Role for the red nucleus in motor control during REM sleep

by

Daniel W. Li

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

© Copyright by Daniel W. Li, 2015
Role for the red nucleus in motor control during REM sleep

Daniel Li

Master of Science
Cell and Systems Biology
University of Toronto
2015

ABSTRACT

During rapid eye movement (REM) sleep the brain exhibits activity similar to waking. Despite this activity, the motor system is forced into a state of paralysis called REM sleep atonia. Though muscle tone is highly suppressed, this atonia is punctuated by phasic twitches. Here, the red nucleus (RN), a predominantly glutamatergic motor center in the midbrain, is identified as a potential region involved in the control of REM sleep twitches. Using a pharmacogenic technique termed Designer Receptors Exclusively Activated by Designer Drugs (DREADDs), the RN was stimulated during REM sleep to determine its effect on muscle tone. I found that activation of the RN and its glutamatergic neurons increases muscle activity during REM sleep by increasing the number of phasic twitches. This increase disrupts the normal pattern of muscle activity seen during REM sleep. These results bring to light a new area of motor control during REM sleep.
ACKNOWLEDGEMENTS

First and foremost, I would like to thank my supervisor and mentor, Dr. John Peever. The lessons that I have acquired from you will never be forgotten. Thank you for all of your guidance and wisdom.

I would also like to acknowledge my committee members, Dr. John Yeomans, Dr. Kaori Takehara, and Dr. Les Buck. Thank you for all the advice and expertise you have given me in shaping my thesis and project over the past two years.

To my lab family and good friends, Victoria Cheung, Dr. Jimmy Fraigne, Wesley Graham, Jennifer Lapierre, Simon Liu, Dillon McKenna, Hinal Patel, Paul Sanghera, Siyao Shi, Dr. Peter Schwarz-Lam, Matthew Snow, Zoltan Torontali, Dr. Nicole Yee, and Wendy Xie. I have been blessed to work with a brilliant group of wonderful people and wish you all the best. I will miss every one of you dearly.

Thank you to my Mom, Dad and sister who have supported me every step of the way. Despite my shortcomings and failures, they were always there to pick me up and push me to be my best.

A special shoutout to my friends NLP, KY, and LD. Thank you for taking me out and listening to me stress whenever I needed. I am forever grateful.

Lastly, to my partners in crime, Sharshi Bulner and Choden Shrestha. No words can express how appreciative I am to the two of you. Thank you for always being there, no matter what the circumstance. I finally made it!
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>iii</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF FIGURES AND TABLES</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>ix</td>
</tr>
</tbody>
</table>

## CHAPTER ONE: INTRODUCTION

1.1 Overview 1

1.2 REM Sleep Generation and Motor Control 2

1.3 REM Sleep Twitches 9

1.4 Red Nucleus and Phasic Activity 12

1.5 DREADDs 15

1.6 Thesis Objectives and Hypothesis 16

## CHAPTER TWO: MATERIALS AND METHODS

2.1 Animals 18

2.1.1 *Mice* 18

2.2 Surgical Protocol 19

2.2.1 *Stereotaxic injection of mice* 19

2.2.2 *Instrumentation of mice* 19

2.3 Sleep Recording and Procedures 20
2.3.1 Recording Environment 20
2.3.2 Data acquisition 20
2.3.3 Sleep recording 21

2.4 Data analysis 22
2.4.1 Sleep scoring 22
2.4.2 EMG analysis 22
2.4.3 Analysis of phasic activity 23

2.5 Statistical Analysis 24
2.5.1 EMG and phasic twitch analysis 24
2.5.2 Temporal phasic twitch analysis 24

CHAPTER THREE: ACTIVATION OF THE RED NUCLEUS INCREASES MUSCLES ACTIVITY DURING REM SLEEP

3.1 Cre-independent activation of all cell types in the red nucleus increases overall muscle activity during REM sleep 25

3.1.1 Injection of 2.5 mg/kg CNO and 5 mg/kg CNO produce similar results in the masseter and neck muscles 26

3.2 Phasic activity during REM sleep is increased with activation of all cell types in the red nucleus 32

3.3 The temporal pattern of twitch activity changes with cre-independent RN activation 34

3.4 Injection of AAV-GFP does not affect muscle activity or sleep-wake states 37
CHAPTER FOUR: STIMULATION OF THE GLUTAMATERGIC NEURONS IN RED NUCLEUS USING A CRE-DEPENDENT STRATEGY INCREASES MUSCLE ACTIVITY DURING REM SLEEP

4.1 Activation of glutamatergic RN cells using a cre-dependent strategy increases overall muscle activity during REM sleep

4.2 Phasic activity during REM sleep increases with cre-dependent activation of glutamatergic RN cells

4.3 The temporal pattern of twitch activity changes with activation of the glutamatergic neurons in the RN using a cre-dependent strategy

CHAPTER FIVE: DISCUSSION

5.1 Red nucleus influences during REM sleep

5.2 Excitatory and inhibitory inputs during REM sleep

5.3 Temporal regulation of REM sleep phasic activity

5.4 Hypothesized model of REM sleep motor control and REM sleep behaviour disorder

CHAPTER SIX: SUMMARY AND FUTURE DIRECTIONS

REFERENCES
# LIST OF FIGURES AND TABLES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Muscle tone across sleep-wake behaviours</td>
<td>1</td>
</tr>
<tr>
<td>1.2</td>
<td>Hypothesized circuitry responsible for generation of REM sleep and motor control</td>
<td>7</td>
</tr>
<tr>
<td>1.3</td>
<td>Hypothesized circuitry for phasic motor control during REM sleep</td>
<td>8</td>
</tr>
<tr>
<td>1.4</td>
<td>Phasic increase in RN activity during rapid eye movements</td>
<td>14</td>
</tr>
<tr>
<td>1.5</td>
<td>RN neuron inhibition during electrical and chemical stimulation of the GiG</td>
<td>14</td>
</tr>
<tr>
<td>1.6</td>
<td>Activation of secondary messenger cascades using excitatory hM3DGq DREADDs</td>
<td>16</td>
</tr>
<tr>
<td>2.1</td>
<td>Experimental time line and protocol</td>
<td>21</td>
</tr>
<tr>
<td>3.1</td>
<td>Immunohistological verification of cre-independent hM3DGq expressing neurons within the RN</td>
<td>27</td>
</tr>
<tr>
<td>3.2</td>
<td>Neurons expressing cre-independent hM3DGq are located within and around the area of the RN</td>
<td>28</td>
</tr>
<tr>
<td>3.3</td>
<td>Activation of the RN using a cre-independent strategy increases number of twitches during REM sleep</td>
<td>29</td>
</tr>
<tr>
<td>3.4</td>
<td>Muscle activity increases with cre-independent RN activation</td>
<td>30</td>
</tr>
<tr>
<td>3.5</td>
<td>Muscle activity remains constant during NREM sleep and waking with cre-independent RN activation</td>
<td>31</td>
</tr>
<tr>
<td>3.6</td>
<td>Frequency of twitch activity increases with cre-independent strategy of RN activation</td>
<td>33</td>
</tr>
<tr>
<td>3.7</td>
<td>RN activation using a cre-independent strategy regulates the distribution of muscle twitches during REM sleep</td>
<td>36</td>
</tr>
</tbody>
</table>
Figure 3.8. Injection of AAV-GFP control does not affect muscle activity

Figure 3.9. Injection of CNO does not affect sleep-wake behaviour or REM sleep

Figure 4.1 Immunohistological verification of glutamatergic, cre-dependent hM3DGq expressing neurons within the RN

Figure 4.2. Neurons expressing cre-dependent hM3DGq are located within and around the area of the RN

Figure 4.3. Activation of the glutamatergic cells in the RN using a cre-dependent strategy increases number of twitches during REM sleep

Figure 4.4. Activation of glutamatergic neurons using a cre-dependent DREADD in the RN increase muscle activity during REM sleep

Figure 4.5. Muscle activity remains constant during NREM sleep and waking with activation of glutamatergic cells in the RN

Figure 4.6. Frequency of phasic activity increases with activation of glutamatergic neurons in the RN

Figure 4.7. Activation of glutamatergic neurons in the RN affects the temporal pattern of twitches during REM sleep

Figure 5.1. Hypothesized model responsible for generation of REM sleep twitches and RBD
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAV</td>
<td>adeno-associated virus</td>
</tr>
<tr>
<td>a.u.</td>
<td>arbitrary units</td>
</tr>
<tr>
<td>AW</td>
<td>active wake</td>
</tr>
<tr>
<td>CNO</td>
<td>clozapine-N-oxide</td>
</tr>
<tr>
<td>CNQX</td>
<td>6-cyano-7-nitroquinoxaline-2,3-dione</td>
</tr>
<tr>
<td>Cre</td>
<td>cre-recombinase</td>
</tr>
<tr>
<td>CTb</td>
<td>cholera toxin subunit B</td>
</tr>
<tr>
<td>dDPM Me</td>
<td>dorsal deep mesencephalic nucleus</td>
</tr>
<tr>
<td>DLPT</td>
<td>dorsolateral pontine tegmentum</td>
</tr>
<tr>
<td>DPGi</td>
<td>dorsal paragigantocellular nucleus</td>
</tr>
<tr>
<td>DREADDs</td>
<td>Designer Receptors Exclusively Activated by Designer Drugs</td>
</tr>
<tr>
<td>DRN</td>
<td>dorsal raphe nucleus</td>
</tr>
<tr>
<td>EEG</td>
<td>electroencephalogram</td>
</tr>
<tr>
<td>EMG</td>
<td>electromyogram</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GiG</td>
<td>gigantocellular reticular nucleus</td>
</tr>
<tr>
<td>Hz</td>
<td>hertz</td>
</tr>
<tr>
<td>kHz</td>
<td>kilohertz</td>
</tr>
<tr>
<td>kg</td>
<td>kilogram</td>
</tr>
<tr>
<td>LC</td>
<td>locus coeruleus</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>LDT</td>
<td>laterodorsal tegmental nucleus</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>mm</td>
<td>millimeter</td>
</tr>
<tr>
<td>QW</td>
<td>quiet wake</td>
</tr>
<tr>
<td>RBD</td>
<td>REM sleep behaviour disorder</td>
</tr>
<tr>
<td>REM</td>
<td>rapid eye movement</td>
</tr>
<tr>
<td>RN</td>
<td>red nucleus</td>
</tr>
<tr>
<td>s</td>
<td>seconds</td>
</tr>
<tr>
<td>NREM</td>
<td>non-rapid eye movement</td>
</tr>
<tr>
<td>PCRt</td>
<td>parvocellular reticular nucleus</td>
</tr>
<tr>
<td>PGO</td>
<td>ponto-geniculo-occipital</td>
</tr>
<tr>
<td>PMnR</td>
<td>paramedian reticular area</td>
</tr>
<tr>
<td>PPT</td>
<td>pedunculopontine tegmental nucleus</td>
</tr>
<tr>
<td>SLD</td>
<td>sublaterodorsal nucleus</td>
</tr>
<tr>
<td>Sub-C</td>
<td>subcoeruleus</td>
</tr>
<tr>
<td>Vglut2</td>
<td>vesicular glutamate transporter 2</td>
</tr>
<tr>
<td>vlPAG</td>
<td>ventrolateral peri-aqeductal grey</td>
</tr>
<tr>
<td>WT</td>
<td>wildtype</td>
</tr>
</tbody>
</table>
CHAPTER ONE: INTRODUCTION

1.1 Overview

This thesis examines the neurochemical mechanisms and brain regions that mediate muscle control and muscle twitches during REM sleep. Muscle activity is highly regulated during sleep where muscle tone decreases from wake into non-rapid-eye-movement (NREM) sleep. This muscle tone is even further suppressed during rapid eye movement (REM) sleep where the muscles enter a state termed REM sleep atonia. Though muscle tone is highly suppressed in this state, this atonia is often punctuated by the appearance of brief phasic muscle twitches (Jouvet, 1967) (Figure 1.1). The purpose and generation of these twitches have still yet to be determined and remain very unclear.

![Graph showing muscle tone across sleep states](image)

**Figure 1.1 Muscle tone across sleep-wake behaviours.** Electroencephalogram and electromyogram recordings of wake, NREM sleep, and REM sleep states in a mouse. It is important to note the disturbance in REM sleep atonia by the punctuation of phasic muscle activity in both masseter and neck muscles.

REM sleep atonia was first described in 1959 by Jouvet et al. where it was determined that though muscle tone is at its lowest and most suppressed in this state, superimposed on this atonia are phasic muscle twitches. Muscle activity in REM sleep can be separated into two main distinct forms of activity; the tonic suppression of muscle tone and brief periods of phasic muscle
activation. Despite it being 60 years since the first description of REM atonia, the mechanisms behind the generation and maintenance of this muscle control still remain unknown. This gap in our understanding of motor activity in REM sleep leads to promising avenues of study, and thus, it is this phasic activation of muscles during REM sleep that is the main focus of this thesis.

1.2 REM Sleep Generation and Motor Control

In order to understand the mechanisms that mediate the muscle twitches during REM sleep, it is important to gain an understanding of the circuitry that underlies the generation of REM sleep. Though mechanisms behind the motor control of REM sleep twitches are not yet known, much work has been done to elucidate the areas and circuitry that govern the entry and maintenance of the REM sleep state (Jouvet, 1962; Siegel et al., 1986; Siegel et al., 1983; Boissard et al., 2002; Lu et al., 2006). Through the use of transections, lesions, unit recordings, and electrical and chemical stimulation, the circuitry involved with the regulation and generation of REM sleep and REM sleep atonia is becoming quite well-defined.

Areas involved in the entrance into REM sleep and the generation of atonia were first defined and localized in the brainstem through transection studies in the cat. Jouvet pioneered this work demonstrating that, in cats, ponto-mesencephalic transections did not abolish REM sleep atonia (Jouvet, 1962). In fact, it was shown that normal REM sleep with atonia still occurred after the forebrain was isolated from the brainstem. Other studies confirmed these findings and further added to knowledge of REM sleep circuitry by demonstrating that caudal pontine-rostral medullary transections abolished REM sleep atonia (Jouvet, 1962; Siegel et al., 1986; Webster et al., 1986). From these findings, it was concluded that the areas essential for REM sleep were located primarily in the rostral pons while those responsible for the generation of REM sleep
atonia were found in the medial medulla (Siegel et al., 1983). These studies not only determined key areas in REM sleep circuitry, but added to the understanding that interactions between the pons and medulla were essential in generating muscle tone suppression and inhibition.

In light of the findings from transections, lesioning studies were carried out to define the critical regions responsible for REM sleep and motor control with more specificity. It was discovered that lesions in the area of the dorsal rostral pons eliminated motor inhibition during REM sleep while leaving all other signs intact, supporting results from transection studies. (Henley and Morrison, 1974; Jouvet and Delorme, 1965). Subsequent research identified an area termed the subcoeruleus in the pons and implicated its role in the generation of REM sleep and atonia (Hendricks et al., 1982). These findings were also confirmed in rats, as lesions of an equivalent pontine region, the sublaterodorsal nucleus (SLD) produced results similar to those seen in cats; REM sleep without atonia (Boissard et al., 2002; Lu et al., 2006). Taken together, these studies extend the earlier transection work demonstrating the dorsolateral pons and medial medulla are key elements in the regulation of muscle tone and the generation of REM sleep. They also implicate a new area in the pons, the subcoeruleus, which may be responsible for REM sleep generation.

Studies using transection and lesioning techniques to identify brain regions important for REM sleep then lead to research into the chemical nature of critical regions and the neurotransmitters involved in REM sleep through stimulation studies. One longstanding hypothesis in REM sleep generation is that entrance and maintenance of this state is through a cholinergic mechanism. This theory stems from research, in cats, showing that microinjections of carbachol, a cholinergic agonist, into the pontine region produced a state similar to REM sleep with atonia (George et al.,
1964; Van Dongen et al., 1978). On the other hand, though a cholinergic hypothesis exists, other research supports a glutamatergic mechanism of REM sleep generation (Boissard et al., 2003; Luppi et al., 2011; Lu et al., 2006). Indeed, it has also been shown that the structures thought to be involved in REM sleep, such as the subcoeruleus, are glutamatergic in nature (Boissard et al., 2002; Lai and Siegel, 1988). Thus, it may be that the control of REM sleep and its corresponding motor activity are affected by both a cholinergic and glutamatergic mechanism. Despite the debate on which neurotransmitters are involved, these studies collectively reveal that key structures in the pontine and medullary regions are responsible for the generation of REM sleep and its corresponding motor atonia.

As these pontine structures seem to be conserved across species, it has been suggested that, in mice, an area in the pons termed the subcoeruleus or Sub-C is responsible for gating the entry into REM sleep and producing REM sleep atonia (Luppi et al., 2011). Further research into this area in rats adds further support to the hypothesis that the Sub-C is involved in triggering REM sleep. Studies applying bicuculline or gabazine, two GABA receptor antagonists, to the Sub-C report the entrance into a REM like sleep state following application of the drugs (Pollack and Mistlberger, 2003; Sanford et al., 2003). Further retrograde tracing studies with CTb injected into the Sub-C showed GABAergic projections from the ventrolateral peri-aqeductal grey (vlPAG) and dorsal deep mesencephalic nucleus (dDPMe) to the Sub-C (Boissard et al., 2003). From these studies, it is thought that, during waking conditions, glutamatergic neurons in the Sub-C are actively inhibited by the vlPAG and dDPMe (Sapin et al., 2009). In order to enter REM sleep, it is hypothesized that wake promoting areas such as the locus coeruleus (LC), the vlPAG and dDPMe are inhibited by areas such as the dorsal paragigantocellular nucleus (DPGi). This then leads to the disinhibition of the Sub-C and subsequent entrance into a REM sleep state. A tonic
glutamatergic drive is hypothesized to activate neurons in the Sub-C which project to and stimulate areas in the medial medulla such as the gigantocellular reticular nucleus (GiG). Indeed, in 2006, Lu et al. reported, through studies with c-fos, a neurological marker of activity, and vglut-2 immunohistochemistry, a marker for glutamatergic neurons, that the area of the Sub-C is glutamatergic in nature. Furthermore, Kodama et al. in 1998 show an increase in glutamate release in the medial medulla during REM sleep. It is hypothesized that the GiG directly inhibits cranial and spinal motoneurons through GABAergic and glycinergic mechanisms causing the motor atonia that is characteristic of REM sleep (Figure 1.2). It has previously been reported that the GiG is a key area for the generation of muscle atonia as lesions of this area produce a REM sleep state without atonia (Lu et al., 2006). It has also been shown that greater than 50% of the GABA/glycinergic neurons in the GiG project directly onto spinal motor neurons (Holstege and Bongers, 1991; Kato et al., 2006). As such, these studies support the hypothesis that the Sub-C and GiG are key areas in the generation of REM sleep and motor control during these states.

Although it is hypothesized that REM sleep atonia is due to GABAergic or glycinergic mechanisms hyperpolarizing motor neurons (Chase et al., 1980; Glenn and Dement, 1981; Kodama et al., 2003), multiple lines of evidence have indicated that this atonia is a result from a disfacilitation of excitatory inputs onto motor neurons (Fenik et al., 2005; Lai et al. 2001; Kubin et al., 1993; Chan et al., 2006). Studies have shown microinjections of bicuculline, a GABA receptor antagonist, has no effect on motor atonia in the hypoglossal motor nucleus during induced REM sleep (Kubin et al., 1993). Further evidence using another GABA/glycine antagonist, strychnine, has demonstrated that REM sleep atonia in the hypoglossal motor nucleus still persists in the absence of GABAergic and glycinergic inhibition (Morrison et al., 2003). Instead, it has been shown that during REM sleep, neurotransmitter levels of serotonin and
noradrenaline are decreased in motor pools which could contribute to the lack of muscle activity seen during this state (Jacobs and Azmitia, 1992; Kubin et al., 1994). Indeed, Fenik et al. (2005) showed that antagonism of noradrenergic and serotonergic inputs together caused no depression of hypoglossal nerve activity during carbachol induced REM sleep, providing support for this hypothesis of disfacilitation. However, no reports to date have succeeded in reversing the phenomenon of REM sleep atonia through application of excitatory neurotransmitters onto motor pools (Burgess et al., 2008; Sood et al., 2005; Jelev et al., Mir et al., 2006). Thus, it may be that a combination of both inhibition and disfacilitation are important to the phenomenon of REM sleep atonia (Figure 1.3). Despite these extensive studies, the generation and maintenance of the phasic activity seen in REM sleep has largely been overlooked and still remains unclear. It is, thus, important to understand the control of muscle activity during this sleep state to add to the current circuit and shed more light on this motor phenomenon.
Figure 1.2. Hypothesized circuitry responsible for generation of REM sleep and motor control. A) During wake, GABAergic neurons in the vIPAG and dDPMe inhibit the Sub-C glutamatergic neurons. B) In REM sleep, glutamatergic neurons in the Sub-C are disinhibited, generating muscle atonia through descending projections to the GiG.
Figure 1.3. Hypothesized circuitry for phasic motor control during REM sleep. A) During wake, motor neurons receive excitatory inputs from numerous regions such as the dorsal raphe nucleus, the LC, and the RN. B) In REM sleep, there is a disfacilitation of excitatory inputs. Concurrently, the disinhibition of the Sub-C activates GABA/glycinergic projections in the GiG leading to a further inhibition of motor neurons.
1.3 REM Sleep Twitches

Muscle activity during REM sleep is characterized not only by a suppression of muscle tone, but also by phasic activation of the muscle that occurs in the form of muscle twitches. It has more recently been suggested that these twitches are essential in the development of organisms in order to fine tune and learn the motor system (Blumberg et al., 2013a). It has been hypothesized that this twitch activity during REM sleep plays a critical role in development of the sensorimotor nervous system in newborns and infants (Blumberg, 2010). As twitches are imposed against a background of motor atonia, it is thought that the nervous system has an easier time making connections and establishing relationships between the motor signal that triggers a twitch and the feedback arising from that twitch (Blumberg et al., 2013b). In this way, it is believed that babies fine tune their nervous systems and lay a foundation for the everyday movements performed during wake. Still, REM sleep twitches remain quite frequent in adults, though not as frequently seen in newborns, and thus, it is difficult to know whether the adolescent or adult brain experiences the same sensory feedback as seen in infants (Blumberg et al., 2013a; Blumberg et al., 2013b). Therefore, it is important to continue research into these movements as the mechanisms surrounding their generation and causes are still poorly understood.

Numerous studies investigating the regulation of muscle control during REM sleep normally overlook the temporal pattern of muscle twitches and treat this state as homogenous (Chase et al., 1989; Lai et al., 2001; Soja et al., 1991). As such, little is known of the causes and regulation of the muscle activity during REM sleep. Previous studies of REM sleep events such as rapid eye movements (Aserinsky, 1971; Salzarulo, 1972), EEG power (Takahara et al., 2006), and ponto-geniculo-occipital (PGO) waves (Marks et al., 1980) have reported a temporal pattern of activity
during REM sleep durations. Thus, it follows that muscle twitches are not uniformly distributed and have a temporal pattern across REM sleep periods. Previous work by Brooks and Peever (2011) showed an uneven distribution of phasic motor activity in the masseter muscle in which twitch frequency progressively increases towards the latter half of REM sleep durations. This temporal pattern of twitches is regulated through inhibitory glycinergic and GABAergic inputs that oppose excitatory glutamatergic inputs onto motor neurons to suppress muscle activity. It has been shown that REM sleep motor twitches are controlled by a glutamatergic mechanism, as antagonism of trigeminal glutamate receptors with CNQX significantly reduced the number of twitches seen during REM sleep (B Burgess et al., 2008). Collectively, these data suggest the potential involvement of a glutamatergic area that can break through the inhibition and atonia of this state to generate muscle twitches during REM sleep. Recent evidence from Anaclet et al. (2010) has hypothesized medullary areas termed the parvocellular reticular nucleus (PCRt) and paramedian reticular area (PMnR) to be responsible for the generation of phasic activity in the masseter muscles. They show that through both cell specific lesions of these areas and blocking glutamate release, there was a reduced amount of phasic activity during REM sleep in the masseter. More research from Karlsson et. al (2005) has implicated another region, the laterodorsal tegmental nucleus (LDT), in the modulation and production of phasic activity during REM sleep. They show through electrophysiological recordings that the LDT contains ‘twitch on’ neurons that exhibit action potentials that precede phasic twitches. Further analysis showed electrolytic lesions of the dorsolateral pontine tegmentum (DLPT), which contains the region of the LDT, significantly reduced the number of twitches seen during REM sleep. This research supports other experiments in cats by Shouse and Siegel (1992) that demonstrate lesions of the pedunculopontine tegmental nucleus (PPT), a region nearby to the LDT, resulted in a loss of twitch activity. These studies imply that numerous areas, such as the PCRt, LDT, and PPT, may
be involved in the generation of phasic activity, yet the upstream sites responsible for the eliciting and timing of this activity remain to be determined. As well, the brain regions and neural circuits responsible for generating twitches in other cranial and postural muscles are unknown (Vetrivelan et al., 2011). It, thus, remains important to elucidate the complex mechanisms and circuitry underlying this phasic activation.

Determining the mechanisms mediating phasic activation during REM sleep can be of major clinical importance as abnormal motor control during REM sleep and the over-exaggeration of twitch activity underlies a major sleeping disorder, REM sleep behaviour disorder (RBD) (Schenk and Mahowald, 2002; Schenck et al. 1988; Olson et al., 2000). Disruption of regular muscle control during REM sleep can lead to abnormal and harmful behaviours such as those exhibited in patients with RBD. These patients exhibit a loss of motor atonia during REM sleep in the form of elaborate motor behaviours, often enacting dreams and harming themselves or others around them (Schenck and Mahowald, 2002; Sfoza et al., 1997). It is hypothesized that it is the disruption of REM sleep motor circuitry that is the cause of this disorder (Siegel et al., 1991). In fact, neuroimaging data from RBD patients have shown that these patients contain damage in the medial medulla or brainstem which is hypothesized to cause the overt motor behaviours seen in REM sleep (Boeve et al., 2007). In order to advance our understanding of the complex mechanisms surrounding REM sleep motor control, it is important to determine the regions that control phasic activity during REM sleep. This can lead to valuable insights into the generation and maintenance of normal motor control and the effects of a dysfunctional REM sleep circuit. By understanding the brain regions that control twitch activity, effective treatments targeting important areas that modulate REM sleep motor activity can be found.
1.4 Red Nucleus and Phasic Activity

While many studies have focused on the control and maintenance of REM sleep atonia, the phasic aspect of muscle activity during REM sleep has received much less attention. Areas involved in the generation of twitch activity remain unknown yet evidence supports the role of the red nucleus (RN) in the involvement of phasic activity during REM sleep. The red nucleus is a predominantly glutamatergic motor center located in the midbrain that has been previously shown to function in the control of muscle movement and acts as a relay center for information from the motor cortex to the cerebellum (Hicks and Onodera, 2012; Massion, 1988). The RN has been shown to mainly function in the motor coordination and control of distal limbs in posture, locomotion, gait, and reflex (Massion, 1967; Hicks and Onodera, 2012). Electrophysiological stimulation of this area was found to evoke discharges of action potentials to motor neurons in the dorsolateral cervical and lumbar regions of the spinal cord. Further anterograde tracing experiments have shown rubrospinal fibres terminating in the laminae at all spinal cord levels, especially at motor neurons that control distal limbs such as the hands and feet (Liang et al., 2012). In addition, stimulation sends signals along projections to the inferior olivary nucleus which acts with the cerebellum to control and coordinate movements (Horn et al., 1998). The RN is important for the regulation of movement and, thus, has been shown to be relatively silent during sleep in order to maintain REM sleep paralysis (Gassel and Pompeiano, 1966).

Although relatively silent, in 1966, Gassel and Pompeiano recorded cells from the RN in cats during periods of REM sleep and discovered an increase in RN activity that preceded the brief periods of phasic muscle activity. Additional lesioning studies of the RN resulted in a decrease, albeit a transient one, of the muscle twitching seen during REM sleep (Figure 1.4). These
experiments provided the foundation for further experiments into the RN as a possible generator of phasic activity during REM sleep.

More evidence supports the role of the RN in the generation of muscle twitches as a decrease in RN activity is seen with chemical or electrical stimulation of the GiG and DPGi, two important areas for muscle inhibition in REM sleep circuity. Using electrical stimulation or kainic acid, a glutamate agonist, into the GiG and DPGi, Mileykovskiy et al. (2002) discovered decreased activity in the RN and a corresponding inhibition of motor function in numerous limbs (Figure 1.5). This research implies a possible inhibitory projection from the GiG to the RN that is activated during REM sleep. As the GiG is hypothesized to be responsible for the generation of REM sleep atonia, it follows that there is an inhibitory projection to the RN and its corresponding projections to motor neurons. Taken together, these studies suggest a possible role for the involvement of the RN in REM sleep circuitry and in the generation of REM sleep twitches.

In this thesis, both the masseter and neck muscles were studied to determine how stimulation of the RN affects muscle activity during REM sleep. Projections to both of these muscles from the RN have been characterized in previous studies. Reciprocal connections to the trigeminal sensory complex from the RN, which in turn innervates the masseter muscle, have been characterized (Godefroy et al., 1998). Similarly, projections from the RN to cervical sections of the spinal cord, which are responsible for neck flexors and extensors, have been characterized (Brown, 1974; Liang et al., 2012). Thus, analysis of changes in activity of these two muscles during REM sleep could lead to important insights into the functional role of the RN during this state.
Figure 1.4. **Phasic increase in RN activity during rapid eye movements.** Intracellular recordings of the RN shows an increase in activity correlated with myoclonic twitches and rapid eye movements in unrestrained, unanaesthetized cats. (Adapted from Gassel et al. 1966)

Figure 1.5. **RN neuron inhibition during electrical and chemical stimulation of the GiG.** Stimulation of the GiG leads to a decrease in RN activity and corresponding decreases in EMG activity of limb muscles. (Adapted from Mileykovskiy et al. 2002)
1.5 DREADDs

While past research supports the role of the RN in the generation of twitches, its functional role during REM sleep has not yet been tested. Thus, the purpose of this thesis was to determine the role of the RN during REM sleep through pharmacogenetically stimulating the area and determining its effects on REM sleep atonia. To do this, a novel technique termed Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) was used. This technique provides the benefits of a reversible, less invasive procedure of controlling neuronal signaling. Whereas electrolytic lesions and cell recordings are less specific and can destroy fibres of passage in the area, DREADDS allows for highly specific, reversible control with very good spatial resolution. DREADDs are engineered, mutated G-protein coupled receptors that are activated solely by an inert synthetic ligand, clozapine-N-oxide (CNO) (Alexander et al., 2009). For this thesis, the excitatory DREADD hM3DGq was used to stimulate the RN. These mutated M3-muscarinic receptors are coupled to the Gq pathway and mediate neuronal signalling through inositol phosphate hydrolysis, calcium release and extracellular signal-regulated kinase activation in order to depolarize neurons and elicit action potentials (Rogan and Roth, 2011) (Figure 1.6). For the purpose of this thesis, two separate DREADD strategies were used; a cre-independent and a cre-dependent strategy. In order to stimulate all cells in the area of the RN a cre-independent strategy was used. Using this, we can determine whether any cells in the RN play a role in muscle twitches during REM sleep. As twitches have previously been reported to be mediated by a glutamatergic mechanism (Burgess et al., 2008), further research of the RN led us to use a cre-dependent strategy in an attempt to target specifically glutamatergic neurons in this area. In this way, it is possible to excite the area of the RN and determine how it affects muscle activity during REM sleep.
Despite extensive research into the circuitry and mechanisms that control REM sleep and motor control, much remains to be discovered regarding the phasic phenomena seen during this state. Several lines of evidence have implicated the RN, a predominantly glutamatergic nucleus in the midbrain, as a potential area for the generation and control of REM sleep twitches. Through electrophysiological recordings of this region, it has been established that there is an increase in activity preceding phasic events. Further lesions of the RN have been reported to result in a decrease in twitch activity (Gassel and Pompeiano, 1966). It has also been hypothesized that the RN may be involved with important areas of REM sleep circuitry and motor control such as the GiG and DPGi (Mileykovskiy et al., 2002). Further research into twitch activity has established that twitches are mediated through a glutamatergic mechanism (Burgess et al., 2008). While evidence supports the role of the RN in the generation of phasic activity during REM sleep, its functional role during this state has not yet been tested. The objective of this thesis is to elucidate...
a functional role for the RN during REM sleep and determine its contributions to phasic muscle activation. I hypothesize that the RN is responsible for mediating REM sleep phasic activity and expect that stimulation of this area during REM sleep would cause an increase in motor activity during this state.

To first determine whether any cells in the RN influence phasic activity in REM sleep, I use a cre-independent DREADD strategy to target the RN. Using this technique, all cells in the RN will express the DREADD receptor and be activated with the use of CNO. Following confirmation of a physiological response to stimulation of all cells in the RN, I then target glutamatergic neurons in the RN using a cre-dependent DREADD strategy in a transgenic line of mice, Vglut2-cre. In this line of mice, all cells that contain the vesicular glutamate transporter vglut-2 also contain the enzyme cre-recombinase. Using a cre-dependent DREADD will target all cells that contain cre-recombinase, thus, in theory, allowing glutamatergic neurons to express the excitatory DREADD receptor. Both masseter and neck muscles will be studied during REM sleep which allows for comparisons to be made across different cranial and spinal motor pools and gives a wider scope of changes in motor neuron activity. It is expected that stimulation of the RN through the use of the DREADD technology will increase muscle activity in the form of twitches during REM sleep.
CHAPTER TWO: MATERIALS AND METHODS

2.1 ANIMALS

This research was conducted on a total of 21 adult male mice from two different lines, wildtype and Vglut2-cre mice (Vong et al., 2011). All of the work performed was on freely moving, naturally sleeping animals. All procedures and protocols were approved by the University of Toronto’s animal care committee. Animals were cared for under the supervision of the staff in the Bioscience Support Facility. Mice were group housed with up to five same-sex siblings on cob bedding in plastic cages and maintained on a 12:12 light dark cycle at a temperature and humidity of 22°C and 50%, respectively. Food and water were readily available at all times.

2.1.1 Mice

Chapter 4 used a C57BL/6 wildtype mouse line in order to activate all neurons within the area of the RN. These mice are used in a non-specific, cre-independent DREADD strategy in which all cell types in the RN are targeted to determine if activation of any and all cells in the RN influences REM sleep motor activity.

In Chapter 5, experiments were performed on a transgenic mouse line maintained on a C57BL/6 background. In order to activate glutamatergic neurons, these mice contain the enzyme cre-recombinase in all cells that contain the vesicular glutamate transporter, Vglut-2 (Vong et al., 2011). These mice are used for a cre-dependant glutamatergic neuron specific DREADD strategy in which the glutamatergic cell populations in the RN are targeted for the expression of the DREADD receptor. In this way, we will determine whether the glutamatergic cell population in the RN plays a role in phasic activity.
2.2 SURGICAL PROTOCOL

Sterile surgery was performed in order to inject and instrument mice for sleep-wake recordings.

2.2.1 Stereotaxic injection of mice

Mice used in Chapter 4 were injected with the virus AAV8-hM3DGq-hsyn-mcherry while those used in Chapter 5 were injected with a cre-dependant virus, AAV8-hM3DGq-DIO-mcherry. Controls were injected with AAV5-eGFP in WT mice and AAV8-DIO-mcherry in Vglut2-cre mice (Blits et al., 2010).

To inject mice, animals were anesthetized using isoflurane (1-2%) and positioned into a stereotaxic apparatus. Cannulas were lowered into the area of the red nucleus at coordinates 3.6 mm dorsal, 3.5 mm posterior ± 0.5mm lateral to bregma. Virus was then injected at a rate of 50nL/min for 2-3 minutes. After injection, the cannula was left in the region for 8-10 minutes before removal.

Post-surgery, mice were given ketoprofen (3mg/kg) and individually housed in a plastic cage on a 12:12 light-dark cycle for 10-14 days. High calorie food pellets and a dietary supplement (i.e. Nutri-Cal) were given a day post operation.

2.2.2 Instrumentation of mice

10-14 days post injection, mice were surgically instrumented with EEG, masseter, and neck electrodes in order to record muscle tone across sleep-wake behaviours.

To instrument mice, animals were again anesthetized using isoflurane (1-2%) and positioned into the stereotaxic apparatus. EEG and EMG electrodes were attached to a micro-strip connector and fixed to the skull with the use of Ketac dental cement. EEG signals were obtained using four
micro-screws at positions 1 mm anterior ± 1.5 mm lateral to bregma and 3 mm posterior ± 1.5 mm lateral to bregma. EMG electrodes were made with the use of micro stainless steel wire (AS 632 Cooner Wire) and sutured into the right masseter muscle and neck muscle.

Post-surgery, mice were given ketoprofen (3 mg/kg) and individually housed in a plastic cage on a 12:12 light-dark cycle for 10-14 days. High calorie food pellets and a dietary supplement (i.e. Nutri-Cal) were given a day post operation. Mice recovered for at least 2 weeks before experimental testing began.

2.3 SLEEP RECORDING AND PROCEDURES

2.3.1 Recording Environment

During experiments, mice were habituated for a week and housed in a plastic chamber with regular cob bedding and a running wheel.

2.3.2 Data acquisition

EEG and EMG recordings were obtained through the use of a lightweight cable attached to the micro-strip connector on the animal’s head. The cable was then connected to a Super-Z head-stage amplifier and BMA-400 AC/DC Bioamplifier (CWE Inc.). EEG signals were amplified by 20 times and band-pass filtered between 1 and 100 Hz while EMG signals were amplified between 20-50 times and band-pass filtered between 30 Hz and 1 kHz. All signals were digitized (Spike 2 Software, 1401 Interface, CED Inc., Cambridge, UK) at 1000 Hz and stored on a computer for future analysis. Along with EEG and EMG recordings, infrared video recordings were captured and synchronized.
2.3.3 Sleep recordings

Two weeks post implantation of EEG and EMG electrodes, mice were tethered and transfer into recording cages. Animals were given a week to habituate to the environment, as well as given habituation injections of lactated ringers in the morning and evening at 9:30 am and 6:30 pm, respectively. After habituation, an undisturbed baseline recording of the mice was taken over 24 hours synchronized with video recordings. Following this baseline recording, mice are injected with different CNO doses of 0mg/kg (saline+DMSO), 2.5 mg/kg and 5 mg/kg over the course of 3 days (Farrell and Roth, 2012) (Figure 2.1). Each day mice received a different dose chosen at random to control for the effects of successive injections. As with the habituation injections, morning injections were given at 9:30am so that the drug may act during a time when propensity for REM sleep is high. Evening injections were given at 6:30pm in preparation for the dark cycle when mice turn active. Again, video is recording alongside EEG and EMG signals.

![Image of experimental timeline and protocol]

**Figure 2.1. Experimental time line and protocol.** Mice are injected with an adeno-associated virus into the area of the RN. Animals are then implanted with EEG and EMG electrodes two weeks post-injection. Following another two week period of recovery, animals are subjected to 3 days of randomized treatments and experimental recording.

<table>
<thead>
<tr>
<th>Day</th>
<th>Treatment*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Baseline Recording</td>
</tr>
<tr>
<td>1</td>
<td>5 mg/kg CNO</td>
</tr>
<tr>
<td>2</td>
<td>Vehicle (Saline)</td>
</tr>
<tr>
<td>3</td>
<td>2.5 mg/kg CNO</td>
</tr>
</tbody>
</table>

*treatments are randomized for each mouse
2.4 DATA ANALYSIS

2.4.1 Sleep scoring

Sleep recordings are segmented into 5 second epochs which are then classified into 4 behavioural states; active wake (AW), quiet wake (QW), non-REM sleep (NREM), and REM sleep. Each state is determined based on a criterion of frequencies and amplitudes of EEG and EMG. Active wake is characterized by high frequency low amplitude EEG activity coupled with high EMG activity while in quiet wake EMG tone is more moderate and low. NREM sleep is distinguished by high-amplitude, low frequency EEG signals with minimal EMG tone. Lastly, REM sleep is distinguished by low amplitude, high frequency theta-rich EEG signals with REM sleep atonia interrupted by periodic muscle twitches similar to criteria used to identify REM sleep in the cat lesion model (Henley and Morrison, 1974; Brooks and Peever, 2012). Files were analyzed for 3 hours post injection following a 30 minute grace period for the drug to take effect (Alexander et al., 2009).

2.4.2 EMG analysis

EMG recordings were full-wave rectified, integrated and quantified in arbitrary units. Average integrated EMG activity was quantified in 5 second epochs for each behavioural state 30 mins post injection. This is due to the fact that the drug, CNO, does not take effect until 30 minutes after injection. As well, it has been noted previously in mice that injections affect behaviour for an average of 30 minutes post injection (Burgess et al., 2010). Thus, files were analyzed 30 minutes after injection to obtain accurate data in freely moving and behaving animals.
2.4.3 Analysis of phasic activity

Muscle activity in REM sleep consists of two components: motor atonia during tonic REM sleep and the periodic muscle twitches that punctuate the atonia. Because manipulation of the red nucleus may have effects on the twitches during REM sleep, a method to identify and quantify the phasic activity was needed. In order to do so, the ten longest REM sleep periods in the first 3 hours post injection were identified and quantified in 10ms epochs. The first 5 seconds of a REM sleep episode where no muscle twitches occurred was then identified in order to differentiate between phasic and tonic activity. Within these 5 seconds, the 99\textsuperscript{th} percentile of EMG activity was calculated. This determined the value at which 99\% of the 500 epoch values (ie. 10ms epochs over 5s) fell below. This value was then used as a threshold to classify phasic activity during REM sleep. In the context of this thesis, muscles twitches were defined as motor activity that exceeded this threshold value.
2.5 STATISTICAL ANALYSIS

2.5.1 EMG and phasic twitch analysis

All statistical analysis used GraphPad Prism and applied a critical two-tailed alpha value of p<0.05. All data was analyzed to test if values followed a Gaussian distribution through a Kolmogorov-Smirnov test. If data was found to have normalized distributions, differences between overall EMG tone during wake, REM sleep, and NREM sleep under baseline (i.e. saline) and drug (i.e. 2.5 mg/kg and 5 mg/kg CNO) treatments were determined using a one-way analysis of variance with repeated measures (1 way RM-ANOVA). Student-Newman-Keuls was used for post hoc comparisons. Data not following a Gaussian distribution instead used a Friedman test with a Dunn’s comparison post hoc test.

Differences between twitch frequency, tonic activity, sleep-wake states, and REM sleep durations under baseline and drug conditions were determined using paired t-tests. Student-Newman-Keuls was used for post hoc comparisons. All data are expressed as mean ± standard error of the mean (SEM).

2.5.2 Temporal phasic twitch analysis

All statistical analysis used GraphPad Prism and applied a critical two-tailed alpha value of p<0.05. All comparisons made between baseline and drug treatment were determined using a two-way analysis of variance with repeated measures (2way RM-ANOVA). Bonferroni tests were used for post hoc comparisons. All data are expressed as mean ± standard error of the mean (SEM).
CHAPTER THREE: ACTIVATION OF THE RED NUCLEUS INCREASES MUSCLES ACTIVITY DURING REM SLEEP

3.1 Cre-independent activation of all cell types in the red nucleus increases overall muscle activity during REM sleep

Although the RN contains many cell types, a first step was to determine if activation of any and all cells in the RN influences motor activity during REM sleep. To do this, we used a cre-independent strategy to activate RN cells by driving expression of the hM3Dq DREADD virus in the RN of WT animals. Using this strategy, all cell populations in the RN would be activated with the use of CNO. If the RN does indeed play a functional role in the generation of REM sleep twitches, then activation of this area during REM sleep should affect muscle activity. After verification of viral expression and spread within the RN, mice were grouped and analyzed for changes in muscle activity (Figure 3.1, Figure 3.2). A total of 6 WT animals were injected with a cre-independent hM3Dq excitatory DREADD into the RN and monitored for changes in masseter and neck muscle activity during sleep and wake states. Data from the neck EMG signals of 3 animals was excluded due to electrical noise artifact. Intraperitoneal injection of both 2.5 mg/kg CNO and 5mg/kg CNO caused an overall increase in muscle activity during REM sleep states compared to an injection of saline (Figure 3.3). Masseter activity increased significantly by a magnitude of 51 ± 19% with 2.5mg/kg of CNO and 43 ± 12% with 5mg/kg of CNO (saline vs. 2.5mg/kg CNO vs. 5 mg/kg CNO, n=6, one-way repeated measure ANOVA, p= 0.0288), respectively during REM sleep (Figure 3.4). Neck muscle activity, though not significant, had a trend towards increasing muscle activity during REM sleep (saline vs. 2.5mg/kg CNO vs. 5 mg/kg CNO, n=6, one-way repeated measure ANOVA, p= 0.1475).

Although RN activation increased masseter muscle activity during REM sleep, this increase in
masseter activity was not seen in either NREM sleep states (saline vs. 2.5 mg/kg CNO vs. 5 mg/kg CNO, n=6, one-way repeated measure ANOVA, p=0.5437), active waking (saline vs. 2.5 mg/kg CNO vs. 5 mg/kg CNO, n=6, one-way repeated measure ANOVA, p=0.0662), or quiet waking (saline vs. 2.5 mg/kg CNO vs. 5 mg/kg CNO, n=6, one-way repeated measure ANOVA, p=0.4328) (Figure 3.5). Likewise, no significant differences were seen in neck muscle activity during NREM sleep states (saline vs. 2.5 mg/kg CNO vs. 5 mg/kg CNO, n=3, one-way repeated measure ANOVA, p=0.8025), active waking (saline vs. 2.5 mg/kg CNO vs. 5 mg/kg CNO, n=3, one-way repeated measure ANOVA, p=0.1474), or quiet waking (saline vs. 2.5 mg/kg CNO vs. 5 mg/kg CNO, n=3, one-way repeated measure ANOVA, p=0.2042) (Figure 3.5). Compared to saline controls, injection of CNO into the 6 animals did not significantly change NREM or waking muscle activity, indicating a REM sleep specific effect of RN activation.

3.1.1 Injection of 2.5 mg/kg CNO and 5 mg/kg CNO produce similar results in the masseter and neck muscles

Analysis of overall muscle activity during REM sleep following the injection of 2.5 mg/kg of CNO and 5 mg/kg of CNO produced no significant differences between either dosage in the masseter (saline vs. 2.5 mg/kg CNO vs. 5 mg/kg CNO, n=6, one-way repeated measure ANOVA, post hoc, p>0.05) and neck (saline vs. 2.5 mg/kg CNO vs. 5 mg/kg CNO, n=3, one-way repeated measure ANOVA, post hoc, p>0.05) (Figure 3.4). As shown previously, both CNO doses produced significant differences in muscle activity in comparison to saline in the masseter muscle, yet, there were no significant differences seen in the neck. However, 5 mg/kg injections of CNO have been conventionally used in our lab, therefore, the data collected from 5 mg/kg dose experiments was used for analysis in the following sections for consistency.
Figure 3.1. Immunohistological verification of cre-independent hM3Dグ expressing neurons within the RN. An example from a single wildtype animal indicates bilateral injection into the area of the RN shows expression of the fluorophore, m-cherry, in neurons that express the cre-independent DREADD, AAV8-hM3Dグ-hsyn-mCherry.
Figure 3.2. Neurons expressing cre-independent hM3DGq are located within and around the area of the RN. Stereotaxic map of hM3DGq expressing neurons in wildtype animals (n=6). Shaded regions indicate the location and extent of spread of viral injection. The insert illustrates an immunohistological example of neurons expressing the cre-independent DREADD, AAV8-hM3DGq-hsyn-mCherry.
Figure 3.3. Activation of the RN using a cre-independent strategy increases number of twitches during REM sleep. Raw data traces of a single wildtype animal under saline and CNO treatments. It is important to note that under RN activation, phasic activity in both the masseter and neck seems to be increased. a.u., arbitrary units
**Figure 3.4. Muscle activity increases with cre-independent RN activation.** Group data depicting an increase in muscle activity in the masseter (n=6) (A) and neck (n=3) (B) during REM sleep. * indicates p<0.05 compared to control; a.u., arbitrary units. All values are mean ± SEM.
A  **MASSETER**

<table>
<thead>
<tr>
<th></th>
<th>Active Wake</th>
<th>Quiet Wake</th>
<th>NREM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>5.0 ± 0.5</td>
<td>3.0 ± 0.3</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>2.5 mg/kg CNO</td>
<td>6.5 ± 1.0</td>
<td>3.5 ± 0.7</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>5 mg/kg CNO</td>
<td>7.0 ± 1.5</td>
<td>4.0 ± 1.2</td>
<td>2.0 ± 0.4</td>
</tr>
</tbody>
</table>

B  **NECK**

<table>
<thead>
<tr>
<th></th>
<th>Active Wake</th>
<th>Quiet Wake</th>
<th>NREM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>4.0 ± 0.4</td>
<td>2.0 ± 0.2</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>2.5 mg/kg CNO</td>
<td>5.5 ± 0.8</td>
<td>3.5 ± 0.6</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>5 mg/kg CNO</td>
<td>6.0 ± 1.0</td>
<td>4.0 ± 0.8</td>
<td>1.5 ± 0.3</td>
</tr>
</tbody>
</table>

**Figure 3.5.** Muscle activity remains constant during NREM sleep and waking with cre-independent RN activation. Group data depicting no change in muscle activity in the masseter (n=6) (A) and neck (n=3) (B) during active waking, quiet waking and NREM sleep. a.u., arbitrary units. All values are mean ± SEM.
3.2 Phasic activity during REM sleep is increased with activation of all cell types in the red nucleus

It is important to note that the phasic activation of muscles is affected by REM sleep duration. Periods of REM sleep with similar duration were identified for both saline and CNO treatments and analyzed for twitch activity to determine the effects of cre-independent RN activation during REM sleep. REM sleep periods were matched based on duration so that any observed changes in phasic activity could be attributed to RN activation, and not differences in the length of REM sleep. Overall, tonic muscle activity during REM sleep was unaffected with CNO treatment in both masseter (saline vs. CNO treatment, n=6, paired t-test, p=0.5397) and neck (saline vs. CNO treatment, n=3, paired t-test, p=0.7719) (Figure 3.6). However, phasic muscle activity was increased in the masseter and neck by 45 ± 22% (saline vs. CNO treatment, n=6, paired t-test, p=0.0479) and 180 ± 56% respectively (saline vs. CNO treatment, n=3, paired t-test, p=0.0397) with RN activation using a cre-independent strategy (Figure 3.6).
Figure 3.6. Frequency of twitch activity increases with cre independent strategy of RN activation. The number of twitches occurring per second during a REM period increases in both the masseter (n=6) (A) and neck (n=3) (B) muscles when the RN is stimulated. In contrast, tonic activity in both muscles is unchanged during REM sleep. * indicates p<0.05 compared to control. All values are mean ± SEM.
3.3 The temporal pattern of twitch activity changes with cre-independent RN activation

As shown in the data above, when the RN is activated through the use of a cre-independent strategy, there is an increase in the rate of muscle twitching during REM sleep. This suggests that the RN may play a functional role in the generation of REM sleep twitches. If the RN is responsible for the generation of phasic activity, it may be possible that the normal pattern of muscle activity during REM sleep is disrupted with constant RN stimulation. To analyze this, each REM sleep period that was identified in the above analysis was divided into quarters and the twitch activity of each quarter was compared within the CNO treatment as well as to the saline treatment. In this way, I was able to analyze the temporal regulation of phasic activity during each REM sleep period.

With activation of the RN, there was a significant increase in the temporal pattern of phasic activity in the first quarter of REM sleep periods in the masseter muscle (saline vs. CNO treatment, n=6, 2way RM-ANOVA, p=0.047). Within the first quarter, CNO caused an increase in twitch frequency of $243 \pm 55\%$ compared to the injection of saline (Figure 3.7). This is in contrast to the latter 3 quarters of the REM sleep periods in which twitch activity returns to a control level of frequency (saline vs. CNO treatment, n=6, 2way RM-ANOVA, post hoc test p>0.05). Therefore, the activity of the RN may play more of a role in the generation of twitches in the masseter within the first half of REM sleep as the regular twitch profile was lost with CNO injection.

In a similar manner to the masseter, the temporal pattern in the neck muscle was analyzed when the RN was activated. Unlike the effect seen in the masseter muscle, the neck responded differently to injection of CNO as there was a significant increase in twitch frequency throughout the duration of REM sleep periods rather than just the beginning half. Frequency of twitches
increased by 147 ± 57%, 307 ± 60%, and 338 ± 90% in the 1st, 3rd, and 4th quarters respectively when the RN was activated compared to control (saline vs. CNO treatment, n=3, 2way RM-ANOVA, p=0.0047) (Figure 3.7). Though the effects on the temporal patterns of the masseter and neck were different, the overall increases seen in the amount of twitches in both muscles suggest that the RN may be playing a role in the generation and timing of phasic activity in REM sleep.
Figure 3.7. RN activation using a cre-independent strategy regulates the distribution of muscle twitches during REM sleep. Group data comparing how phasic twitch activity changes across REM sleep periods in the masseter (n=6) (A) and the neck (n=3) (B). Compared to a saline control (blue line), activation of the RN (red line) increases twitch activity in the masseter in the first quarter of REM sleep (A). In the neck (B), activation of the RN increased twitch frequency across the majority of REM sleep periods. * indicates statistically significant difference between treatments (p<0.05). All values are mean ± SEM.
3.4 Injection of AAV-GFP does not affect muscle activity or sleep-wake states

In order to determine whether the effects presented above were in fact due to the manipulation of the RN, a total of 4 mice were injected with a control virus (AAV-GFP) into the area of the red nucleus. This viral construct does not contain the genetic coding for the excitatory DREADD and instead only expresses the fluorescent protein, e-GFP in the injected area. Thus, it can be certain that the effects shown previously were indeed due to RN activation and not simply due to viral injection in the RN. Experimental procedures and data analysis followed the same protocols as mice injected with DREADDs. Due to noise artifact in the neck muscle of one animal, this data was excluded from the neck analysis. Injection of CNO into these animals produced no effects on muscle activity in REM sleep that were significantly different from saline injections in the masseter (saline vs. CNO treatment, n=4, paired t-test, p=0.6296) or neck (saline vs. CNO treatment, n=3, paired t-test, p=0.0598) (Figure 3.8). This verifies that the results presented above are a true reflection of the manipulations of the RN rather than from the injection of a virus into the RN.
Figure 3.8. Injection of AAV-GFP control does not affect muscle activity. Group data showing that muscle activity in the masseter (n=4) (A) and the neck (n=3) (B) is unaffected when mice are injected with a control virus. All values are mean ± SEM.
To verify that the previous results are due to the activation of the RN and not due to changes in sleep-wake behavior, the percent of time spent in different states was analyzed to ensure that there were no significant differences between saline and CNO treatments, as an increased overall time spent in REM sleep would result in a greater number of twitches. The frequency of different REM sleep durations was also compared between treatments to ensure that different durations of REM sleep occurred in similar frequencies, as a higher frequency of REM sleep periods with longer duration would also result in a greater number of twitches. It was found that injection of CNO compared to saline caused no significant differences in the percent of time spent in total wake (saline vs. CNO treatment, n=4, paired t-test, p=0.3616), active wake (saline vs. CNO treatment, n=4, paired t-test, p=0.3314), quiet wake (saline vs. CNO treatment, n=4, paired t-test, p=0.2548), NREM sleep (saline vs. CNO treatment, n=4, paired t-test, p=0.2098), and REM sleep (saline vs. CNO treatment, n=4, paired t-test, p=0.6969). As well, the frequencies of REM sleep durations under CNO conditions were not significantly different for durations of <30 seconds (saline vs. CNO treatment, n=4, paired t-test, p=0.8925), 30-60 seconds (saline vs. CNO treatment, n=4, paired t-test, p=0.3120), 60-90 seconds (saline vs. CNO treatment, n=4, paired t-test, p=0.6638), 90-120 seconds (saline vs. CNO treatment, n=4, paired t-test, p=0.2152), 120-150 seconds (saline vs. CNO treatment, n=4, paired t-test, p=1.000), 150-180 seconds (saline vs. CNO treatment, n=4, paired t-test, p=0.3910), and >180 seconds (saline vs. CNO treatment, n=4, paired t-test, p=0.3910) when compared with saline controls (Figure 3.9). From this, we can be sure that the effects presented above are indeed due to the manipulation of the RN and not from the generation of longer REM sleep durations.
Figure 3.9. Injection of CNO does not affect sleep-wake behaviour or REM sleep. A) Injection of CNO does not affect the time mice spend in any state (n=4). Behaviours are unchanged across all states. B) The relationship between the frequency of REM periods and their durations is not affected with treatment of CNO compared to saline (n=4). All values are mean ± SEM.
CHAPTER FOUR: STIMULATION OF THE GLUTAMATERGIC NEURONS IN RED NUCLEUS USING A CRE-DEPENDENT STRATEGY INCREASES MUSCLE ACTIVITY DURING REM SLEEP

One aspect of this thesis that remains to be completed is to ensure via immunohistochemistry that the excitatory DREADD, hM3Dq, expresses in glutamatergic neurons. With some reliability, we can be sure that the cre-dependent excitatory DREADD expresses in glutamatergic neurons due to the use of the cre-lox system in vglut-2 cre animals. Due to the genetic coding for the cre-dependent hM3Dq DREADD, the receptor will not be expressed in cells without the presence of cre-recombinase. As the vglut-2 cre animals do express cre-recombinase in glutamatergic neurons (Vong et al., 2011), we can state with some certainty that glutamatergic neurons were infected with the excitatory DREADD. Therefore, the following results are from the activation of presumably glutamatergic neurons using a cre-dependent DREADD strategy and will be stated as such. Still, it should be considered that it remains to be quantified through the use of immunohistochemistry to confirm co-localization of the DREADD receptor with glutamate neurons.

4.1 Activation of glutamatergic RN cells using a cre-dependent strategy increases overall muscle activity during REM sleep

As previous results show, activation of the neurons in the RN of WT mice using a cre-independent strategy increases muscle activity during REM sleep. Thus, it is hypothesized that activation of the glutamatergic neurons in this area with a cre-dependent strategy should produce similar increases in muscle activity as it has been shown that glutamate stimulation affects phasic muscle activity in REM sleep (Burgess et al., 2008). Again, after verification of viral injection of the excitatory cre-dependent DREADD into the RN, EEG and EMG of the mice were analyzed
for changes in muscle activity (Figure 4.1, Figure 4.2). In a total of 7 vglut2-cre animals, masseter and neck activity were recorded and analyzed for changes across sleep-wake states (Figure 4.3). In one animal, neck EMG signal was excluded from the group data due to electrical noise artifact. Similar to results in the WT mice, there was an increase in overall masseter muscle activity of 40 ± 18% during REM sleep with injection of 5 mg/kg of CNO compared to saline controls (saline vs. 2.5mg/kg CNO vs. 5 mg/kg CNO, n=7, Friedman test, p= 0.0003) (Figure 4.4). As well, results from the neck muscle show a non-significant change in muscle activity across treatments of saline and CNO (saline vs. 2.5mg/kg CNO vs. 5 mg/kg CNO, n=6, one-way repeated measure ANOVA, p= 0.1284). Further analysis of masseter muscle activity during active waking (saline vs. 2.5mg/kg CNO vs. 5 mg/kg CNO, n=7, one-way repeated measure ANOVA, p= 0.0858), quiet waking (saline vs. 2.5mg/kg CNO vs. 5 mg/kg CNO, n=7, one-way repeated measure ANOVA, p= 0.4968) and NREM sleep (saline vs. 2.5mg/kg CNO vs. 5 mg/kg CNO, n=7, one-way repeated measure ANOVA, p= 0.1107) shows no significant change (Figure 4.5). This was also seen in the neck muscle in active waking (saline vs. 2.5mg/kg CNO vs. 5 mg/kg CNO, n=6, one-way repeated measure ANOVA, p= 0.7678), quiet waking (saline vs. 2.5mg/kg CNO vs. 5 mg/kg CNO, n=6, one-way repeated measure ANOVA, p= 0.3635) and NREM sleep states (saline vs. 2.5mg/kg CNO vs. 5 mg/kg CNO, n=6, one-way repeated measure ANOVA, p= 0.2766) (Figure 4.5). This data shows that activation of glutamatergic neurons in the RN increases muscle activity during specifically REM sleep and adds support for the hypothesis that the RN plays a role in the control of phasic activity.
Figure 4.1. Immunohistological verification of glutamatergic, cre-dependent hM3DGq expressing neurons within the RN. An example from a single vglut2-cre animal indicates bilateral injection into the area of the RN shows expression of the fluorophore, m-cherry, in neurons that express the cre-dependent DREADD, AAV8-hM3DGq-DIO-mcherry.
Figure 4.2. Neurons expressing cre-dependant hM3Dq are located within and around the area of the RN. Stereotaxic map of hM3Dq expressing neurons in vglut2-cre animals (n=7). Shaded regions indicate the location and extent of spread of viral injection. The insert illustrates an immunohistological example of neurons expressing the DREADD, AAV8-hm3Dq-Isyn-mCherry.
Figure 4.3. Activation of the glutamatergic cells in the RN using a cre-dependent strategy increases number of twitches during REM sleep. Raw data traces of a single vglut2-cre animal under both saline and CNO treatments. As previously displayed in WT animals, phasic activity in both the masseter and neck seems to increase with RN activation. a.u., arbitrary units.
Figure 4.4. Activation of glutamatergic neurons using a cre-dependent DREADD in the RN increases muscle activity during REM sleep. Group data depicting an increase in muscle activity during REM sleep in the masseter (n=7) (A) and the neck (n=6) (B) with injection of CNO. * indicates p<0.05 compared to control; a.u., arbitrary units. All values are mean ± SEM.
Figure 4.5. Muscle activity remains constant during NREM sleep and waking with activation of glutamatergic cells in the RN. Group data depicting no change in muscle activity in the masseter (n=7) (A) and neck (n=6) (B) during active waking, quiet waking and NREM sleep. a.u., arbitrary units. All values are mean ± SEM.
4.2 Phasic activity during REM sleep increases with cre-dependent activation of glutamatergic RN cells

As with the WT mice, periods of REM sleep with similar duration were time matched between saline and CNO treatments and analyzed for phasic activity to determine the effects of activating glutamatergic neurons in the RN during REM sleep. Analysis of vglut-2 cre animals yielded similar results to those seen in the WT mice in which tonic muscle activity in both the masseter (saline vs. CNO treatment, n=7, paired t-test, p=0.2017) and neck muscles (saline vs. CNO treatment, n=6, paired t-test, p=0.1758) remained unchanged while phasic activity significantly increased with CNO injection compared to saline. The number of muscle twitches per second during REM sleep increased by a magnitude of 25 ± 8% (saline vs. CNO treatment, n=7, paired t-test, p=0.048) and 68 ± 29% (saline vs. CNO treatment, n=6, paired t-test, p=0.0064) in the masseter and neck, respectively (Figure 4.6). This data provides more evidence for the hypothesis that glutamatergic neurons in the RN are responsible for control of phasic REM sleep activity.
Figure 4.6. Frequency of phasic activity increases with activation of glutamatergic neurons in the RN. Group data showing that, in comparison to a saline control, the number of twitches per second during REM sleep is increased with the activation of the RN in both the masseter (n=7) (A) and the neck (n=6) (B) muscles. In contrast, tonic activity remains unchanged. * indicates p<0.05 compared to saline. All values are mean ± SEM.
4.3 The temporal pattern of twitch activity changes with activation of the glutamatergic neurons in the RN using a cre-dependent strategy

The increases seen in phasic activity in the masseter and neck during REM sleep provide more support for the hypothesis that the RN, a glutamatergic nucleus, is responsible for mediating REM sleep twitches. As such, the temporal patterns of twitch activity may be affected through the stimulation of glutamatergic cells in the RN. Again, all previously identified REM sleep periods were divided into quarters and phasic activity was analyzed for each quarter across treatments. Much like the WT mice, the temporal pattern of twitch activity in the masseter of vglut-2 cre animals was significantly affected in the first half of REM sleep episodes when activating glutamatergic RN neurons (saline vs. CNO treatment, n=7, 2way RM-ANOVA, p=0.025). Injection of CNO caused an increase in twitch frequency in the masseter by a magnitude of $93 \pm 50\%$ and $195 \pm 94\%$ in the first and second quarters respectively compared to saline (Figure 4.7). In the latter half of REM sleep periods, twitch frequency in the masseter returns to saline levels with no significant differences (saline vs. CNO treatment, n=7, 2way RM-ANOVA, post hoc test p>0.05).

In contrast to the masseter, the neck muscle responded differently to activation of the glutamatergic RN neurons. Instead of an increase in twitch frequency in the first half of REM sleep periods, injection of CNO caused an increase in twitch frequency in the latter half of REM sleep periods in the neck. In the 3rd and 4th quarters of REM sleep periods phasic activity was significantly increased by $88 \pm 42\%$ and $91 \pm 52\%$ (saline vs. CNO treatment, n=6, 2way RM-ANOVA, p=0.042, Figure 4.7). Activity in the first 2 quarters were not statistically different when activation of the RN was compared with saline (saline vs. CNO treatment, n=6, 2way RM-ANOVA, post hoc test p>0.05). Though the effects of RN activation on masseter and neck
muscles differ, the changes in the temporal pattern of REM sleep phasic activity suggest the RN may play a role in not only generation of REM sleep twitches but the timing as well.
Figure 4.7. Activation of glutamatergic neurons in the RN affects the temporal pattern of twitches during REM sleep. In comparison to a saline control (blue line), the distribution of twitches is disproportionately affected with glutamatergic cell activation (red line). (A) Twitch frequency is increased in the first half of REM sleep in the masseter (n=7) with RN activation. (B) Phasic activity is increased in the neck (n=6) in the latter half of REM sleep periods when the RN is activated. * indicates statistically significant difference between treatments (p<0.05). All values are mean ± SEM.
CHAPTER FIVE: DISCUSSION

The data presented here support the hypothesis that the RN is partly responsible for the generation of phasic activity during REM sleep. Upon confirmation that the excitatory DREADD injected into the area of the RN was expressing well, it was demonstrated that activation of these receptors using both a cre-independent and cre-dependant strategy increased the amount of muscle twitches during REM sleep. Though convincing, these results do not completely explain the causes of REM sleep twitches. Still, this data sheds more light on the mechanisms and areas involved in the generation of phasic activity during REM sleep.

5.1 Red nucleus influences during REM sleep

Several studies have investigated the role of the red nucleus in the modulation of muscle control during waking states (Hicks and Onodera, 2012; Massion, 1988; Horn et al. 1998); however few have looked at its role during REM sleep. Due to its role in the generation of muscle tone and movement during wake, it follows that the RN remains relatively silent during REM sleep. Indeed, previous recordings of the RN during REM sleep have shown only an increase in activity in the RN that precedes phasic activity in the eyes and muscles (Gassel et al., 1966). The data presented here is in agreement with their findings in which phasic bursts of muscle activity are shown to increase with the activation or stimulation of the RN. Unlike previous studies on the red nucleus during REM sleep, this data provides a direct functional role for the red nucleus in the augmentation in phasic activity during this sleep state. As the RN has been shown to function in the control of muscles, it is consistent that stimulation of the area leads to an increase in muscle activity. It, therefore, appears that the RN may be in part responsible for the generation of REM sleep twitches that interrupt the tonic activity of motor atonia. The increase in overall
muscle activity and number of twitches per second during REM sleep strengthen the notion that the RN plays a role in the generation of phasic activity.

5.2 Excitatory and inhibitory inputs during REM sleep

To further characterize the role of the RN during REM sleep and determine its effect on phasic activity, glutamatergic neurons were stimulated in the area of the RN using a cre-dependent strategy in vglut 2-cre animals. Glutamatergic neurons in the RN have been implicated in motor activity and, thus, activation of these neurons, during wake, increases muscle activity (Massion, 1988; Hicks and Onodera, 2012). It has previously been shown that the muscle twitches in REM sleep are the result of an excitatory glutamatergic drive onto motor neurons through the heavy GABAergic and glycinergic inhibition (Burgess et al., 2008). It was, therefore, expected that an increase in muscle activity would be seen. Similar to results from the WT mice, stimulation of the glutamatergic neurons in the RN increases both muscle tone and muscle twitches, breaking through the potent muscle inhibition characteristic of REM sleep. These findings, again, provide more support for the observation that the RN plays a role in generating muscle activity during REM sleep and may be responsible for the phasic twitches seen in REM sleep.

5.3 Temporal regulation of REM sleep phasic activity

Numerous studies have investigated the areas and circuitry that are involved in the regulation of muscle control during REM sleep yet many overlook the time course and patterns of the muscle activity (Chase et al., 1989; Lai et al., 2001; Soja et al., 1991). It has commonly been assumed that the mechanisms regulating muscle activity remain constant and homogenous throughout the state. However, more recent evidence has shown that motor control is not uniformly regulated during REM sleep but has a more dynamic pattern of regulation (Brooks and Peever, 2012;
Fraigne and Orem, 2011). In fact, it has been shown in the masseter muscle that phasic activity during REM sleep progressively increases over the duration of a REM sleep period (Brooks and Peever, 2012). According to this model of muscle activity, excitation is more strongly opposed in the early stages of REM sleep as opposed to later.

The experiments done in this thesis show that when the RN is stimulated through the use of the DREADDS, phasic activity is increased during REM sleep periods compared to controls. In particular, twitch activity in the masseter is increased during the early stages (ie. the first half) of REM sleep periods. These data show the RN may be playing a role in the generation of phasic activity at different time points during REM sleep. As motor neurons are very highly inhibited during the beginning parts of REM sleep, the excitation of the RN may provide enough stimulation onto motor neurons to break through the atonia and generate phasic activity. It is interesting to note that the elevation in twitch activity in the masseter is seen only during the beginning portions of REM sleep as levels return to normal in the latter half of REM sleep. This may imply that the RN has a more significant role in the generation of masseter twitches early on in REM sleep periods while other systems may take over in the latter periods. Indeed, recent evidence has implicated areas such as the PCRt-PMnR (parvocellular reticular formation-paramedian reticular area) to regulate masseter phasic activity via glutamatergic mechanisms (Anaclet et al., 2010). In accordance with this data, it may be possible that the RN is working in tandem with the PCRt-PMnR area as projections from the RN to the PCRt have been characterized. This initial increase in twitching also indicates the possible role the RN has in generating twitches in the latter half of REM sleep periods. By stimulating the area with DREADDS, the RN may be overcoming the potent inhibition that is lost in the latter half of
REM sleep. It is possible that the return to control levels may be at a maximal firing rate and, thus, unable to exceed normal twitch frequencies nearer the end of REM sleep periods.

The pattern of muscle activity associated with the masseter is quite different from that seen in the neck muscle. Unlike the masseter, phasic activity in the neck does not progressively increase over the course of REM sleep periods (Fraigne and Orem, 2011). Instead, the probability for muscle twitches to occur is much more random throughout REM sleep durations, leading to the hypothesis that inhibition is unchanged throughout REM sleep in the neck muscle. Consequently, analysis of the temporal pattern of phasic activity in the neck muscle showed an overall increase in twitch frequencies throughout whole REM sleep durations. This implies that the RN has differential activation of muscles as the neck and masseter activity profiles differ. This difference in twitch activity during REM sleep may be attributed to the differential innervation of the neck muscle (Kuchler et al., 2002). As inhibition is unchanged in the neck, the activation of the RN increases the probability for phasic activity to occur in any subdivision of REM sleep. Thus, RN activation allows motor neurons in the neck to overcome the inhibition of REM sleep increasing the frequency at which phasic activity is seen. This, in combination with data from the masseter, provides evidence that the RN could play a role in the generation of phasic activity during REM sleep.
5.4 Hypothesized model of REM sleep motor control and REM sleep behaviour disorder

The current model of motor control and regulation during REM sleep hypothesizes that, along with disfacilitation, the medial medulla or GiG is responsible for the potent inhibition of muscle activity that produces the atonia seen during this state (Luppi et al., 2011). Despite this potent inhibition and lack of excitation, brief phasic periods of muscle activation occur in the form of muscle twitches. With the activation of the RN, increases in muscle activity and the number of twitches during REM sleep are seen. This supports the idea that the RN plays a role in the generation of REM sleep Twitches and, thus, may play a key role in the circuitry of motor regulation during REM sleep. Studies done by Mileykovskiy et al. (2002) support hypothesized projections from the medial medulla to the RN as electrical and chemical stimulation of the GiG leads to a decrease in activity within the RN. This, in combination with the data presented in this thesis, provides more support for the involvement of the RN in REM sleep circuitry. Under normal REM sleep conditions, it is hypothesized that the activation of the GiG, through the disinhibition of the Sub-C, would inhibit the RN and decrease its activity during REM sleep. As inhibition has been shown to gradually decrease during REM sleep, it is possible that the projections to motor neurons by the RN are more easily excited allowing for the appearance of muscle twitches.

This, in turn, may provide implications for the potential involvement of the RN in REM sleep behaviour disorder. RBD is a sleep disorder characterized by the lack of muscle atonia during REM sleep manifested in the form of abnormal muscle activity. It is hypothesized that it is the breakdown of REM sleep circuitry that accounts for these abnormal muscle behaviours during REM sleep. In fact, numerous studies have shown that patients with RBD contain structural damage in the medial medulla or brainstem which may cause the overt motor behaviours seen in
REM sleep (Boeve et al., 2007). In accordance with this new hypothesized model, it may be that the elimination or damage of GiG neurons allows areas such as the RN to trigger the muscle behaviours seen in RBD patients. With the removal of areas that are responsible for the potent atonia of REM sleep, motor neurons become more easily excitable from areas like the RN causing an overexpression of twitch behaviour and motor activity (Figure 5.1).
Figure 5.1. Hypothesized model responsible for generation of REM sleep twitches and RBD. A) During REM sleep, the disinhibition of glutamatergic neurons in the Sub-C activates GABAergic and glycineric neurons in the GiG. This then generates atonia through ascending and descending projections to motor centers and motor neurons, respectively. Because inhibition weakens over REM sleep durations, projections from the RN are able to overcome GiG inhibition and generate phasic activity. B) In patients with RBD, the loss of the GiG removes inhibition from both the RN and motor neurons. This may allow the RN to stimulate motor neurons and cause the overt muscle behaviours seen in RBD.
CHAPTER 6: SUMMARY AND FUTURE DIRECTIONS

Taken together, the data presented in this thesis provide much support for the role of the RN in the generation of REM sleep twitches and shed more light on motor control during REM sleep. It was demonstrated here that pharmacogenetic stimulation in the area of the RN produced a significant effect in which muscle activity was increased during REM sleep in the form of a greater number of phasic twitches. It was further discovered that the RN plays differential roles in the activation of the neck and masseter muscles, augmenting twitch activity nearer the beginning of REM sleep periods in the masseter and overall in the neck. Though the pattern of activity is differentially affected by RN stimulation, this data still indicates that the role of the glutamatergic neurons RN in generating phasic activity during REM sleep may be important, still, for different motor systems.

This thesis provides evidence that shows a functional correlation between stimulation of the RN and an increase in phasic activity during REM sleep. This furthers our current understanding of the modulation and regulation of motor activity during REM sleep and leads to further hypotheses in REM sleep circuitry and motor control. Further investigations into the inactivation of the RN through the use of pharmacogenetics and/or optogenetics are needed to fully understand the intricate mechanisms responsible for generating phasic activity during REM sleep.
References


