li et al (2002) describe an endogenous glutamatergic drive onto Hcrt neurons. The mechanism for this excitation appears to be Hcrt-mediated excitation of local glutamatergic neurons that regulate Hcrt neuron activity. No direct effect of Hcrt on the membrane properties of Hcrt neurons was detected (Li, Gao et al. 2002). Therefore, modulation of Hcrt neurons is mediated by a glutamatergic mechanism.

1.2.4.1. Glutamate

Glutamate is the primary fast excitatory neurotransmitter in the CNS. It exerts its effects by acting at two types of postsynaptic ionotropic receptors: α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and N-methyl-D-aspartate (NMDA)
receptors (Collingridge and Lester 1989; Nakanishi 1992). Activation of these receptors generates inward currents that result in excitatory postsynaptic potentials of varying amplitudes and initiate the release of neurotransmitters (Westbrook and Mayer 1984; Ouardouz and Durand 1994; Rekling, Funk et al. 2000).

Ampakines are drugs structurally derived from aniracetam that potentiate currents mediated by AMPA-type glutamate receptors (Danysz 2002). These drugs slow deactivation and attenuate desensitization of AMPA receptor currents and increase synaptic responses. Ampakines do not compete in binding assays and thus presumably act through separate sites (Arai and Kessler 2007).

Ampakines have previously been shown to alleviate opioid-induced respiratory depression by increasing the drive on respiratory motoneurons (Ren, Poon et al. 2006; Greer and Ren 2009; Ren, Ding et al. 2009). These class of drugs have also been used to bolster the effect of anti-depressant medication (Lynch G 1999) as well as rectify the effects of sleep deprivation in non-human primates (Porrino LJ 2005).

1.2.4.2. GABA

γ-Aminobutyric acid (GABA) is one of the primary fast inhibitory neurotransmitters of the CNS. In the Hcrt field, GABA\(\alpha\) receptors are concentrated on postsynaptic membranes of Hcrt neurons (Li, Gao et al. 2002; Backberg, Ultenius et al. 2004). Hcrt neurons are in an intrinsic state of membrane depolarization that promotes their spontaneous activity (Eggermann, Bayer et al. 2003). Release of GABA onto these Hcrt neurons promotes their inactive state that is seen during bouts of sleep; c-Fos studies have shown decreased staining after perfusion of muscimol (GABA\(\alpha\) agonist) into the
Hcrt field (Alam, Kumar et al.; Alam, Kumar et al. 2005). Furthermore, GABA_A currents on Hcrt neurons have been confirmed to be greatest during NREM sleep in rodents (Alam, Kumar et al.). These results together suggest that GABA_A receptor-mediated increased inhibitory tone contributes to the suppression of Hcrt neurons during sleep bouts. In this thesis, we used muscimol as a tool to pharmacologically promote the endogenous state of Hcrt neuron inactivity to investigate the effect on muscle tone levels.

1.3. The motor system

The motor system is organized in a fashion whereby different regions in the CNS orchestrate the control, coordination and execution of muscle activity. Areas in the cortex and sub-cortex, regions of the brainstem, and the spinal cord together comprise the motor system. In a hierarchical organization, the highest levels in the motor system influence the levels below them with reciprocal connections between levels (Sherrington, 1947). Muscle activity, in turn, consists of both voluntary movements as well as involuntary processes such as reflexes, postural maintenance and respiratory control. These processes range in their complexity; a simple reflex occurs autonomous of higher order neural inputs, whereas complex movements require the coordinated integration of inputs originating from numerous brain regions.

The motor and premotor cortices, comprising the upper tier of the hierarchy, plan and program complex movements; neurons in the motor cortex, in turn, send projections to brainstem neurons that integrate these signals with one another as well as with sensory afferents. Occupying the bottom of the hierarchy are the alpha-motoneurons (hereafter referred to as motoneurons) of both the brainstem and spinal cord. These receive synaptic
inputs from both cells in the motor cortex as well as brainstem nuclei such as premotor neurons, and serve as the final common effectors of motor output (Sherrington, 1947). Other brain regions existing outside of the hierarchy, such as the basal ganglia and cerebellum, make further adjustments to refine motor output (see reviews by Sherwood, 2003 and Rhoades et al., 2003 for details of the motor system).

1.3.1. The motoneurons

A motoneuron is any neuron in the motor system with an axon terminating on skeletal muscle fibres. Collectively, the motoneuron and all of the muscle fibres it innervates are termed the motor unit. Motoneurons can originate either in the ventral horn of the spinal cord or in a group within a nucleus in the brainstem. Motoneurons receive numerous afferents that could alter their membrane potential; once the threshold potential is reached by excitatory postsynaptic potentials, motoneurons generate action potentials (APs) which traverse the length of the axon to produce APs in each of its target muscle fibres. The frequency of APs determines the level of force produced in a muscle. In short, increased excitation of motoneurons leads to an increase in muscle activity, while an inhibitory drive on motoneurons will result in reduced muscle contraction. Motoneurons, therefore, act as the final pathway through which the CNS can access and control skeletal muscles. All neural commands to modify movements must be directed through the motoneuron (Sherrington, 1947).

Neural commands onto motoneurons originate from a variety of presynaptic terminals that release neurotransmitters onto receptors present on the membrane of
motoneurons. The resulting changes in motoneuron activity are due to the integrative effect of all premotor inputs. Glutamate is the principal excitatory neurotransmitter and directly elicits excitatory postsynaptic potentials, whereas GABA and glycine are the major inhibitory neurotransmitters and cause inhibitory postsynaptic potentials (Rekling, Funk et al. 2000).

Although glutamate is vital for the activity of motoneurons, it is not the only source of motoneuron excitation. Motoneurons express a range of additional receptors that are activated by different monoaminergic, neuromodulatory neurotransmitters. For example, serotonin and noradrenalin receptor subtypes have been identified on motoneurons (Ridet, Tamir et al. 1994; Wada, Hasegawa et al. 1997). Activation of these receptors on motoneurons increases membrane resistance and thereby result in depolarization and increased motoneuron excitability (Berger, Bayliss et al. 1992; Kubin, Tojima et al. 1992; Parkis, Bayliss et al. 1995).

Hcrt is also active at the level of the motoneuron. Studies have mapped both Hcrt receptor subtypes on the membranes of motoneurons (Cutler, Morris et al. 1999; Fung, Yamuy et al. 2001). As described above, there is strong evidence for a direct excitatory role for Hcrt in motoneuron activity (Fung, Yamuy et al. 2001; Peever, Lai et al. 2003). In addition to the direct innervations of motoneurons, Hcrt neurons also strongly innervate the locus coeruleus- the main source of noradrenaline in the brain (Cutler, Morris et al. 1999; Bourgin, Huitron-Resendiz et al. 2000; Kukkonen, Holmqvist et al. 2002). Therefore, Hcrt could act through both direct and indirect pathways to modulate and control the excitation of cranial motoneurons.
1.3.2. The hypoglossal and trigeminal motor systems

In our laboratory, the hypoglossal and trigeminal motor systems are used as a model to study the effects of modulating motoneuron activity on muscle tone. From a clinical perspective, these motor systems play a vital role in the regulation of airway muscle activity.

The GG muscle is innervated by the hypoglossal motor nucleus and helps maintain an open pharyngeal airspace for effective passage of air into the lungs; the improper regulation of GG motor tone is implicated in OSA (Remmers, deGroot et al. 1978; Horner 1996; Steenland, Liu et al. 2006). Therefore, elucidating the effects of neuromodulation of hypoglossal motor nucleus motoneurons is clinically important in the development of effective therapies. Furthermore, the neuroanatomical location of this system allows unhindered access to the motor pool for the purpose of pharmacological manipulation. Lastly, the dichotomous nature of GG EMG activity provides a convenient means for assessing the effects of pharmacological manipulation on muscle tone: GG muscle is characterized by both a tonic and a phasic component. As will be stated in our objectives, we sought to investigate the effects of inhibiting Hcrt activity at the motor pool on muscle tone. Clear phasic variations in GG activity occur under anaesthesia and the basal levels of these twitches is intrinsically at a higher tone than the basal levels of tonic GG activity and masseter muscle tone. This characteristic of phasic GG activity would make it possible to investigate the effect of decreased excitation on basal muscle tone. The low basal tone of tonic GG activity, and masseter activity, makes it difficult to investigate decreases in excitation when recording these two parameters.
The masseter muscle is a target of the trigeminal motor nucleus and helps to maintain jaw position and airway patency during sleep; the masseter muscle is implicated in sleep disorders including bruxism (Gastaldo, Quatrale et al. 2006). The masseter muscle follows the stereotypical pattern of muscle activity across the sleep-wake cycle, being highest in wake, reduced in NREM sleep and further reduced in REM sleep. Finally, masseter muscle activity is successfully recorded in our laboratory. Therefore, like the GG, the masseter muscle is a suitable and relevant muscle to investigate in terms of its excitability and neuronal regulation.

Finally, both of these motor systems have been shown to be a target of Hcrt neurons and express Hcrt receptors (Peyron, Tighe et al. 1998; Fung, Yamuy et al. 2001; Kukkonen, Holmqvist et al. 2002; Yamuy, Fung et al. 2004). They are therefore an ideal index to investigate the endogenous role of Hcrt in muscle tone control in an anaesthetised mouse model.

1.4. Summary

The neural control of motoneuron excitability dictates the stereotypical changes in muscle tone across the sleep-wake cycle. Numerous neurotransmitters have been shown to play different roles in controlling the activity of motoneurons in both sleep and wakefulness (Rekling, Funk et al. 2000; Kayama and Koyama 2003; Jones 2005). Although glutamate is the primary excitatory neurotransmitter on motoneurons, other excitatory neurotransmitters have also been shown to contribute to basal muscle tone levels (Chan, Steenland et al. 2006; Burgess, Lai et al. 2008). Hcrt neurons reside exclusively in the Hcrt field of the lateral hypothalamus and project to both cranial
motoneurons as well as important premotor centers (Hagan, Leslie et al. 1999; Bourgin, Huitron-Resendiz et al. 2000; Fung, Yamuy et al. 2001; Hwang, Chen et al. 2001; Burlet, Tyler et al. 2002). Hcrt activity is positively correlated with levels of muscle tone and waking behaviour (Kiyashchenko, Mileykovskiy et al. 2002; Lee, Hassani et al. 2005). Furthermore, the loss of Hcrt function leads to disorders of motor dysregulation (Nishino, Okura et al. 2000; Siegel JM 2006; Sansa, Iranzo et al. 2009). To date, the endogenous role of Hcrt in muscle tone control has not been determined and accordingly, the major goal of this study was to determine the extent of such a role using an anaesthetised mouse model (Figure 1-3).
Figure 1-3: Schematic of hypothesis: Hypocretin (Hcrt) neurotransmission elicits changes in motoneurons excitability and muscle tone. Hcrt neurons increase motoneuron activity either directly or through an indirect pathway. Motoneurons, in turn, stimulate muscles and lead to changes in muscle tone. Abbreviations: LC, locus coeruleus.
CHAPTER 2:
AIMS AND OBJECTIVES
Chapter 2: AIMS AND OBJECTIVES

2.1 Aims and hypotheses

The overall aim of this project was to investigate the role of Hcrt in controlling the excitation of cranial motoneurons within the hypoglossal and trigeminal motor pools. We hypothesized that the modulation of Hcrt activity would lead to changes in the activity of motoneurons and affect the muscle tone of both GG and masseter muscles in anaesthetized mice (Figure 1-3).

2.2 Specific objectives

The following are a list of specific questions we sought to address in this thesis:

1. What is the effect of activating hypocretin neurons on the muscle tone of the genioglossus and masseter muscles?

2. Is there an excitatory endogenous hypocretinergic drive onto motoneurons?

3. Is the effect of hypocretin through a direct circuit or via other mechanisms?
CHAPTER 3:

MATERIALS AND METHODS
Chapter 3: MATERIALS AND METHODS

A detailed outline of the surgical techniques, drug protocols and electrophysiological recording methods comprising this study is presented in this chapter. All experimental interventions were acute procedures carried out in anaesthetized mice and approved by the Animal Care Committee at the University of Toronto and were in accordance with the Canadian Council on Animal Care.

3.1. Animals

C57BL/6J wild-type (WT; n=83) mice, ages 12 to 13 weeks (mean weight: 28 ± 3g), were used in these experiments. In one study, prepro-hypocretin knockout (KO; n=6) mice were used. Mice were genotyped using PCR with a neo primer, 5'-CGCTATCAGGACATAGCGTTGGC, or a genomic primer, 5'-GACGACGGCCTCAGACTTCTTGGG, and a genomic primer, 3'-TCACCCCCCTTGGGATAGCCCTTCC, common to KO and WT. Animals were bred and cared for under the supervision of the Animal Care Facility in the Department of Cell and Systems Biology (University of Toronto) until 24 hours prior to each experiment, at which point they were relocated to the laboratory to allow them to acclimate to their new environment and minimize stress. All mice were maintained at 20 ± 1°C room temperature and 60% humidity on a 12-hour light/dark cycle (lights on 7:00a.m. and off at 7:00p.m.) and housed in plastic cages (Nalgene Labware, Nalge Nunc International, Rochester, NY, U.S.A.) equipped with standard cob bedding. Water and mouse chow (Lab Diet; PMI Nutrition International, St. Louis, MO, U.S.A.) were available ad libitum.
3.2. Experimental procedure

Mice were initially placed into a sealed plastic container and received a gas consisting of 100% O₂; this gas was maintained throughout the experimental protocol. Gas flow was regulated via a variable flowmeter system (Model FM-1050, Matheson Tri-Gas, Newark, CA) and calibrated to a flow rate of 600 mL/min. Gas was delivered via a silicone rubber inlet hose and flowed out of the container through an outlet hose connected to an activated-carbon scavenging canister for halogenated anaesthetic (A.M. Bickford, Wales Center, NY). Inhalation anaesthesia was induced by introducing 1.25 to 2% isoflurane (1-chloro-2,2,2-trifluoroethyl ether) into the gas mixture, and animals were monitored until they appeared to lose consciousness (after approximately 3 minutes). Mice were then removed from the container and placed in a supine position on to an acrylic operating platform with a heating pad connected to a temperature controller that samples the animal’s temperature via a rectal temperature probe (TC-1000 Temperature Controller; CEW, INC; Mntrl, QC). Gaseous anaesthetic was delivered by way of a custom-made, sealed nosepiece that was secured to both the platform and to the animal’s snout. Mice were allowed to breathe spontaneously throughout the procedure. Mice were shaved in all areas for operation (i.e. scalp) using animal clippers. Effective depth of anaesthesia was determined by the abolition of the pedal withdrawal and blink reflexes; constant checks for the depth of anaesthesia were performed throughout experiments and levels of anaesthesia were adjusted accordingly.

The goal of the studies was to determine the role of Hcrt in the control of muscle tone; therefore, electromyogram (EMG) activity of muscles innervated by motor pools known to be a target of Hcrt neurons, namely the trigeminal and hypoglossal motor
nuclei, were chosen as a suitable index of muscle tone. EMG activity of the masseter (innervated by trigeminal motoneurons) and GG (innervated by hypoglossal motoneurons) muscles were recorded using bipolar recording electrodes. Incisions of the skin and fascia caudal to the jaw exposed the masseter muscles for implantation of thin-strand looped wire electrodes using a 6-0 nylon suture (Deknatel, Mansfield, MA). In order to record the GG muscle, incisions of the skin and fascia ventral to the jaw exposed the muscle layers superficial to the GG muscle. Bipolar needle electrodes (Grass Technologies, West Warwick, RI) were inserted into the ventral surface of the jaw to implant into the GG muscle. The electrodes were then secured to the surrounding skin of the animal using 4-0 braided silk suture (Deknatel, Mansfield, MA); the make of this suture allowed for friction and a stronger knot that was able to secure the electrodes in place with minimal movement.

In order to pharmacologically manipulate the Hcrt field, a stereotaxic method was employed to precisely insert a custom-made double tipped probe delivery system into the Hcrt field and bilaterally apply drugs as needed. From their supine position, mice were turned into the prone position and placed into a stereotaxic frame (David Kopf Instruments, Chatsworth, CA). The animals were secured with blunt ear bars and a snout piece. The head was maintained at proper inclination using a stereotaxic head leveler (David Kopf Instruments).

A midline incision was made from the frontal bone to the caudal base of the skull. A bone scraper was used to pull away the overlying connective tissue and expose the surface of the skull, while 3% hydrogen peroxide was applied to clean away excess blood and tissue covering the surface of the skull. Bleeding was controlled using topical
application of adrenaline and/or thrombostat, which act as a blood vessel constrictor and a clotting agent, respectively. The reference point bregma was located by identifying the junction of the sagittal and coronal sutures. Using a flexible-shaft drill (Model 732, Dremel, Racine, WI), burr holes were drilled bilaterally in the skull to expose the brain. The custom-made, double tipped probe held by a stereotaxic micromanipulator (David Kopf Instruments) was inserted into the brain precisely 1.86 mm caudal from bregma, 0.78 mm lateral from the midline (in either direction) and 4.65 mm ventral from the surface of the skull, ensuring accurate placement into the Hcrt field consistent with the stereotaxic brain atlas (Paxions & Watson (2004)). In studies targeting the hypoglossal motor pool, burr holes were drilled unilaterally in the skull to expose the brain. A bevelled 1μL microsyringe (Hamilton, Reno, NV) held by a stereotaxic micromanipulator (David Kopf Instruments) was inserted into the medulla roughly 7.2 mm caudal to bregma, 0.18 mm lateral to the midline and 4.2 mm ventral from the surface of the skull, ensuring accurate placement into the motor pool (Paxions & Watson (2004)).

In place of lowering a microsyringe, 2 experimental aims used a mouse microdialysis probe to exogenously perfuse pharmacological agents into the hypoglossal motor pool. The microdialysis probe (Bioanalytical Systems West Lafayette, IND; 1 mm semi-permeable membrane; MD-2211) was lowered into the hypoglossal motor nucleus. The inlet end of the probe was connected to Teflon tubing (inside diameter, 0.12 mm; EiCom, Kyoto, Japan). The tube connected to the inlet was connected to a 500μL gastight syringe (MD00500 Bee Stinger Gastight Syringes; BASi) via a liquid switch (UniSwitch; BASi). The probe was continuously perfused with filtered (0.22 μm PVDF, Fisher Scientific, Toronto, ON.) saline or candidate drugs via a syringe pump driver and
controller (MD-1020 Bee Give Pump Controllers; BASi) at a rate of 5μl/min. At this rate of perfusion, it takes perfusate exactly 3 minutes to reach the semi-permeable membrane of dialysis probes. (Figure 3-1) (Proper placement of the probe trips was further verified using a number of checkpoints (see section 3.5.4)).

Figure 3-1: Schematic illustrating experimental setups. A: Axial section of mouse head with masseter highlighted. B: Sagittal section of mouse head depicting placement of a bilateral (custom made) probe onto the PeF regions for microinjection, and a microdialysis probe into the hypoglossal for perfusion (in one set of experiments a microsyringe was used instead of a microdialysis probe). Genoglossus muscle is tagged. C: Raw recording trace depicting EMG of masseter and genoglossus.
3.3. Electrophysiological recording

Masseter EMG signals were amplified between 1000-5000 times using a Super-Z High Impedance Head Stage and BMA-400 AC/DC Bioamplifier (CWE Inc., Ardmore, PA), bandpass filtered between 100Hz and 1kHz. GG EMG signal was amplified 2000 times and bandpass filtered 100Hz and 3kHz (Figure 3-2). All signals were sampled at 250Hz (Spike2 software, 1401 Interface; CED, Cambridge, UK) and stored on a computer for offline analysis.

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**Figure 3-2:** Sample raw experimental trace. This illustration depicts an example of recorded output from the Spike2 data acquisition software. The recorded parameters include left masseter EMG activity and both integrated/rectified and raw genioglossus EMG activity. Scales are seconds on the x-axis and arbitrary units (AU) on the y-axis.
3.4. Drugs

All drugs were dissolved in either saline (0.9% NaCl) or ethanol (100%) (vehicle. AMPA (α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid hydrobromide; formula weight (FW): 267.08), was purchased from Tocris Bioscience (Ellisville, MO) and dissolved to a final stock concentration of 700mM in saline. Ampakine CX546 (1-(1,4-Benzodioxan-6-ylcarbonyl) piperidine; FW: 247.29; Sigma, St. Louis, MO) an AMPA receptor potentiator, was prepared to a final stock concentration of 100mM in ethanol. The stock solution was then diluted and used at a concentration of 500µM in experiments. This concentration was validated using calcium imaging (See Figure 1-2). Lidocaine (Lidocaine Hydrochloride; 2%; AstraZeneca, Mississauga, ONT) was used at a concentration of 2% and stored at room temperature (dissolved in saline). Muscimol (3-Hydroxy-5-aminomethyl-isoxazole, 5-Aminomethyl-3-hydroxy-isoxazole, 5-Aminomethyl-3-isoxazolol; FW: 114.10; Sigma), a GABA_A receptor agonist, was dissolved in saline to a stock solution of 1mM and stored at 4°C. SB 408214 (N-(6,8-Difluoro-2-methyl-4-quinolinyl)-N’-[4-(dimethyl amino)phenyl]urea; FW: 356.37; Tocris), a Hcrt-1 antagonist, was dissolved in saline to a final stock concentration of 1mM. The stock solution was then diluted into aliquots of 30µM and stored at -20°C. TCS OX2 ((2S)-1-(3,4-Dihydro-6,7-dimethoxy-2(1H)-isoquinoliny)-3, 3-dimethyl-2-[(4-pyridinylmethyl)amino]-1-butanone hydrochloride; FW: 433.97; Tocris), a selective Hcrt-2 antagonist, was dissolved to a final stock concentration of 100mM in ethanol. The stock solution was then diluted into aliquots of 100nM and stored at 4°C (Hirose, Egashira et al. 2003; Langmead, Jerman et al. 2004). All drugs were vortexed and filtered (0.22 µm PVDF, Fisher Scientific, Toronto, ON) before use.
3.5. Experimental protocol

A general microinjection protocol was used for all experimental aims:

Upon probe insertion, preparations were left to stabilize for 10 to 15 minutes. Candidate drugs or vehicle control were then applied on to the targeted area (0.1μL) over the course of 120 seconds.

EMG activity was monitored for a minimum of 20 min after a microinjection was complete. The probe was then gently removed, flushed thoroughly with filtered ethanol and double-distilled water, and loaded with a second compound. Subsequent microinjections were accomplished in an identical manner.

In studies where microdialysis was employed, a microdialysis probe was used to exogenously perfuse pharmacological agents into the hypoglossal motor nucleus. The microdialysis probe (Bioanalytical Systems West Lafayette, IND; 1mm semi-permeable membrane; MD-2211) was lowered into the hypoglossal motor nucleus. The inlet end of the probe was connected to Teflon tubing (inside diameter, 0.12mm; EiCom, Kyoto, Japan). The tube was connected to a 500μL gastight syringe (MD00500 Bee Stinger Gastight Syringes; BAS Inc) via a liquid switch (UniSwitch; BAS Inc). The probe was continuously perfused with control vehicle or candidate drug via a syringe pump driver and controller (MD-1020 Bee Hive Pump Controllers; BAS Inc) at a flow rate of 5.0μL/min.

In one study, a combination of microdialysis at the hypoglossal motor pool, and bilateral microinjection into the Hcrt field was employed. To do this, bilateral burr holes were drilled to the coordinates of the Hcrt field. A third burr hole was drilled corresponding to the stereotaxic location of the hypoglossal motor pool. The bilateral
probes were then lowered into the Hcrt field and secured onto the skull using a vial of 3M Ketac Cement Aplicap (the vial was unsealed and mixed using an ESP RotoMix; St. Paul, MN). After allowing the cement to dry and confirming the probe is secured onto the skull, the micromanipulator was carefully removed and loaded with a microdialysis probe. The probe was then lowered onto the hypoglossal motor pool. Following this preparation, microinjection and microdialysis protocols were similar to the previously described protocols.

3.6. Individual studies:

3.6.1. Study 1: What is the effect of activating hypocretin neurons on muscle tone?

Hcrt neurons are excited via a glutamatergic drive and so to address the question of activating Hcrt neurons, AMPA was microinjected into the Hcrt field of anaesthetized mice while recording GG and masseter EMG activity. Dosages of 0.1mM, 0.01mM and 0.001mM AMPA were tested.

3.6.2. Study 2: Do hypocretin neurons play an endogenous role in the control of muscle tone?

To answer this question, we conducted three different studies that looked at changes in muscle tone when Hcrt neuron activity was potentiated, and when it was inhibited.

1. To increase the endogenous glutamatergic excitatory drive on Hcrt neurons, we microinjected 500µM of ampakine CX546 in the Hcrt field of anaesthetized mice.
2. To inhibit Hcrt neurons, we microinjected 2% lidocaine into the Hcrt field of anaesthetized mice.

3. To increase the inhibitory drive on Hcrt neurons, we microinjected 1mM muscimol into the Hcrt field of anaesthetized mice.

3.6.3. Study 3: Does hypocretin have a direct effect on motoneurons?

To investigate if Hcrt directly contributes to the control of motoneuron activity, we antagonized Hcrt-1 and Hcrt-2 in the hypoglossal motor pool using both microinjection and microdialysis. To antagonize Hcrt-1, SB 408214 was applied at 30μM (Wang, You et al. 2009). The Hcrt-2 antagonist TCS OX2 was applied along with SB 408214 at a dose of 100nM. According to the literature, application of TCS OX2 has not been used regularly in the mouse and therefore, dosage was approximated based on a study investigating the affinity of TCS OX2 to Hcrt-2 in the rat (Hirose, Egashira et al. 2003).

3.6.4. Study 4: Does the antagonism of hypocretin receptors in the motor pool abolish the excitatory effects of hypocretin neurons?

To answer this question, we combined two previous protocols in order to antagonize Hcrt receptors at the hypoglossal motor pool and to activate Hcrt neurons through microinjection of AMPA (0.1mM) into the Hcrt field.

3.6.5. Study 5: Does the loss of hypocretin ligands abolish the excitatory effects of hypocretin neurons on muscle tone?
To investigate this question, we repeated our AMPA injection protocol (0.1mM AMPA) in prepro-hypocretin KO mice where the Hcrt ligand is abolished, but Hcrt neurons are otherwise functional. We measured any changes in muscle tone that may be elicited by the activity of co-localized and co-released neurotransmitters in Hcrt neurons.

3.7. Target checkpoints and controls

Four procedures were used to demonstrate that microsyringe needles and microdialysis probes were both functional and located in the targeted area of the mouse brain:

3.7.1. Microinjection probe test

Before every experiment involving microinjections, the injection probes were tested to insure the tips were not clogged. Post-injection and removal of microinjection probes, the tips were again tested to insure no tissue or crystallized compounds had clogged the tips. If there appeared to be a clog, the data from that subject was not used in the analysis.

3.7.2. Insertion activation

In aims targeting the hypoglossal motor pool, proper placement of the microsyringe needle, or microdialysis probe, was ensured during experimentation by observing an insertion-induced burst of GG muscle EMG activity above baseline. Pharmacological interventions would be performed once this insertion activation subsided.
3.7.3. Histology

At the conclusion of experiments, mice were overdosed with isoflurane (5%) until ventilation and cardiac activity ceased. Mice were immediately decapitated and brains were dissected out and placed in chilled 4% paraformaldehyde in 0.1M phosphate buffered saline (PBS) at 4°C over 24-72 hours for fixation. Brains were then transferred to 30% sucrose in 0.1M PBS for cryoprotection over several days until they sank in solution. Brains were then transferred to a freezing-microtome (Leica Microsystems, Wetzlar, De) and cut into 40μm coronal sections. Sections were classified and separated based on whether they contained the probe lesion or immediately flanked the lesion site, 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, Canada) in order to identify the insertion tract of the microsyringe needle in the Hcrt field and the hypoglossal motor pool. Images were photographed using a high resolution digital camera (3.3 RTV Micro Publisher, QIMAGING, Burnaby, Canada). The location of insertion lesion tracts were plotted on standardized brain maps (Paxions & Watson, 2004).

3.7.4. Immunohistochemistry

In studies targeting the Hcrt field, we sought to confirm the location of our probes within the vicinity of the Hcrt field. To do this, the previously described histology protocol was performed to fix a brain. The brain was then cut into 30μm coronal sections collecting the probe lesion sites. On the first day of the immunohistochemistry protocol,
brain tissue slices were placed in wells and washed in 0.1mM PBS. Slices were then incubated for 1 hr in a solution of 10% goat serum, 0.4% Triton-X and 0.1mM PBS. The slices were then placed into a similar solution containing an anti-Orexin A rabbit pAb (Calbiochem, La Jolla; CA) at a dilution of 1:1000. These slices were placed onto an agitator at a temperature of 4°C for 48 hrs. On day 3 of the protocol, slices were washed in a solution of goat serum in PBS then incubated in a solution containing a biotinylated anti-rabbit IgG secondary anti-body (Vector Labs, Burlingame; CA) at a dilution of 1:200 for 1 hr at room temperature. After incubation in the secondary anti-body solution, slices were placed in a solution containing the avidin biotinylated enzyme complex (ABC kit; Vector labs) for an additional hr at room temperature. To visualize the antibody complex, we incubated the slices in a solution containing 0.05% 3,3’-Diaminobenzidine (DAB) (Sigma, St. Louis, MO) and hydrogen peroxide (oxidizes the DAB and promotes its brown pigment). The slices were then mounted on uncharged slides (Fisher Scientific), visualized, and photographed using the same equipment described in section 3.6.4. This immunohistochemistry protocol was adapted from the laboratory of Dr. Clifford Saper.

3.8. Time control

In one group of mice, previous interventions were repeated with only injections of saline to determine the natural effect of anaesthesia and time on the EMG activity of the masseter and GG muscles. The animal was allowed to breath under anaesthesia without any pharmacological intervention. The resulting timeline of changes in EMG activity were then used to insure that periods of collected data were not significantly influenced by the natural decrease due to anaesthesia use.
Data analyses were performed both on-line and off-line using Spike 2 software (CED, Cambridge, UK). Raw EMG signals were full-wave rectified, integrated and quantified into arbitrary units (AU). GG EMG signals were classified as either phasic (inspiratory) or tonic (expiratory) activity and analyzed independently. Characterization of phasic activity entailed application of a peak-detection algorithm to an integrated EMG channel. In order to analyze tonic activity, a search cursor coupled to the peak-detection algorithm was used to filter out phasic bursts and isolate mean raw tonic activity. The output of analyzed waveform data was converted to numerical data in a text file and then imported as a spreadsheet into Excel (Microsoft, Redmond, WA) for subsequent analysis. Masseter tonic EMG activity was averaged using epochs equal to those used in analyzing the tonic GG activity; this was done in order to avoid including “noise” from the phasic bursts of GG activity in masseter analysis. This also ensured high temporal resolution.

To designate basal muscle tone (i.e. baseline), EMG activity was averaged during the 5-min period immediately before drug intervention. Duration of response to drug application was designated as the period during which EMG activity exceeded and remained 2 standard deviations (SD) above the baseline mean value; wherever EMG activity dropped below this threshold briefly (15-20 seconds) but then rose again, response duration extended over this period until EMG activity dropped below threshold and was maintained at this level for a continuous period of 5 minutes. Accordingly, amplitude of response to a microinjection or microdialysis was calculated as the mean EMG activity throughout the response duration. Latency to response was regarded as the
period between onset of microinjection and the point at which EMG activity had risen 2 SD above baseline mean. Negative control (saline, or ethanol) injections were analyzed over the same response duration and latency as their corresponding drug injections in a given mouse. To insure that pressure injections were not causing significant changes in recorded EMG activity, we randomised the delivery of drug and control vehicle. We found mean EMG activity in both the GG and masseter muscles did not change significantly with control vehicle injections at any time during the experiment. We therefore quantified our baseline as the 10-min period of stable EMG activity prior to drug intervention and analyzed our data to investigate any significant changes from that baseline. The percentage change of EMG activity during drug intervention from baseline was calculated by dividing the difference of baseline and drug-evoked increase by baseline values and multiplying this factor by 100.

3.10. Statistical analyses

The specific statistical tests used for analysis are included within the results section. Comparisons between groups were made using either a paired *t*-test (parametric) or Wilcoxon’s matched-pairs sign-ranked tests (nonparametric). In groups where percent change from baseline was compared, 1-Way ANOVA was performed. Statistical analyses were performed electronically using Sigmastat (SPSS Inc., Chicago, IL) and applied a critical 2-tailed alpha value of P<0.05. Data are presented as means ± standard error of the mean (SEM).
CHAPTER 4:

RESULTS
Chapter 4: RESULTS

4.1. Pharmacological modulation of hypocretin neurons elicits changes in muscle tone.

4.1.1. Activation of hypocretin neurons increases muscle tone.

The purpose of this study was to determine if the exogenous excitation of Hcrt neurons elicits changes in muscle tone. To test this, different doses of AMPA were microinjected into the Hcrt field while GG and masseter EMG activities were recorded.

Microinjection of 0.1mM AMPA into the Hcrt field increased muscle tone (Figure 4-1). Relative to baseline, phasic GG activity increased by 40 ± 7% (n=6; 1-Way ANOVA on ranks; p< 0.001), and tonic GG activity increased by 20 ± 5% (p<0.001). Duration and latency of these changes in GG activity were 994 ± 96 s and 15 ± 1 s respectively (Figure 4-2 B). Masseter muscle tone also increased compared to baseline by 25 ± 4% (p<0.001). Duration and latency of these changes in the masseter muscle were 988 ± 117 s and 19 ± 1 s respectively. Masseter EMG activity developed a rhythmic phasic twitch that was maintained throughout the duration of drug effect as illustrated in figure 4-1.
To determine if the effects elicited by AMPA injection into the Hcrt field on muscle tone are dose-dependent, a second group of animals received microinjections of
0.01mM AMPA. The lower dose of AMPA increased muscle tone relative to baseline. Phasic GG activity increased by 25 ± 5% (n=5; 1-Way ANOVA on ranks; p<0.001), and tonic GG activity increased by 11 ± 3% (p<0.001). Duration and latency of these effects were 658 ± 260 s and 43 ± 1 s respectively (Figure 4-2 B). The masseter muscle tone also increased with AMPA injections by 22 ± 4% (p<0.001) (Figure 4-2 A-D). Duration and latency of changes in the masseter muscle were 580 ± 78 s and 39 ± 1 s respectively. Masseter EMG activity developed a rhythmic phasic twitch that was maintained throughout the duration of drug effect as illustrated in figure 4-1.

A third dose of AMPA was injected into the Hcrt field. Microinjection of 0.001mM AMPA into the Hcrt field (n=6) caused no changes in muscle tone compared to baseline (1-Way ANOVA on ranks; p>0.05 in phasic GG, tonic GG and masseter muscle tone) (Figure 4-2 A-D).

Although there appeared to be a trend towards a dose-dependent increase in EMG activity with the excitation of Hcrt neurons, there was no significant difference in the magnitude of change when comparing the two higher doses in either GG or masseter muscles (1-Way ANOVA on ranks; p>0.05). Duration of drug effects were shorter when comparing the 0.1mM and 0.01mM doses although the decrease in duration was not significant (t-test; p>0.05 for both GG and masseter muscles). The latencies to drug effects were significantly longer when comparing the 0.1mM and 0.01mM doses (t-test; p<0.001 for both GG and masseter muscles).
Figure 4.2: Microinjection of AMPA into the Hcrt field exerts a dose-dependent increase in muscle tone. **A**: Average group data depicting an increase in phasic GG activity with AMPA (0.1mM (n=6) and 0.01mM (n=5)) injection. Injection of AMPA (0.001mM (n=6)) had no significant effect on muscle tone. **B**: Average group data depicting the difference in latency duration of drug effect of phasic GG activity when AMPA 0.01mM and 0.1mM were injected. Latency was measured after the volume of 0.1μL was injected over 120 seconds * (t-test, p<0.05). **C**: Average group data depicting effect of AMPA injection on tonic GG activity. **D**: Average group data depicting effect of AMPA injection on masseter muscle tone. * Significantly different compared to baseline (1-Way ANOVA on ranks; p < 0.001). Group data are normalized and expressed as percent change from baseline (100) +/- s.e.m.
4.1.2. Hypocretin neurons endogenously excite motoneurons.

4.1.2.1 Microinjection of CX546 into the hypocretin field increases muscle tone.

Here, we sought to determine whether endogenously activating Hcrt neurons increases muscle tone. To do this, we used a group of animals to pharmacologically potentiate the endogenous glutamatergic drive on Hcrt neurons.

Microinjection of CX546 (500µM) into the Hcrt field increased muscle tone. Relative to baseline, phasic GG activity increased by 33 ± 5% (n=8; paired t-test; p=0.016). This same intervention caused a 24 ± 6% (paired t-test; p=0.030) increase in tonic GG activity. Duration and latency of these effects were 1081 ± 64 s and 16 ± 1 s respectively. Masseter muscle tone also increased compared to baseline by 28 ± 6% (paired t-test; p=0.022) with the development of a rhythmic phasic twitch in response to drug effect (Figure 4-3 A-C). Duration and latency of these changes in the masseter muscle were 1111 ± 79 s and 18 ± 1 s respectively.
Figure 4-3: Microinjection of CX546 into the Hcrt field exerts an increase in muscle tone. A: Raw trace (i) and average group data (ii, n=8) depicting an increase in phasic genioglossus (GG) activity with CX546 (500μM) microinjection. B: CX546 microinjection induced an increase in expiratory GG activity as depicted by (i) raw trace and (ii, n=8) average group data. C: Raw trace (i) and average group data (ii, n=8) depicting an increase in masseter muscle tone with CX546 microinjection. * Significantly different compared to baseline (paired t-test, p <0.05); Group data are expressed in arbitrary units +/- s.e.m
4.1.2.2. **Microinjection of lidocaine into the hypocretin field decreases phasic genioglossus muscle tone.**

To further investigate the excitatory endogenous role of Hcrt neurons in muscle tone control, we sought to inhibit the activity of Hcrt neurons. To do this, we initially injected lidocaine into the Hcrt field.

Microinjection of 2% lidocaine into the Hcrt field attenuated phasic GG activity. When compared to baseline, phasic GG activity decreased by 29 ± 3% (n=7; paired t-test; p=<0.001). The latency to drug effect was 28 ± 2 s and the potent suppression of phasic GG activity remained throughout the duration of experimental recordings (1200 s). Recordings of tonic GG activity and masseter activity showed no changes with lidocaine application into the Hcrt field (Figure 4-4 A-C).

4.1.2.3. **Microinjection of muscimol into the hypocretin field decreases phasic genioglossus muscle tone.**

Here, we sought to use a second pharmacological compound to inhibit Hcrt neurons. To do this, we injected muscimol into the Hcrt field.

Microinjection of 1.0mM muscimol into the Hcrt field attenuated phasic GG activity compared to baseline levels. Phasic GG activity decreased by 26 ± 23% (n=6; paired t-test; p=0.005). The latency to drug effect was 24 ± 1 s and the potent suppression of phasic GG activity was maintained throughout the period of experimental recording (1200 s). Analysis of tonic GG activity and masseter activity recordings showed no changes in these parameters of muscle activity (Figure 4-5 A-C).
Together, our data from these studies show that modulating the endogenous activity of Hcrt neurons elicits changes in muscle tone. Hcrt is an excitatory ligand and could therefore facilitate the activation of somatic motoneurons controlling the GG and masseter muscles. Our studies suggest that a tonic hypocretinergic drive exists on motoneurons. To investigate the specificity of our studies in the Hcrt field, and further elucidate the existence of a tonic hypocretinergic drive on motoneurons, we sought to antagonise Hcrt activity at the level of the motoneuron in the brainstem.
Figure 4.4: Microinjection of lidocaine into the Hcrt field induces a decrease in phasic genioglossus activity. A: Raw trace (i) and average group data (ii, n=7) depicting a decrease in phasic genioglossus (GG) activity with lidocaine (2%) microinjection. B: Lidocaine microinjection has no effect on expiratory GG activity as depicted by (i) raw trace and (ii) average group data. C: Raw trace (i) and average group data (ii, n=7) depicting no effect on masseter muscle tone with lidocaine microinjection. *Significantly different compared to baseline (paired t-test; p < 0.05); Group data are expressed in arbitrary units +/- s.e.m.
Figure 4-5: Microinjection of muscimol into the Hcrt field induces a decrease in phasic genioglossus activity. A: Raw trace (i) and average group data (ii, n=6) depicting a decrease in phasic genioglossus (GG) activity with muscimol (1.0 mM) microinjection. B: Muscimol microinjection has no effect on expiratory GG activity as depicted by (i) raw trace and (ii, n=6) average group data. C: Raw trace (i) and average group data (ii, n=6) depicting no effect on masseter muscle tone with muscimol microinjection. *Significantly different compared to baseline (paired t-test, p < 0.05); Group data are expressed in arbitrary units +/- S.E.M.
4.2. Antagonism of hypocretin receptors in the hypoglossal motor pool decreases phasic genioglossus activity.

4.2.1. Microinjection of hypocretin receptor antagonists in the hypoglossal motor pool decreases phasic genioglossus activity.

Here, we sought to determine the effects of antagonizing both Hcrts present on hypoglossal motoneurons. We found that the microinjection of the Hcrtr-1 and Hcrtr-2 antagonists, SB-408124 (30μM) and TCS OX2 (100nM) respectively, into the hypoglossal motor pool decreased phasic GG activity when compared to baseline. Phasic GG activity decreased by 28 ± 3% (n=6; paired t-test; \( p=0.003 \)) (Figure 4-6 A-B). The latency to drug effect was 12 ± 2 s and the potent suppression of phasic GG activity was maintained for 833 ± 54 s. EMG recordings of tonic GG activity did not indicate any changes with Hcrtr antagonism in the hypoglossal motor pool.

4.2.2. Microdialysis of hypocretin receptor antagonists in the hypoglossal motor pool decreases phasic genioglossus activity.

Antagonism of Hcrtr results in decreased phasic GG activity as is indicated by the previous study. Here, we sought to investigate if the continuous perfusion of Hcrtr antagonists could maintain a continuous attenuation of phasic GG activity.

Perfusion of the Hcrtr-1 and Hcrtr-2 antagonists, SB-408124 (30μM) and TCS OX2 (100nM) respectively, into the hypoglossal motor pool decreased phasic GG activity by 26 ± 1% (n=7; paired t-test; \( p=0.001 \)) compared to baseline. The latency to drug effect was 23 ± 4 s and the potent suppression of phasic GG activity was maintained throughout
the period of experimental recording (1200 s). The intervention did not cause any significant changes in tonic GG activity (Figure 4-6 A and C).

Together, the two different approaches to antagonize Hcrtrs describe an endogenous tonic release of Hcrt onto motoneurons in the hypoglossal motor pool.
Figure 4-6: Antagonism of hypocretin receptors in the hypoglossal motor pool attenuates phasic genioglossus activity.

A: (i) Representative genioglossus (GG) EMG and masseter EMG raw traces illustrating the effect of Hcrtr antagonism (horizontal black line) at the level of the motor pool. (ii) Illustrates GG activity in an expanded time scale, and latency duration using microinjection vs microdialysis.

B: Microinjection of Hcrtr-1 and Hcrtr-2 antagonists, SB-408124 (30µM) and TCS OX2 (100nM) respectively, at the hypoglossal motor pool attenuated phasic GG activity but not tonic activity (n=7).

C: Microdialysis of Hcrtr antagonists at the hypoglossal motor pool attenuated phasic GG activity but not tonic activity (n=7). *Significantly different compared to baseline (paired t-test, p<0.05), Group data are expressed in arbitrary units +/- s.e.m.
4.3. Antagonism of hypocretin receptors in the hypoglossal motor pool attenuates the effects of AMPA injection into the hypocretin field on genioglossus activity, but not masseter activity.

Here, we sought to determine if antagonism of Hcrtrs in the hypoglossal motor pool abolishes the excitatory effects of activating Hcrt neurons in the Hcrt field. Combining our previous protocols, we activated Hcrt neurons in the Hcrt field, while continuously antagonizing Hcrtrs in the hypoglossal motor pool.

Perfusion of the Hcrtr-1 and Hcrtr-2 antagonists, SB-408124 (30μM) and TCS OX2 (100nM) respectively, into the hypoglossal motor pool decreased phasic GG activity of 22 ± 3% (n=5; paired t-test; p=0.012) compared to baseline. Microinjection of 0.1mM AMPA into the Hcrt field increased phasic GG activity by 17± 2% (p=0.011). The duration and latency of this effect was 338 ± 69 s and 18 ± 3 s respectively (Figure 4-7 A-C).

Although the increase in phasic GG activity was significant, the duration of drug effect was significantly shorter when compared to AMPA injections without Hcrtr antagonism (338 s vs. 994 s respectively) in the motor pool (t-test; p<0.001) (Figure 4-7 C). The magnitude of increase in phasic GG activity is also significantly lower when compared to the study where no Hcrt antagonists were perfused into the hypoglossal motor pool (t-test; p=0.042) (Figure 4-8).
Figure 4-7: Antagonism of hypocretin receptors in the hypoglossal motor pool attenuates the excitatory effects of AMPA injection into the Hcrt field on genioglossus activity, but not masseter activity. A: Representative raw genioglossus (GG) and masseter EMG traces illustrating the effect of AMPA (0.1mM, upward arrows) injection into the Hcrt field while antagonizing Hcrt at the hypoglossal motor pool (horizontal black bar). B: i-iii illustrate GG and masseter EMG activities in expanded time scale. C: (i) Average group data (n=5) depicting the effect of AMPA injection into the Hcrt field on phasic GG activity. (ii) Average group data depicting the difference in duration and latency of drug effect on phasic GG activity when AMPA was injected during antagonism of Hcrt activity at the hypoglossal motor pool vs. no antagonism. *Significantly different compared to baseline (paired t-test; p<0.05). Group data are expressed in arbitrary units (A.U.) +/- s.e.m.
Figure 4-8: Antagonism of hypocretin receptors in the hypoglossal motor pool attenuates the effects of AMPA injection into the Hcrt field on genioglossus activity when compared to no antagonism. The effect elicited by AMPA injection into the Hcrt field on phasic genioglossus (GG) activity is attenuated with the antagonism of Hcrt at the hypoglossal motor pool. * Significantly different compared to baseline (t-test; p < 0.05); Group data are normalized and expressed as percent change from baseline (100) +/- s.e.m.
4.4. Microinjection of AMPA into the hypocretin field of hypocretin knockout mice has no effect on muscle tone.

Prepro-hypocretin KO mice lack the precursor gene to produce the Hcrt ligand. These mutant mice still possess the neuronal network of Hcrt neurons, and synthesize other co-localized excitatory glutamate. Therefore, exciting Hcrt neurons in these mice would allow us to investigate whether co-localized and co-released glutamate plays a role in the control of muscle tone. Furthermore, the use of these mice would further support the specificity of previously presented results to the activity of Hcrt neurons and not other neurons present within the Hcrt field.

Microinjection of 0.1mM AMPA into the Hcrt field of Hcrt KO mice caused no changes in phasic or tonic GG activity compared to baseline (n=6; paired t-test; \( p=0.388 \) and \( p=0.928 \) respectively). Masseter muscle tone did not change with AMPA application into the Hcrt field (paired t-test; \( p=0.888 \)) (Figure 4-9 A-C).
Figure 4-9: Microinjection of AMPA into the Hert field of Hert knockout mice has no effect on muscle tone. A: Raw trace (i) and average group data (ii, n=5) depicting no effect on phasic genioglossus (GG) activity with AMPA (0.1mM) injection. B: AMPA injection had no effect on expiratory GG activity as depicted by (i) raw trace and (ii, n=5) average group data. C: Raw trace (i) and average group data (ii, n=5) depicting no effect on masseter muscle tone with AMPA injection. Group data are expressed in arbitrary units +/- s.e.m.
4.5. Histology confirms probe insertion into target areas of the hypothalamus and brainstem.

4.5.1. Histology and immunohistochemistry confirms location of probes in the hypocretin field.

Post-mortem histological analyses allowed the visual verification of probe insertion tracts in mouse brain sections and confirmed that the tips of injection probes were within the Hcrt field. Anatomical location of probes was confirmed for a total of 57 out of 65 mice, and in animals were probes were not located in the Hcrt field (n= 8), no EMG data was used in the results of the studies (Figure 4-10).

To further confirm the presence of probes within the Hcrt field, brain slices from select mice were labelled with Hcrt-1 anti-body using a DAB-tagged immunohistochemistry protocol (Figure 4-11).

4.5.2. Histology confirms location of probes in the hypoglossal motor pool.

Post-mortem histological analyses allowed the visual verification of probe insertion tracts in mouse brain sections and confirmed that the tips of injection probes were within the hypoglossal motor pool. Anatomical location of probes was confirmed for a total of 20 out of 28 mice, and in animals were probes were not located in the Hcrt field (n=8), no EMG data was used in the studies (Figure 4-12).
Figure 4-10: Probe tips were located in the Hcrt field of subjects. Panel A is a photograph illustrating a lesion made by a probe lowered into the Hcrt field. B: Black filled circles represent the location of probe tips in the Hcrt field of 52 subjects for which histology was available. A second probe tip is located at the same coordinates on the contralateral side.
Figure 4-11: Probe tips were located in the Hcrt field of subjects. A: A schematic illustrating a section of the mouse brain where the Hcrt field exists surrounding the perifornical region (PeF). Panel B is a photograph illustrating a lesion made by a probe lowered into the Hcrt field and the Hcrt neurons within the field. C: Photographs illustrating a close-up of the Hcrt neurons.
Figure 4-12: Probe tips were located in the hypoglossal motor pool of subjects. Panel A is a photograph illustrating a lesion made by a probe lowered into the hypoglossal motor pool. B: A photograph illustrating a close-up of the hypoglossal motor pool. C: Black filled circles represent the location of probe tips in the hypoglossal motor pool of 20 subjects for which histology was available.
CHAPTER 5:

DISCUSSION
Chapter 5: DISCUSSION

5.1. Summary

This thesis project investigated the endogenous role of Hcrt in modulating hypoglossal and trigeminal motor outflow in an intact animal. Here, we provide evidence that the hypocretinergic system plays an excitatory role in modulating somatic motoneuron activity, and thereby, muscle tone. Hcrt is one of a number of excitatory neurotransmitters responsible for levels of muscle tone across the sleep-wake cycle. In summary, we show that:

1. Hypocretin neurons are excited via exogenous AMPA application and elicit increases in masseter and genioglossus muscle tone.
2. The modulation of endogenous hypocretin neuronal activity elicits changes in masseter and genioglossus muscle tone.
3. Antagonism of hypocretin receptors on hypoglossal motoneurons attenuates hypoglossal muscle tone.
4. Hypocretin neurons act on hypoglossal motoneurons through the direct release of Hcrt onto motoneurons.
5. Hypocretin may indirectly modulate muscle tone through the activation of excitatory premotor centers.

5.2. Exogenous activation of hypocretin neurons facilitates genioglossus and masseter muscle tone.

Several lines of evidence exist in support of a role for Hcrt in the control of muscle tone. First, Hcrt neurons project from the perifornical region of the lateral
hypothalamus to every region of the CNS including directly innervating premotor centers, the spinal cord and somatic motoneurons (Cutler, Morris et al. 1999; Bourgin, Huitron-Resendiz et al. 2000; Fung, Yamuy et al. 2001; Kukkonen, Holmqvist et al. 2002). Somatic motoneurons, in turn, express Hcrt receptors and can be excited by the local application of Hcrt (Peever, Lai et al. 2003). Second, the activity and neurotransmitter release of Hcrt neurons is positively correlated with muscle tone levels across the different states of the sleep-wake cycle (Yoshida, Fujiki et al. 2001; Kiyashchenko, Mileykovskiy et al. 2002). Finally, loss of Hcrt activity underlies disorders characterized by the dysregulation of motor control across different behavioural states, such as cataplexy/narcolepsy and an increased occurrence of OSA (Busquets, Barbe et al. 2004; Nishino 2007; Sansa, Iranzo et al. 2009).

Given the existing evidence of a hypocretinergic drive onto centers responsible for regulating muscle tone, we sought to investigate, in an intact animal, the effect of exogenously activating Hcrt neurons on muscle tone. Our hypothesis stems from the observation that AMPA can excite and increase the activity of Hcrt neurons. Hcrt neurons are in an intrinsically depolarized state during periods of active behaviour when muscle tone levels are maximal (Eggermann, Bayer et al. 2003). This maintained state of excitation is mediated via a glutamate-dependent positive feedback system: presynaptic glutamate interneurons innervate Hcrt neurons and are receptive to Hcrt excitation (Li, Gao et al. 2002; Hettes, Gonzaga et al. 2003; Hettes, Heyming et al. 2007; Mallick BN 2008; Schlicker and Kathmann 2008). Hcrt neurons themselves are not auto-excited by the Hcrt ligand (Li, Gao et al. 2002).
Under anaesthesia, exciting Hcrt neurons would allow a direct measurement of Hcrt’s effect on the muscle tone of both the masseter and GG muscles. Accordingly, the results of this study demonstrate that focal application of AMPA into the Hcrt field increases EMG activity of the masseter as well as the GG muscle in a dose-dependent manner. The potent increases in both masseter and GG muscle tone lasted for several minutes and were triggered following a relatively short latency period from injection.

Although glutamate appears to be the main excitatory neurotransmitter driving motoneuron excitation during active waking, antagonism of glutamate activity in the trigeminal and hypoglossal motor pools does not suppress muscle tone to levels seen during sleep (Burgess, Lai et al. 2008; Steenland, Liu et al. 2008). Therefore, Hcrt could be an additional neuromodulator contributing to the waking levels of muscle tone. Other possible sources of excitatory wake-related drives onto motoneurons include inputs from serotonergic and noradrenergic cell groups, which not only project to and facilitate motoneuron excitation (Kubin, Tojima et al. 1992; Parkis, Bayliss et al. 1995; Fenik, Davies et al. 2005; Schwarz, Yee et al. 2008), but also discharge maximally during periods of active waking (McGinty and Harper 1976).

The focal application of Hcrt into the trigeminal motor pool has already been shown to increase motoneuron activity (Peever, Lai et al. 2003). A second study focally applied Hcrt into the locus coeruleus and observed an increase in muscle tone associated with increased monoaminergic activity at the motor pool (Horvath, Peyron et al. 1999; Kiyashchenko, Mileykovskiy et al. 2001). The locus coeruleus is the main source of noradrenalin in the CNS and has been shown to increase motoneuron excitability (Parkis, Bayliss et al. 1995; Chan, Steenland et al. 2006). Therefore, a number of different
neuronal pathways exist through which Hcrt may have an excitatory effect on muscle tone.

While increased Hcrt release elevated both phasic and tonic GG muscle tone, the frequency of phasic activity remained unaffected. This finding suggests that Hcrt is driving the excitation of hypoglossal motoneurons through a pathway that is independent of respiratory centres and is direct onto the motoneurons. This observation does not rule out the possible role of Hcrt in respiratory control. Our protocol was primarily aimed at investigating the effects of Hcrt on muscle tone and not respiration and therefore we cannot draw any conclusions on the role of Hcrt in respiration. Due to the evidence supporting such a role (Redgate and Gellhorn 1958; McDowall, Horiuchi et al. 2007; Dampney, Horiuchi et al. 2008; Kuwaki 2008; Terada, Nakamura et al. 2008; Fortuna, Stornetta et al. 2009), it is imperative to expand our protocol for future studies to examine the role of Hcrt in respiratory control.

An interesting observation made in this study is the development of rhythmic, phasic twitches in masseter muscle activity that are parallel to augmented GG activity. In reviewing the literature, it appears that it has not been previously shown that the masseter muscle in mice displays a respiratory-related rhythm, however, glutamate mediated phasic twitches do occur during REM sleep bouts (Anaclet, Pedersen et al.; Soja, Lopez-Rodriguez et al. 1995; Burgess, Lai et al. 2008). Furthermore, the masseter muscle is an important feeding behaviour muscle; our interventions could have unmasked a rhythmic activity in the masseter muscle that is associated with the muscle’s activity during feeding. It is currently unknown what stimulus elicits the release of glutamate onto trigeminal motoneurons during phasic twitches of REM sleep. Hcrt neurons are known to
fire during the phasic twitches of REM sleep (Kiyashchenko, Mileykovskiy et al. 2002) and they may, therefore, play a role in stimulating the release of glutamate onto trigeminal motoneurons during REM sleep bouts. It is also possible that our observed effect on masseter muscle tone could be due to the increased excitatory neuronal pathway that elicited the changes in phasic GG muscle tone. The exact mechanism is unknown and should be further investigated.

5.3. Endogenous hypocretin activity facilitates genioglossus and masseter muscle tone.

The Hcrt system is vital in coupling behavioural state with normal muscle tone. Malfunction of the Hcrt system leads to a number of disorders that are characterized by a dysregulation of muscle tone in different behavioural states. This suggests an endogenous role for Hcrt in regulating muscle tone and coupling it to the different behavioural states.

Through three different approaches, we found that there indeed is an endogenous regulation of somatic motoneurons by Hcrt neurons. We sought to pharmacologically alter endogenous excitatory and inhibitory currents responsible for the activity pattern of Hcrt neurons across the sleep-wake cycle.

5.3.1. Ampakine mediated increase of hypocretin neuron activity elicits increases in genioglossus and masseter muscle tone.

As previously stated, an intrinsically depolarized state is maintained by Hcrt neurons via a glutamatergic current (Li, Gao et al. 2002; Eggermann, Bayer et al. 2003). We found that potentiating this glutamate current through the use of focally applied
Ampakine CX546 elicits an increase in both masseter and GG muscle tone. The potent increases in both masseter and GG muscle tone lasted for several minutes and were triggered following a relatively short latency period after injection. These observations are consistent with our results indicating that increases in Hcrt activity elicit increases in muscle tone.

Ampakines have been developed over the past few years as a potentially powerful clinical therapy for a number of physiological issues. Ampakines can cross the blood-brain barrier and act at a number of different centers in the brain, potentiating the activity of endogenous glutamate already present at synapses. Ampakines have been shown to recover and protect against opioid-induced respiratory depression in rats (Lorier, Funk et al.; Ren, Poon et al. 2006; Ren, Ding et al. 2009). We have also found in mice that CX546 does indeed recover the depressed activity of the GG muscle when administered systemically and focally at the hypoglossal motor pool post-fentanyl (µ-opioid agonist) treatment (Saleh and Peever, unpublished data). The inspiratory-depressant actions of opioid analgesics include presynaptic inhibition of hypoglossal motoneuron output; ampakines counter this depression without affecting the analgesia of opioids, making this drug a potentially powerful pharmacological therapy during surgeries and treatments requiring analgesics (Ren, Poon et al. 2006; Greer and Ren 2009).

Evidence suggests that ampakines may only have a positive effect when the homeostatic state of a system is dysregulated. Studies looking at the detrimental effects of sleep loss in non-human primates found that ampakine administration facilitates task performance and alleviates the effects of sleep deprivation (Porrino LJ 2005). In our laboratory, however, we have shown that the systemic application of ampakines does not
affect behavioural state nor muscle tone (Gillis and Peever, unpublished data). This suggests that systemic ampakine application only has an effect when countering a depressed state of excitation.

Loss of Hcrt excitation is an underlying cause of narcolepsy and possibly OSA (Busquets, Barbe et al. 2004); both of these disorders are caused by dysregulation of muscle tone. Therefore, ampakines could potentially be developed as a pharmacological therapy to rectify or protect against decreases in muscle tone associated with decreased Hcrt activity on somatic motoneurons. Ampakines may be able to effectively treat such disorders by potentiating the activity of glutamate already present in motor pools. The interesting nature of systemically applied ampakines to alter only perturbed systems would make this drug a particularly useful pharmacological treatment. This pharmacological agent should therefore be investigated as a treatment to rectify the loss of Hcrt excitation on motoneurons.

5.3.2. Inhibition of hypocretin neuron activity decreases genioglossus muscle tone.

We show that when lidocaine, a general anaesthetic, is applied into the Hcrt field, phasic GG activity decreases from baseline levels. We also show that focally applied muscimol, a GABAergic agonist, decreases phasic GG activity from baseline levels. This muscimol-induced suppression of activity is comparable to the general inhibition of activity produced by lidocaine. This suggests that an inhibitory GABAergic drive regulates neuronal activity in this region. Importantly, muscimol has been previously shown to only affect the activity of Hcrt neurons and not other neurons that exist in the same field (Alam, Kumar et al. 2005). Thus, muscimol decreases phasic GG activity by
increasing the GABAergic drive specifically onto Hcrt neurons. This notion is supported by the observation that a GABAergic drive inhibits the intrinsically depolarized state of Hcrt neurons during sleep bouts (Alam, Kumar et al.; Eggermann, Bayer et al. 2003; Alam, Kumar et al. 2005). Taken together, these data support a role for GABA in the endogenous regulation of somatic motoneurons by Hcrt neurons.

Our studies use three muscle tone indices to investigate the effect of Hcrt on muscle activity; phasic and tonic GG, and masseter muscle tone. Our results indicate that only phasic GG activity is potently attenuated after drug injection into the Hcrt field. Our results are consistent with many studies attempting to decrease endogenous motor tone: the basal tone of tonic GG activity and masseter tone did not decrease. The lack of any decreases could be a result of the low basal levels of muscle tone seen under baseline conditions. It is extremely difficult to discern if further decreases occur from the already low basal levels and therefore, the phasic GG activity was the only indicator in this set of studies. Nevertheless, the potent suppression of phasic GG activity we observe supports the notion that increasing the inhibitory drive onto Hcrt neurons attenuates muscle tone.

To the best of our knowledge, our studies are the first to define an endogenous role for Hcrt neurons in modulating muscle tone by directly measuring motor output. Numerous studies have investigated the effects of Hcrt application at different neuronal centers and many have characterized excitatory effects in premotor and motor centers (Bourgin, Huitron-Resendiz et al. 2000; Kiyashchenko, Mileykovskiy et al. 2001; Burlet, Tyler et al. 2002; Peever, Lai et al. 2003), but none have manipulated the endogenous activity of Hcrt neurons and directly measured the effects on motor output. Our protocol allowed us to manipulate endogenous Hcrt neuronal activity using a number of different
stimuli. The protocol is also ideal to investigate the pathway through which Hcrt may play its regulatory role of somatic motoneuron excitability.

5.4. Antagonism of hypocretin receptors in the hypoglossal motor pool decreases genioglossus muscle tone.

Hypoglossal motoneurons express Hcrtrs and are excited via Hcrt application (Marcus, Aschkenasi et al. 2001; Volgin, Saghir et al. 2002; Peever, Lai et al. 2003). In this study, we show that the antagonism of both Hcrtrs on hypoglossal motoneurons attenuates GG muscle tone. Antagonism of Hcrtrs causes a potent, continuous attenuation of phasic GG activity that is relatively short in latency after drug application. This suggests there is an endogenous hypocretinergic excitatory drive on hypoglossal motoneurons: it is a direct drive and contributes to the excitation of hypoglossal motoneurons. This is consistent with our data demonstrating that inhibition of Hcrt neuronal activity in the Hcrt field suppresses GG activity. Taken together, this study demonstrates that an endogenous Hcrt drive excites hypoglossal motoneurons through a predominantly direct innervation of motoneurons.

The mechanism through which Hcrt excites motoneurons is not fully characterized. It has previously been shown that in the trigeminal motor pool, Hcrt-1 excites motoneurons via an NMDA dependent pathway (Peever, Lai et al. 2003). Hcrt-1 appears to excite glutamatergic interneurons that release glutamate onto motoneurons. Here, we antagonised both Hcrtrs since both have an affinity for Hcrt-1. By doing so, we have demonstrated that such antagonism attenuates GG muscle tone. This is consistent with a direct innervation of hypoglossal motoneurons by Hcrt neurons. However, we also
wanted to determine if this direct innervation was the only Hcrt-mediated drive onto motoneurons. To do this, we developed a protocol whereby we continuously perfused Hcrt antagonists into the motor pool while activating Hcrt neurons in the Hcrt field. If blockade of Hcrt activity at the motor pool was sufficient to prevent any increases in muscle tone when Hcrt neurons were activated, this would indicate only a direct innervation of motoneurons. However, if an increase in tone was observed, this would suggest the involvement of indirect pathways as well.

5.5. Hypocretin receptor antagonism attenuates excitation of hypoglossal motoneurons elicited by AMPA application into the hypocretin field.

We have described how the microinjection of AMPA into the Hcrt field of anaesthetized mice elicits increases in muscle tone. These findings are consistent with a previous study that found the disinhibition of neurons in the Hcrt field of anaesthetized rats elicits increases in hypoglossal nerve activity (Lu, Fenik et al. 2007). Excitation of hypoglossal motoneurons by Hcrt neurons may be an important mechanism through which adequate muscle tone is maintained across the different states of the sleep-wake cycle. Loss of Hcrt excitation is detrimental and underlies disorders of inadequate muscle tone such as cataplexy/narcolepsy and increased occurrence OSA in narcoleptic patients (Busquets, Barbe et al. 2004; Nishino 2007; Sansa, Iranzo et al. 2009).

Although glutamate plays a dominant role in regulating basal muscle tone, it is not the sole excitatory modulator of muscle tone (Burgess, Lai et al. 2008; Steenland, Liu et al. 2008). Therefore, it is imperative to investigate and develop pharmacological therapies aimed at enhancing the neurotransmission of other excitatory modulators, such
as Hcrt, serotonin and noradrenalin, at the level of motoneurons. Elucidating the pathway through which Hcrt acts on motoneurons is vital for the development of such effective therapies.

We found that the co-antagonism of Hcrtr-1 and Hcrtr-2 prevents the long-term activation of hypoglossal motoneurons elicited by AMPA microinjection into the Hcrt field. This suggests that the neuronal pathway of Hcrt activity onto motoneurons is through a predominantly direct pathway. These findings suggest that the excitatory effect of Hcrt neurons onto hypoglossal motoneurons are independent of the respiratory centers and are directly responsible for muscle tone levels. The study also confirms the specificity of recorded changes to Hcrt activity and not the excitation of other neurons that reside in the same neuro-anatomical area as Hcrt neurons. Masseter muscle tone was facilitated with AMPA microinjection into the Hcrt field further supporting the evidence for a direct role of Hcrt on motoneuron excitability.

An interesting finding of this study was the short lasting increase in GG muscle activity immediately following AMPA application into the Hcrt field. Because of the Hcrtr antagonism in the hypoglossal motor pool, this increase must be Hcrt-independent or through an indirect pathway. Hcrt neurons synthesize and co-release glutamate (Torrealba, Yanagisawa et al. 2003). Therefore, this increase could be a result of glutamate release onto motoneurons. The increase may also be the result of a hypocretinergic drive onto other premotor centers such as the locus coeruleus and/or dorsal raphe nucleus that synthesize and release noradrenaline and serotonin respectively. Both serotonin and noradrenaline have been shown to facilitate the excitatory state of motoneurons (McGinty and Harper 1976; Parkis, Bayliss et al. 1995; Horvath, Peyron et
al. 1999). We sought to examine the possibility of an additional source of excitation onto motoneurons that is driven by Hcrt neuronal activity on premotor, monoaminergic centers or by the co-release of glutamate from Hcrt neurons.

5.6. Loss of the hypocretin ligand abolishes any changes in muscle tone elicited by hypocretin neurons.

Prepro-hypocretin KO mice lack the precursor gene to produce the Hcrt ligand. These mice still possess the neuronal network of Hcrt neurons, and synthesize other co-localized neurotransmitters, i.e. glutamate. Prepro-hypocretin KO mice are narcoleptic, displaying all of the characteristics of the narcolepsy phenotype. In this thesis, they have provided an invaluable tool for investigating the specificity of our pharmacological manipulations in the Hcrt field on Hcrt neurons, as well as assist in answering our question of the extent to which synthesized and co-released glutamate in Hcrt neurons can contribute to the excitation of target neurons.

We have shown that in prepro-hypocretin KO mice, the role of Hcrt neurons in regulating muscle tone is abolished. This finding is consistent with all of our previous studies attributing a role for Hcrt in the regulation of muscle tone. What is interesting from these observations is the lack of any facilitation of muscle tone in both the masseter and GG EMG activities.

Together with the findings of the previous study, this study elucidates the role of co-synthesized glutamate in the excitatory effects of Hcrt neurons. It appears that the degree of glutamate co-release from Hcrt neurons is not significant enough to elicit the excitation of motoneurons. Therefore, all of the excitatory effects of Hcrt neurons are due to the excitatory effect of the Hcrt ligand and not co-synthesized neurotransmitter.
In the previous study, we described a short lasting increase in muscle tone elicited by exciting Hcrt neurons while antagonising Hcrtrs in the hypoglossal motor pool. It is evident from using the prepro-hypocretin KO mice that this increase is not mediated by synthesized and co-released glutamate and must be the result of a hypocretinergic drive on other premotor centers. Possible sources of excitatory neuromodulatory drives onto motoneurons include inputs from serotonergic and noradrenergic centers in the brain.

Kopell et al. (2008) describe a delay in Hcrt activity when looking at its role in modulating wakefulness (Diniz Behn, Kopell et al. 2008). They found that other monoaminergic centers, specifically those that synthesize and release serotonin and noradrenalin, are facilitated by Hcrt activity and are responsible for a fast excitation of target centers (Diniz Behn, Kopell et al. 2008). In the case of their study, they found that this fast acting monoaminergic activity is responsible for the inhibition of the ventrolateral preoptic nucleus which is the primary sleep-on center in the brain. This pattern of monoaminergic activity in response to a hypocretinergic drive may also underlie our observed results. It is possible that our results are first initiated by the faster acting monoaminergic drive, followed by the delayed effect of Hcrt directly on motoneurons.

A second possible pathway for the short duration increase in muscle tone elicited by increased Hcrt activity is the release of presynaptic glutamate in the hypoglossal motor pool. Peever et. al. (2003) found that Hcrt activity in the trigeminal motor pool is mediated via the excitation of presynaptic glutamatergic interneurons (Peever, Lai et al. 2003). Although we hypothesize that antagonising Hcrtrs in the motor pool should also antagonise the receptors found on glutamatergic interneurons, it is possible that the effect
did not apply to the entire motor pool and Hcrt still maintained an excitatory effect through acting on a small subset of these interneurons.

The role of Hcrt in regulating muscle tone could be mediated by a number of pathways that all terminate onto motor pools. We describe here a predominantly direct excitatory effect of Hcrt on somatic motoneurons. However, there appears to also be an *indirect* excitatory drive on motoneurons that is also Hcrt mediated. This is consistent with another study that investigated the role of Hcrt excitation on hypoglossal nerve activity: Fenik et. al. (2009) found that the antagonism of serotonergic and adrenergic receptors on hypoglossal motoneurons could not prevent the excitatory effect elicited by the disinhibition of Hcrt neurons in the Hcrt field (Fenik, Rukhadze et al. 2009). Our study found that Hcrtr antagonism could abolish most of the Hcrt neuron-mediated excitatory drive on motoneurons. The neuronal pathway is most likely a combination of all of these excitatory neuromodulators.

**5.7. Technical considerations**

Since this study sought to elucidate one of many diverse roles Hcrt plays in the CNS, it was necessary to control the confounding effects of animal behaviour and state-dependent changes in Hcrt neuronal activity. The use of a reduced, anaesthetized preparation enabled a controlled and systematic analysis of the role of Hcrt in regulating somatic motoneuron activity. Nonetheless, the use of anaesthesia is a potential concern because of its ability to alter the physiological state of an animal by influencing the neurotransmission of other neuromodulators that influence muscle tone. The systemic effects of anaesthesia include enhancing GABAergic transmission as well as reducing the
presynaptic release of glutamate (Franks and Lieb 1994; Haseneder, Kurz et al. 2004; Ranft, Kurz et al. 2004). To minimize the potential confounding effects of anaesthesia in our preparation, we developed our protocols to analyze recordings over a restricted period of time for every treatment. In doing so, we controlled for the longitudinal depressing effects of anaesthesia on basal muscle tone.

Another concern is the presence of melanin concentrating hormone (MCH) containing neurons within the Hcrt field. These neurons are exclusive to the lateral hypothalamus and have been shown to project in a very similar pattern to Hcrt neurons throughout the CNS (Torterolo, Sampogna et al. 2006). The function of MCH appears to be inhibitory and is hypothesized to have a role in the promotion of quiescence (Guyon, Conductier et al. 2009). To the best of our knowledge, no evidence describes any role for MCH in regulating muscle tone and therefore, if our pharmacological manipulations did alter MCH containing neurons’ activity, it is unlikely that it influenced muscle tone recordings.

5.8. Conclusions and clinical implications

To summarize, our data demonstrate that Hcrt regulates the activity of somatic motoneurons. We found that the exogenous excitation of Hcrt neurons facilitates masseter and GG muscle tone. Through modulating both excitatory and inhibitory endogenous currents onto Hcrt neurons, we show that Hcrt plays an endogenous role in regulating muscle tone. Moreover, the antagonism of Hcrtr-1 and Hcrtr-2 at the hypoglossal motor pool attenuates phasic GG activity, further supporting an endogenous hypocretinergic drive onto somatic motoneurons. Our findings suggest that Hcrt neurons
contribute to the regulation of muscle tone through both indirect as well as direct neuronal pathways.

Findings from this study may provide insight into the pathophysiology of a number of clinical disorders that involve dysregulation of hypocretinergic neurotransmission. One example with direct relevance to muscle tone regulation is narcolepsy, a disorder in which Hcrt function is lost (Nishino 2007). This disorder is characterized by, amongst other symptoms, an abnormal regulation of muscle tone during periods of wakefulness - also known as cataplexy. In keeping with the results from this study, it is possible that loss of Hcrt deprives motoneurons from an endogenous excitatory drive that couples behavioural activity with appropriate muscle tone levels.

For OSA, muscles of the upper airway become overly relaxed during sleep, which narrows the airway and leads to cessation of breathing (Horner 1996). It is now becoming evident that the loss, or decrease, of Hcrt function increases the incidence of OSA in narcoleptic patients (Busquets, Barbe et al. 2004; Sansa, Iranzo et al. 2009). We show that Hcrt has a direct excitatory effect on the GG, a pharyngeal muscle responsible for the patency of the upper airway. Although Hcrt neurons decrease their firing rates during sleep, they are not completely suppressed (Kiyashchenko, Mileykovskiy et al. 2002). Therefore, our data suggests that the lack of Hcrt excitation on hypoglossal motoneurons in narcoleptic patients could contribute to the occurrence of OSA during sleep.

5.9. Future directions

This study demonstrated that Hcrt can regulate muscle tone in anaesthetised animals. These results have widespread implications in understanding the neural
modulation of muscle tone and in gaining insight into a myriad of motor-related and sleep-related disorders. Future studies expanding on our investigation into Hcrt’s role in regulating muscle tone should be conducted. A logical extension of this thesis would be to investigate whether hypocretinergic modulation of motoneuron excitability is operative in behaviour. Hcrt is vital for the regulation of normal behaviour and muscle tone. Pharmacological manipulations would allow the inhibition of Hcrt neurons during wakefulness and sleep to determine whether hypocretinergic control of motoneuron excitability can meaningfully influence muscle tone.

Furthermore, it is important to elucidate the possible role Hcrt may play in respiratory control. Studies have already described a respiratory function for perifornical area dating back to the 1950s (Redgate and Gellhorn 1958). More recently, Hcrt has been implicated in the control of ventilation and respiratory function across vigilance states (Young, Wu et al. 2005; McDowall, Horiuchi et al. 2007; Dampney, Horiuchi et al. 2008; Kuwaki 2008; Fortuna, Stornetta et al. 2009). One study of special interest to our laboratory is the role of Hcrt in ventilatory long-term facilitation across the sleep-wake cycle (Terada, Nakamura et al. 2008). Therefore, using our protocols, we could define the contribution of Hcrt to the respiratory control of an intact animal.

Finally, this study has demonstrated that ampakines can be a powerful pharmacological agent and a potential effective therapy. Characterization of ampakines’ potential to influence the occurrence of cataplexy is a logical avenue to investigate since a dysregulation of motor excitation underlies the disorder and our laboratory possesses the necessary prepro-hypocretin KO model to investigate such a treatment.
CHAPTER 6:

APPENDIX
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6.1. Control vehicle microinjection into the hypocretin field did not affect muscle tone.

Comparisons between control vehicle microinjections and baseline levels immediately prior to injection demonstrate that control vehicle has no effect on skeletal EMG activity when looking at the phasic and tonic GG activity (paired t-test; \( p = 0.937 \) and \( p = 0.924 \), respectively). The masseter muscle tone also did not show any changes with saline microinjection (paired t-test; \( p = 0.805 \)) (Figure 6-1 A-C).

These data demonstrate that the effects observed using pharmacological agents are not a result of microinjection of fluid \textit{per se} into the Hcrt field, but are rather mediated via pharmacological pathways.

6.2. Under anaesthesia, muscle tone decreases with time.

A natural decrease in muscle tone is observed when recording EMG activity under continuous anaesthesia and 100% \( \text{O}_2 \). This decrease in muscle tone is most apparent when observing phasic GG activity because of its high basal tone level. We found that after 40 minutes of recording, phasic GG activity significantly decreased below initial baseline levels (\( n = 6 \); 1-Way ANOVA on ranks; \( p < 0.001 \)) (Figure 6-2 A-B). Because of this change in muscle that is independent of pharmacological intervention, we developed our protocol to control for these changes. Our analysis uses 5 minutes prior to any injection as the baseline muscle tone and the following 20 minutes is the duration of time during which we record any changes due to injection. The same protocol is followed
when control vehicle is injected. In this manner, we constrict our analysis to a period of time during which changes due to our experimental model are controlled for and do not influence our results.
Figure 6-1: Microinjection of control into the Hcrt field has no effect on muscle tone. A: Raw trace (i) and average group data (ii, n=8) depicting no effect on phasic genioglossus (GG) activity with control microinjection. B: Control microinjection had no effect on expiratory GG activity as depicted by (i) raw trace and (ii, n=8) average group data. C: Raw trace (i) and average group data (ii, n=8) depicting no effect on masseter muscle tone with control microinjection. Group data are expressed in arbitrary units +/- s.e.m.
Figure 6-2: Phasic GG activity decreased over the duration of control recording. A: Representative raw trace of the decrease in phasic GG activity over the duration of an experiment, such decrease is not observed in masseter tone. B: Average data (n=6) depicting the percent change in phasic GG activity at BL, 40, 60 and 80 minutes during recording. * Significantly different compared to baseline (p < 0.05). Group data are normalized and expressed as percentage change from baseline (100) +/- s.e.m.
CHAPTER 7:

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