INHIBITORY CONTROL OF MUSCLE ACTIVITY IN SLEEP

by

Patricia Lynn Brooks

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

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Doctor of Philosophy, 2011
Patricia Lynn Brooks
Graduate Department of Cell & Systems Biology
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ABSTRACT

In this thesis, I examined the inhibitory control of REM sleep motor activity using both a pharmacological rat model and a genetic mouse model. I characterized the role for GABA and glycine in mediating the REM-specific suppression of muscle activity as well as their involvement in regulating the phasic muscle twitches that punctuate this atonia. Based on four specific research objectives, the following conclusions were drawn:

1. REM atonia is not directly mediated by glycinergic or GABA$_A$-mediated inhibition. These data refute the prevailing hypothesis that REM atonia is caused by glycinergic inhibition. These receptors are, however, important in the regulation of phasic muscle twitch activity.

2. GABA$_B$ receptors can modulate REM atonia but only when acting in concert with GABA$_A$ and glycine receptors. Blockade of all three receptor types results in a partial reversal of REM atonia, suggesting a functional interaction is occurring between these receptors during REM sleep.

3. The phasic glycinergic/GABA$_A$-mediated inhibitory drive present in REM sleep regulates the temporal pattern of phasic twitch activity that is seen across this state. I hypothesize that this progressively decreasing inhibitory input counteracts
a gradually increasing excitatory input to shape the temporal distribution of
muscle twitches across REM sleep.

4. A loss of normal inhibitory function may play a causal role in the pathology of
REM sleep behaviour disorder (RBD), the sleep disorder characterized by
excessive motor activity in REM sleep.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>°C</td>
<td>degree Celsius</td>
</tr>
<tr>
<td>aCSF</td>
<td>artificial cerebral spinal fluid</td>
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<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid</td>
</tr>
<tr>
<td>a.u.</td>
<td>arbitrary units</td>
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<tr>
<td>AW</td>
<td>active wake</td>
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<tr>
<td>Cl⁻</td>
<td>chloride</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
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<tr>
<td>EEG</td>
<td>electroencephalographic</td>
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<tr>
<td>EMG</td>
<td>electromyographic</td>
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<tr>
<td>FW</td>
<td>formula weight</td>
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<td>g</td>
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<td>GABA</td>
<td>γ-aminobutryic acid</td>
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<td>Hz</td>
<td>hertz</td>
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<td>K⁺</td>
<td>potassium</td>
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<td>L</td>
<td>litre</td>
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<tr>
<td>LM</td>
<td>left masseter</td>
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<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
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<tr>
<td>IPSP</td>
<td>inhibitory postsynaptic potential</td>
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<td>µ</td>
<td>micro</td>
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<td>metre</td>
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<tr>
<td>M</td>
<td>molar</td>
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<tr>
<td>NREM</td>
<td>non-rapid eye movement</td>
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<td>OSA</td>
<td>obstructive sleep apnea</td>
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<tr>
<td>PGO</td>
<td>ponto-geniculo-occipital</td>
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<td>RBD</td>
<td>rapid eye movement sleep behavior disorder</td>
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<td>REM</td>
<td>rapid eye movement</td>
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<td>RM</td>
<td>right masseter</td>
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<td>Abbreviation</td>
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<tr>
<td>QW</td>
<td>quiet wake</td>
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<td>seconds</td>
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<td>SEM</td>
<td>standard error of the mean</td>
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<td>Tg</td>
<td>transgenic</td>
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<td>W</td>
<td>wake</td>
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<td>wildtype</td>
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CHAPTER ONE: CONTROL OF REM SLEEP MOTOR ATONIA

1.1 OVERVIEW

This thesis examines the neurochemical mechanisms that mediate muscle control during natural sleep. Skeletal muscle tone is suppressed in sleep, particularly during rapid eye movement (REM) sleep. Muscle activity follows a stereotypical pattern across the sleep-wake cycle: activity is highest in waking, decreased in non-rapid eye movement (NREM) sleep, and further decreased in REM sleep (Jouvet, 1967; Figure 1.1). It is REM sleep, the state characterized by a complete abolition of muscle tone, that will be the main focus of this thesis.

The phenomenon of REM sleep muscle atonia was first described by Jouvet et al. in 1959. A striking paradox of REM sleep is that while muscle tone is at its lowest during this state, superimposed on this background of atonia are phasic muscle twitches. Thus, REM sleep is characterized by both a tonic inhibition of muscle tone as well as brief periods of phasic activation, manifesting in the form of muscle jerks and twitches, that punctuates the atonia. Although it has been over 50 years since the first description of REM atonia, the neural mechanisms responsible for this pattern of muscle control are not completely understood.

Determining the mechanisms that mediate muscle tone in sleep is important in understanding the basic neural control of muscles. Furthermore, a disruption of muscle control underlies, at least in part, most of the major sleep disorders, including REM sleep behaviour disorder (RBD), obstructive sleep apnea (OSA), periodic limb movement disorder, bruxism, and narcolepsy. RBD, for example, involves the loss of normal REM motor control: RBD patients exhibit elaborate nocturnal motor behaviours, often with violent or injurious results to themselves or their bedpartner (Schenck and Mahowald, 2002). In OSA, upper airway patency is compromised by the loss of muscle tone that occurs during sleep (Horner, 1996); hypotonia together with a structural instability in the upper airway of OSA patients (Isono et al., 1997) results in repetitive airway occlusions throughout the night, with a wide range of negative health consequences (Malhotra and White, 2002). To develop effective pharmacological treatments for these sleep-related
Fig. 1.1. Muscle tone across the sleep-wake cycle. Polysomnographic recordings of wake (A), NREM sleep (B) and REM sleep (C) in a cat. Note the state-specific reduction in EMG activity: neck EMG activity is highest in waking, reduced in NREM sleep and abolished in REM sleep. The rapid eye movements (YEUX) and regular theta activity (HIPP), other hallmarks of REM sleep, are also shown. (Adapted from Jouvet, 1967).
motor disorders, it is imperative to first understand the fundamental neurochemical mechanisms that mediate muscle control during sleep.

1.2 BRAINSTEM REGULATION OF REM SLEEP MOTOR CONTROL

While the biochemical substrates responsible for REM sleep motor control are not completely understood, much work has been done to elucidate the brain regions involved in this state-specific motor suppression.

1.2.1 Transection studies

Transection studies first localized the region critical for REM atonia to the brainstem. Jouvet pioneered this work with the demonstration that ponto-mesencephalic transections in the cat did not abolish REM atonia (Jouvet, 1962): REM sleep complete with REM atonia still occurred after the forebrain was separated from the brainstem at the level of the midbrain. Others confirmed that REM atonia remained intact following these rostral pontine transections (Matsuzaki, 1969; Villablanca, 1966). Caudal pontine-rostral medullary transections, on the other hand, essentially abolished REM atonia (Jouvet, 1962; Siegel et al., 1986; Webster et al., 1986). From this, it was concluded that the substrates underlying REM atonia are located primarily in the rostral pons (Jouvet, 1962).

Additional studies elaborated on this seminal work by defining a role for the medial medulla in the control of REM atonia. It has long been known that stimulation of the medial medulla results in bilateral muscle atonia (Magoun, 1944; Magoun and Rhines, 1946). However, as described above, separation of the pons from the medulla eliminates atonia, indicating that, by itself, the medulla cannot produce REM atonia (Jouvet, 1962; Siegel et al., 1986; Webster et al., 1986). Not only is medullary neuronal activity disrupted by ponto-medullary transection (Siegel et al., 1986), but the atonia generating effects of medullary stimulation are lost when pontine input to the medulla is prevented (Kohyama et al., 1998; Siegel et al., 1983). Therefore, to generate atonia, the medullary motor inhibitory system must interact with the pons. Taken together, these transection studies establish the ponto-medullary brainstem as the region critical in the generation of REM atonia and suggest that the pons interacts with medulla to generate muscle tone suppression in this state.
1.2.2 Lesion studies

After transection studies identified the pons and medial medulla as the areas responsible for REM sleep motor control, lesion studies were used to further define these critical regions. Jouvet and Delorme (1965) were the first to describe a behavioural state in the cat known as REM sleep without atonia. This condition presented after bilateral lesions were made in the dorsal rostral pons: these lesions eliminated the motor inhibition of REM sleep while leaving all other signs of the state intact (Henley and Morrison, 1974; Jouvet and Delorme, 1965). Subsequent studies demonstrated that the specific location of the lesions could determine the degree of behavioural manifestation that occurred during REM sleep (Hendricks et al., 1982; Hobson, 1965). In particular, the subcoerulus was implicated as a key area responsible for REM atonia (Hendricks et al., 1982). Lesions in the medial medulla also produce REM sleep without atonia (Holmes and Jones, 1994; Schenkel and Siegel, 1989). Taken together, these studies support and extend the earlier transection work by demonstrating that both the dorsolateral pons and medial medulla are important in the regulation of muscle tone and inhibition of movement during REM sleep.

An equivalent pontine region responsible for REM atonia has been identified in the rat (Boissard et al., 2002; Hajnik et al., 2000). Lesioning of the sublaterodorsal nucleus (SLD) causes REM sleep without atonia in rats (Lu et al., 2006), just as lesions to the subcoeruleus do in cats. Lesion and pathological data from RBD patients, the sleep disorder characterized by excessive muscle activation in REM sleep (Schenck and Mahowald, 2002), indicate analogous regions are responsible for the maintenance of REM motor control in humans as well (Boeve et al., 2007). This suggests a universal location of regions mediating REM atonia in mammals (Siegel, 2006).

1.2.3 Chemical stimulation studies

While transection and lesioning studies identified the brain areas that REM atonia cannot exist without, stimulation studies have determined the chemical nature of the regions that, when activated, induce atonia. Microinjections of carbachol, a cholinergic agonist, into the dorsolateral pons of conscious cats produces a REM-like state, complete with a generalized atonia, that persists for up to an hour after the injection (George et al., 1964;
Van Dongen et al., 1978). Depending on the exact site of injection, REM sleep or just the atonia of REM can be triggered (Katayama et al., 1984). Not surprisingly, the sites most effective in inducing atonia are the same critical atonia-mediating areas identified in lesioning studies (Henley and Morrison, 1974; Van Dongen et al., 1978; Vannimercier et al., 1989). Thus, the dorsolateral pons can be activated via a cholinergic mechanism to trigger muscle suppression.

In addition to cholinergic processes, the pontine structures responsible for generating REM atonia are sensitive to glutamate (Boissard et al., 2002; Lai and Siegel, 1988). Furthermore, it has been shown that the medullary mechanisms that trigger atonia consist of both glutamate- and acetylcholine-sensitive neurons localized in the rostral and caudal regions of the medial medulla, respectively (Lai and Siegel, 1988). This chemical and anatomical organization of the pontomedullary atonia-generating region has been confirmed by microdialysis studies (Kodama et al., 1992, 1998). Therefore, both cholinergic and glutamatergic neurons are involved in the control of REM sleep motor tone.

γ-aminobutyric acid (GABA) has also been implicated in the brainstem regulation of REM atonia. Blockade of GABAergic transmission in the dorsolateral pons in both cats and rats induces a REM-like state complete with atonia (Boissard et al., 2002; Xi et al., 1999), suggesting a disinhibition of GABAergic processes contributes to the generation of this motor phenomenon.

Interestingly, although equivalent atonia-generating pontine sites have been identified in cats and rats, microinjections of carbachol into the SLD of rats does not have the same REM-generating effects as it does in the subcoeruleus of cats (Boissard et al., 2002; Deurveilher et al., 1997; Kubin, 2001). This suggests that the neurons responsible for REM sleep are localized in the same area in these species; however, they may differ in their neurochemical control.

While the chemical nature of the mechanisms responsible for the brainstem control of REM atonia is still being debated, these studies collectively demonstrate that chemical manipulation of the key pontine and medullary regions implicated in REM sleep motor control can induce REM atonia.
1.2.4 Unit recording studies

Neurons involved in the control of REM atonia should show increased discharge rates during this state. The pontine and medullary regions implicated in the generation of REM atonia contain neurons that show state-specific increases in firing during REM sleep (Chase et al., 1981; Hobson et al., 1975; Kanamori et al., 1980; McCarley and Hobson, 1971; Netick et al., 1977; Siegel et al., 1979). These neurons display high levels of tonic activity throughout each REM period, with activity being positively correlated with both the onset and duration of REM sleep (Chase et al., 1981). The increase in neuronal firing of both pontine and medullary cells occurs in conjunction with the atonia of REM sleep as well as during waking postures associated with reduced muscle tone (Hoshino and Pompeiano, 1976; Siegel et al., 1979). This selectivity of neuronal activity during REM sleep in the pontine and medullary atonia-generating regions is an important criterion for establishing these areas as responsible for the motor suppression seen during this state. These findings support the notion of state-dependent circuitry involving a REM-specific inhibition of muscle tone mediated by the medial medulla that has been activated selectively in REM sleep by upstream pontine structures.

1.2.5 Stimulation of pons or medulla causes hyperpolarization of motoneurons

The involvement of pontine and medullary structures in the suppression of motor tone during REM sleep is further supported by the demonstration that electrical stimulation of these regions abolishes muscle activity. As stated earlier, stimulation of the medial medulla reduces bilateral muscle tone as well as the excitability in response to reflex stimulation (Magoun, 1944; Magoun and Rhines, 1946). Thus, the medulla is capable of exerting a general inhibitory influence on motor output. The effect of medullary inhibition on muscle tone is mediated by a sustained inhibition of motoneurons as electrical stimulation of the medullary reticular formation induces a hyperpolarization of lumbar motoneurons (Jankowska et al., 1968; Llinas and Terzuolo, 1964, 1965).

Examining the effects of medullary stimulation in waking and sleep reveals that this inhibitory effect is not constant across the sleep-wake cycle. During REM sleep, medullary stimulation produces large hyperpolarizing inhibitory postsynaptic potentials (IPSPs) in all lumbar motoneurons recorded, whereas during waking and NREM sleep,
only occasionally are small-amplitude potentials seen (Chase et al., 1986). Thus, medullary stimulation results in a state-specific postsynaptic inhibition of motoneurons. A similar finding has been reported after pontine stimulation: while electrical stimulation of the pons hyperpolarizes motoneurons, this effect is specific to REM sleep (Fung et al., 1982).

1.2.6 Brainstem regulation of REM sleep motor control

Through the use of transections, lesions, electrical and chemical stimulation as well as unit recordings, the brainstem regulation of REM motor control has been well-defined. These important works point to the medial pontomedullary region as the critical brain area responsible for the suppression of motor tone in sleep. Based on these studies, it is hypothesized that in REM sleep, pontine inputs activate medullary structures, which excite descending inhibitory projections that impinge on motor pools resulting in the hyperpolarization of motoneurons and thus motor suppression (Siegel, 2005; Figure 1.2A). Perturbation of this brainstem circuitry results in a loss of normal REM motor control, as has been well characterized by the studies outlined above (Figure 1.2B,C). The control of motoneuron hyperpolarization, however, is not clear; the neurochemical inputs responsible for the REM-specific control of motoneuron excitability will be investigated in this thesis.

1.2.7 REM atonia vs. phasic muscle twitches

In addition to dissecting the mechanisms involved in mediating REM atonia at the level of the motoneuron, this thesis will also examine the regulation of phasic muscle activity in REM sleep. Much work has focused on the control of REM atonia; the phasic muscle twitches that punctuate this atonia, however, have received less attention. The loss of normal REM motor control in the aforementioned transection and lesioning studies is assumed to be due to a loss of REM atonia, however it is not actually clear from these studies if the lack of motor suppression in REM sleep is due to the abolition of REM atonia per se, or is due to an increase in the amount of phasic muscle twitch activity. In fact, Henley and Morrison (1974) report that after pontine lesions, the pattern of behavioural manifestations that emerge in REM sleep closely parallel the pattern of alternating phasic muscle twitches and periods of atonia that are characteristic of normal
Fig. 1.2. Control of REM sleep motor atonia. A, In REM sleep, pontine inputs activate medullary structures, which excite inhibitory interneurons that impinge on motor pools resulting in the hyperpolarization of motoneurons and thus motor suppression. Lesions to the pons or medulla result in a loss of motor suppression in REM sleep (B,C). Excitatory projections are represented by plus signs (+) and inhibitory projections are represented by minus signs (-). See text for details.
REM sleep, with the bursts of activity increasing as REM sleep progresses. This suggests that the phasic portion of motor activity is augmented in these animals, while REM atonia remains intact (Figure 1.3). Thus, it may be that the excessive motor activity that is observed in REM sleep after pontine lesions is due to an unmasking of the excitatory phasic drive that is normally suppressed during this state. REM atonia may not in fact be altered in these animals. Whether it is an abolishment of REM atonia or the augmentation of phasic muscle twitches, the end result would be similar, with animals displaying excessive motor activation throughout REM sleep. Differentiating between these two effects, however, is necessary to elucidate the underlying cause of each as it is unclear if these two components of REM sleep are regulated by the same mechanisms or if each are controlled separately. Some work has begun to dissect the neural circuits involved in mediating phasic and tonic events of REM sleep (Vetrivelan et al., 2009), however this important differentiation between the two components of REM sleep motor control is still in its infancy and requires further attention. Thus, in addition to examining the control of REM atonia, this thesis will also consider the control of phasic muscle activity in REM sleep.

1.3 MOTOR CONTROL AT THE LEVEL OF THE MOTONEURON

1.3.1 Postsynaptic inhibition of motoneurons during REM sleep

Motoneurons undergo a state-specific hyperpolarization after pontine or medullary stimulation (Chase et al., 1986; Fung et al., 1982; Jankowska et al., 1968; Llinas and Terzuolo, 1964, 1965). While these IPSPs occur in REM sleep, they are generated by electrical stimulation. If this ponto-medullary-generated inhibition is responsible for REM atonia, it must occur naturally during REM sleep.

The polarity of motoneurons in waking and sleep has been studied extensively. Intracellular recordings of lumbar motoneurons during natural sleep in cats reveals that the membrane potential gradually hyperpolarizes as the animal passes from waking to NREM sleep (Glenn et al., 1978). This gradual hyperpolarization continues throughout NREM sleep, following a roughly linear course, increasing in polarity as NREM continues. A rapid hyperpolarization of motoneurons is observed from NREM to REM sleep (Glenn and Dement, 1981a; Glenn et al., 1978; Morales and Chase, 1978). Arousal
Fig. 1.3. REM sleep without atonia in a cat. A continuous polysomnographic recording of a cat after bilateral pontine lesions. A, During NREM sleep, EMG activity is low. B, EMG tone increases during the transition into REM sleep. C, In REM sleep, while some atonia is present, excessive EMG activity is observed. Note that although EMG activity is elevated, other polysomnographic criteria of REM sleep, including a theta-rich EEG and rapid eye movements (EOG), are present throughout the state. (Adapted from Hendricks et al. 1982).
from REM sleep is accompanied by a reduction in motoneuron polarity. A similar pattern of polarization is observed in trigeminal motoneurons (Chase et al., 1980; Nakamura et al., 1978). The polarity of motoneurons, therefore, appears to follow a pattern that is similar to the stereotypical pattern of motor suppression that occurs across the sleep-wake cycle: motor output gradually decreases from waking to NREM sleep and is then potently suppressed in REM sleep (Figure 1.4).

Together, these studies indicate that motoneuron excitability is tightly controlled across the sleep-wake cycle. It is important to note, however, that these studies were strictly observational in nature. To address this, additional studies quantified the excitability of motoneurons across the different sleep-wake states. Using orthodromic activation to examine state-dependent variations in reflex excitability as well as antidromic activation to determine tonic changes in excitability, it was demonstrated that both lumbar and trigeminal motoneurons are less excitable in REM sleep (Chandler et al., 1980; Glenn and Dement, 1981a, b; Morales and Chase, 1981). This reduced excitability translates into a diminished probability of activation and a reduced responsiveness of motoneurons to depolarizing currents: lumbar motoneurons, for example, require 200% more depolarizing current to discharge in REM sleep than in NREM sleep or waking (Glenn and Dement, 1981b). The changes in excitability observed in REM sleep are indicative of increased membrane conductance, signifying that the inhibitory input responsible for REM atonia is an IPSP-generating mechanism (Chandler et al., 1980). These studies therefore confirm the observed reduction in motoneuron excitability during REM sleep (Glenn and Dement, 1981a; Glenn et al., 1978; Morales and Chase, 1978) and further these observations by attributing the robust hyperpolarization seen in REM sleep to postsynaptic inhibition.

1.3.2 Coactivation of excitatory and inhibitory synaptic drives

In addition to the potent suppression of basal muscle tone that is observed in REM sleep, this state is also characterized by phasic muscle twitches that break through the background atonia. Intracellular studies reveal changes in the membrane potential of motoneurons that coincide with these phasic muscle twitches (Glenn et al., 1978;
Fig. 1.4. Motoneurons are hyperpolarized in REM sleep. Intracellular recording of a lumbar motoneuron during sleep and wake. The membrane potential of motoneurons gradually decreases from waking to NREM and then is further decreased in REM sleep. (Adapted from Morales and Chase 1978).
Motoneurons are subjected to brief depolarizing events during rapid eye movement periods of REM sleep (Morales and Chase, 1978). The outward manifestation of these depolarizing events is muscle twitches. The majority of these depolarizing events are preceded by a well-defined hyperpolarizing shift in membrane potential (Chase and Morales, 1982, 1983; Morales and Chase, 1981). As such, it appears that postsynaptic inhibitory processes operate at the same time as postsynaptic excitatory processes to reduce or eliminate movement in this state. Thus, a phasic enhancement of inhibition occurs in conjunction with the tonic hyperpolarization during periods of phasic activity. These increases in the postsynaptic inhibition of motoneurons during phasic periods of REM sleep may reflect an enhancement of the inhibitory processes already present that are responsible for the atonia of this state or an additional mechanism at work.

1.3.3 Motor control at the motoneuron

Taken together, intracellular recordings reveal that the sleep-wake pattern of motoneuronal polarization corresponds well with the variation in EMG activity observed. This pattern of polarization occurs in both cranial and lumbar motoneurons and is associated with the reduction in motoneuron excitability observed in REM sleep. These data, together with electrical stimulation studies (Chase et al., 1986; Fung et al., 1982), support the notion that active motoneuronal inhibition is responsible, at least in part, for the suppression of motor tone during REM sleep. The cause of this inhibition, however, is not clear and will be the focus of this thesis.

In addition to the tonic hyperpolarization present throughout REM sleep, motoneurons are also subjected to phasic hyperpolarizing shifts in membrane potential (Chase and Morales, 1982, 1983; Morales and Chase, 1981). Muscle twitches are hypothesized to result when large increases in synaptic excitation overcome both the tonic and phasic inhibition present throughout this state. It is not known, however, if REM atonia and phasic muscle twitches are regulated by the same mechanism or if these two components of REM motor activity are controlled by two distinct mechanisms. This question will be examined in this thesis.
Finally, it is important to note that these intracellular studies only recorded a small subset of motoneurons continuously across multiple states (Glenn et al., 1978; Nakamura et al., 1978). The pattern of motoneuron polarity was primarily inferred from a composite of different cell impalements recorded over different sets of states, not from recordings of consecutive states. Therefore, how the excitability of motoneurons changes across REM sleep is not clear; while these studies indicate that the membrane potential changes during periods of phasic REM sleep (Glenn et al., 1978; Nakamura et al., 1978), the temporal pattern of motoneuron polarity across REM sleep is not known. This thesis will address this aspect by examining how motoneuron output changes across a REM period.

1.4 INHIBITION OF MOTONEURONS

It is thought that REM atonia is generated through the activation of descending inhibitory projections which act on motoneurons, resulting in motoneuron hyperpolarization and therefore motor suppression (Siegel, 2005). GABA and glycine are the principal fast inhibitory transmitters in the mammalian CNS, making them likely candidates responsible for this neurochemical suppression of muscle tone.

1.4.1 GABA and glycine

GABA and glycine exert their inhibitory effects by activating chloride (Cl⁻) ion channels, resulting in a local hyperpolarization of the neuronal membrane (Nicoll et al., 1990). While GABA and glycine both cause a rapid increase in Cl⁻ conductance, they act on functionally distinct receptors. GABA binds to GABA-specific Cl⁻ channels, known as GABA_A receptors, which are reversibly blocked by bicuculline and picrotoxin (Curtis et al., 1971). Glycine binds to its own specific ligand-gated Cl⁻ channel that is antagonized by strychnine (Werman et al., 1967). Together, GABA and glycine modulate neuronal excitability through the activation of these discrete postsynaptic Cl⁻ ion channels (Nicoll et al., 1990).

In addition to the classic GABA_A receptor, GABA also acts on two other pharmacologically distinct receptor subtypes, GABA_B and GABA_C receptors. GABA_B receptors are metabotropic receptors coupled to potassium (K⁺) channels via G proteins and second messenger systems: activation of GABA_B receptors results in an outward K⁺
current that is inhibitory in nature (Bormann, 1988; Mody et al., 1994; Nicoll et al., 1990). GABA\textsubscript{B} receptors mediate a slow onset, prolonged hyperpolarization in contrast to the faster IPSPs mediated by GABA\textsubscript{A} receptors (Kerr and Ong, 1995). GABA\textsubscript{C} receptors, like GABA\textsubscript{A} receptors, are ligand-gated Cl\textsuperscript{−} channels that mediate fast synaptic neurotransmission but they are insensitive to drugs that modulate GABA\textsubscript{A} and GABA\textsubscript{B} receptor function (Enz, 2001).

1.4.2 GABAergic and glycinergic inhibition of motoneurons

Iontophoretic application of glycine hyperpolarizes the membrane of motoneurons, inhibits orthodromic and antidromic activation and decreases cell firing (Curtis et al., 1968; Werman et al., 1967, 1968). Application of GABA onto motoneurons produces the same inhibitory effects (Curtis et al., 1968; Curtis et al., 1959). Therefore, motoneurons are inhibited by GABAergic and glycinergic mechanisms. This supports a possible role for GABA and/or glycine in the control of motoneuron excitability during sleep.

GABA\textsubscript{A}, GABA\textsubscript{B} and glycine receptors are ubiquitous to both spinal and cranial motoneurons (Rekling et al., 2000). While GABA\textsubscript{C} receptors occur on motoneurons, they appear to have little role in the regulation of motoneuron excitability (Rozzo et al., 2002) and therefore will not be considered in this thesis. Like GABA\textsubscript{A} receptors (Curtis et al., 1971), GABA\textsubscript{B} receptors have been shown to postsynaptically inhibit motoneurons (Haji and Takeda, 1993; Lalley, 1986), however, the literature focuses primarily on the role for GABA\textsubscript{A} receptors in the control of motoneuron excitability (Rekling et al., 2000). This is particularly true for investigations into the GABAergic control of motoneurons during sleep. Therefore, in addition to glycine receptors, both GABA\textsubscript{A} and GABA\textsubscript{B} receptors will be examined in this thesis for their role in the control of motoneurons during sleep.

1.5 THE ROLE FOR GABA AND GLYCINE IN REM SLEEP MOTOR CONTROL

GABA and glycine are the main inhibitory transmitters in the CNS. Both have been shown to hyperpolarize motoneurons and thus are possible candidates in the control of motoneurons during REM sleep. After ponto-medullary activation of descending GABAergic and/or glycinergic projections onto motoneurons, these transmitters would
act to hyperpolarize motoneurons, resulting in REM atonia (Siegel, 2005). A role for these inhibitory transmitters in mediating REM atonia is supported by the demonstration that there is an increase in the release of GABA and glycine in both cranial and spinal motor pools during pontine-induced REM sleep (Kodama et al., 2003). Pharmacological studies have further examined the roles for GABA and glycine in the control of REM sleep motor tone; the results from these studies, however, are difficult to interpret, as discussed below.

1.5.1 Glycinergic inhibition of motoneurons during REM sleep

Soja et al. (1987a) reported that microinjections of strychnine, the glycinergic antagonist, onto trigeminal motoneurons can prevent the suppression of the masseteric reflex that is normally seen in REM sleep (Chase et al., 1968). Building on these results, it was demonstrated that the REM-specific hyperpolarizing potentials generated in lumbar motoneurons after medullary stimulation (Chase et al., 1986) are suppressed by glycinergic antagonism (Soja et al., 1987b). The IPSPs that occur spontaneously during REM sleep can also be blocked by strychnine (Chase et al., 1989). Further examination of the effects of glycine on the membrane properties of motoneurons in REM sleep demonstrated that strychnine not only decreases IPSP activity but also reduces the degree of hyperpolarization and prevents the increase in rheobase and decrease in input resistance normally seen in REM sleep, thus preventing the REM sleep-related reduction in motoneuronal excitation (Soja et al., 1991). In addition to tonic hyperpolarization, enhancement of inhibition occurring during the phasic portions of REM sleep is also blocked by strychnine (Chase and Morales, 1982, 1983; Lopez-Rodriguez et al., 1992; Lopez-Rodriguez et al., 1990; Morales and Chase, 1981). From these studies, it was therefore concluded that glycine plays a key role in the control of both trigeminal and lumbar motoneurons during REM sleep.

Closer examination of this intracellular work, however, suggests that glycine may not play as important of a role in mediating REM atonia as previously thought. First, while glycinergic antagonism did block spontaneously occurring IPSPs, this only occurred in 32% (6/19) of motoneurons tested (Chase et al., 1989). The remaining 68% of recorded cells still had residual IPSP activity after blockade with strychnine.
Furthermore, motoneuron hyperpolarization was reduced with strychnine, however it was not prevented (Soja et al., 1991): a small but significant reduction in polarity still occurred in REM sleep in the presence of strychnine. This variability in motoneuron response as well as the inability to completely prevent motoneuron hyperpolarization with strychnine indicates that glycine is not the exclusive factor responsible for REM atonia.

1.5.2 GABAergic inhibition of motoneurons during REM sleep

Intracellular studies also examined the role for GABAₐ-mediated inhibition in regulating REM sleep motor control. GABAergic antagonists, like the glycinergic antagonist, prevent the suppression of the masseteric reflex normally seen in REM sleep (Soja et al., 1987a). The authors of this study concluded, however, that GABA is not involved in REM atonia based on the observation that GABAergic antagonism produced a nonspecific increase in reflex amplitude across the sleep-wake cycle in 7 out of 9 experiments, whereas the effects of glycine were said to be specific to REM sleep (Soja et al., 1987a). While the effects of the glycinergic antagonism were specific to REM sleep in 5 out 10 experiments, the remaining 5 experiments showed quantitatively similar increases in reflex amplitude in waking as seen with GABAergic antagonism. Thus, if GABAergic mechanisms were ruled out based on the non-specific effects of the antagonists, it seems the role for glycine may also need to be reconsidered. Furthermore, it is reported that GABAergic antagonism, unlike glycinergic antagonism, has no effect on REM-specific IPSPs in lumbar motoneurons (Chase et al., 1989). However, as discussed above, glycinergic antagonism only completely blocked IPSPs in 32% of motoneurons tested (Chase et al., 1989). Therefore, while intracellular studies do not support a role for GABA in the control of muscle atonia in REM sleep, the role for a glycinergic mechanism also does not appear to be conclusive.

1.5.3 Inhibition at a respiratory motor pool during REM sleep

Studies examining the biochemical nature of respiratory motor control in sleep argue against a role for GABA and glycine in the control of REM atonia. Hypoglossal motoneuron activity is suppressed during carbachol-induced atonia (Kimura et al., 1990). Microinjections of bicuculline, a GABAₐ receptor antagonist, into the hypoglossal motor
nucleus during carbachol-induced atonia has no effect on motoneuron activity (Kubin et al., 1993). This is in agreement with intracellular studies indicating that GABAergic mechanisms are not responsible for REM atonia in cranial and lumbar motoneurons (Chase et al., 1989; Soja et al., 1987a). The effect of glycinergic antagonism on the carbachol-induced suppression of hypoglossal motoneuron activity is less clear. While one study reports that strychnine can reverse the carbachol-induced depression of hypoglossal motor activity (Yamuy et al., 1999), others argue that glycinergic antagonism does not abolish, or even reduce, this suppression in activity (Fenik et al., 2005a; Kubin et al., 1993). Further evidence against a role for GABA and glycine in mediating REM atonia comes from studies in the intact, naturally sleeping rat. Studies in which bicuculline, strychnine, or both are perfused into the hypoglossal motor nucleus across the sleep-wake cycle demonstrate that REM atonia persists in the absence of GABAergic and/or glycinergic inhibition (Morrison et al., 2003a; Morrison et al., 2003b). These latter studies are of particular importance because they, unlike the intracellular studies, quantified changes in motor output resulting from manipulations at the motor pool. Without this causal physiological verification, the mechanisms underlying REM-specific motor suppression can only be correlated with state-specific changes in motoneuron excitability. Taken together, these studies argue against a role for GABAergic and glycinergic inhibition of motoneurons in the motor suppression of REM sleep, at least at a respiratory motor pool.

1.5.4 Role for inhibition during phasic REM sleep

While in vivo studies at the hypoglossal motor pool indicate no role for inhibitory amino acids in the control of REM atonia, there is an increase in extracellular levels of GABA and glycine in and around motor nuclei during REM sleep (Kodama et al., 2003). If GABA and glycine are not involved in mediating REM atonia, their REM-specific increase in release may indicate their involvement in a different aspect of motor output. One possibility is in the phasic inhibition of motoneurons that is thought to oppose excitatory inputs in REM sleep (Chase and Morales, 1982, 1983; Morales and Chase, 1981). In support of this, Kubin et al. (1993) report a strong disinhibitory effect of bicuculline and strychnine on motoneuron activity evoked by lingual nerve stimulation in
the carbachol model. Thus, when strong excitatory inputs impinge on motoneurons, inhibition may act to counteract these inputs, as when inhibition is removed these inputs garner larger responses. In further support of a role for GABA and glycine in regulating phasic muscle activity in REM sleep, removal of inhibition at the motor pool during REM sleep in behaving animals results in an increased proportion of time spent in phasic REM sleep (Morrison et al., 2003a). In other words, removal of inhibitory inputs increases the amount of phasic muscle activity in REM sleep. Collectively, these findings suggest the potential involvement of GABA and glycine in the modulation of phasic REM activity.

1.5.5 Role for GABA and glycine in REM sleep motor control

Together, these studies give insight into the neurochemical mechanisms that mediate muscle control in sleep; it is clear, however, that the biochemical substrates of REM atonia are not completely understood. While most studies seem to agree that GABA is not involved in mediating REM atonia, there is not a consensus in the literature on the role for glycine in the control of muscle tone during REM sleep. Further work examining the roles of GABA and glycine in mediating REM atonia is needed. In addition, the contribution of GABAergic and glycinergic mechanisms in the control of phasic muscle activity should be investigated. Finally, investigations into the role of GABA in REM sleep motor control have only considered GABA_A receptors; the involvement of GABA_B receptors should also be examined.

1.6 THESIS OVERVIEW

As seen above, there are conflicting reports on the roles of GABAergic and glycinergic inhibition in the control of muscle activity in REM sleep. Both GABA and glycine increase extracellularly in and around motor nuclei during pontine-induced REM sleep (Kodama et al., 2003), implicating these inhibitory amino acids in REM sleep motor control. Intracellular studies examining the trigeminal and lumbar motor pools, however, have concluded that only glycine is involved in mediating REM atonia (Chase et al., 1989; Soja et al., 1987a; Soja et al., 1991; Soja et al., 1987b). This has become the predominate hypothesis in the literature (Chase and Morales, 2005). Challenging this are
studies at the hypoglossal motor nucleus: this work does not support a role for either GABAergic or glycinergic mechanisms in the control of REM atonia (Fenik et al., 2005a; Kubin et al., 1993; Morrison et al., 2003a; Morrison et al., 2003b). As such, further investigation into the roles of GABA and glycine in mediating REM atonia is needed.

1.6.1 The trigeminal-masseteric motor system

This thesis will investigate what role, if any, GABA and glycine have in the neurochemical control of muscle tone during REM sleep. To do this, trigeminal motor nuclei, which innervate the masseter muscle, will be used. This system is advantageous because the REM-specific inhibitory control of the trigeminal-masseteric motor system has been well documented by intracellular work (Chandler et al., 1980; Chase et al., 1968; Nakamura et al., 1978; Soja et al., 1987a). By using this system, comparisons can be made with these other studies utilizing the trigeminal system. Furthermore, a similar experimental system will be used to study motor control at the trigeminal motor nucleus as was used to study motor control at the hypoglossal motor nucleus (Morrison et al., 2003a; Morrison et al., 2003b); by using similar methods at a different motor pool, insight into mechanistic differences that may exist between these non-respiratory and respiratory motor nuclei will be gained. This is important because there are multiple motor systems with multiple functions and it is not known if all systems are controlled by similar mechanisms in sleep. For example, while a differential regulation of motor tone has been shown in different muscle groups (Lu et al., 2005), it has been demonstrated that lumbar and trigeminal motor pools are subjected to comparable inputs during REM sleep (Burgess et al., 2008; Soja et al., 1995). This thesis will therefore provide further insight into the REM-specific control of different muscles.

Technically, the trigeminal-masseteric motor system is an ideal system for studies of this nature. Masseter muscle activity follows the stereotypical pattern of muscle tone across the sleep-wake cycle, being highest in waking and lowest in REM sleep. As well, masseter activity in naturally behaving animals is successfully recorded in our laboratory (Brooks and Peever, 2008a; Burgess et al., 2008) and the trigeminal motor nucleus is large and easily accessed with a microdialysis probe.
1.6.2 Objectives of thesis

This work will be performed in freely moving, naturally sleeping animals and thus will provide a physiologically relevant account of the mechanisms at work in natural REM sleep. Both a pharmacological rat model and a genetic mouse model will be used to examine the contribution of inhibitory amino acids to motor control in sleep. In addition to the masseter muscle, the neck and hindlimb muscles will also be studied; this will allow for comparisons of GABAergic and glycinergic control of motor output by cranial and spinal motor pools, giving a broader scope on REM-mediated changes in motoneuron activity. Collectively, the goal of this thesis is to provide a detailed description of the involvement of inhibitory influences in REM sleep motor control. The specific research objectives are as follows:

Objective 1 (Chapter 3): To determine the contribution of glycinergic and GABA$_A$-mediated inhibition to REM sleep motor control.

Objective 2 (Chapter 4): To examine the role that GABA$_B$ receptors play in the regulation of REM atonia.

Objective 3 (Chapter 5): To describe the time course of motor events occurring across REM sleep with specific focus on the contribution of inhibition to the temporal pattern of muscle activity.

Objective 4 (Chapter 6): To describe the sleep motor phenotype of a transgenic mouse model in which glycinergic and GABA$_A$-mediated transmission is severely downregulated.
CHAPTER TWO: EXPERIMENTAL METHODS

2.1 ANIMALS

All of the work presented in this thesis was performed in freely moving, naturally sleeping animals. All procedures and experimental protocols were approved by the University of Toronto’s animal care committee and were in accordance with the Canadian Council on Animal Care. Animals were cared for under the supervision of the support staff of the Bioscience Support Facility (University of Toronto, Toronto, Canada). All animals were maintained at a room temperature of 20 ± 1°C with 60% humidity on a 12:12 light dark cycle (lights on 7:00, lights off 19:00). Food (Lab Diet; PMI Nutrition International, St. Louis, MO, USA) and water were available ad libitum. Rats were housed individually in plastic cages (dimensions 47.5cm x 25.5cm x 20cm, Nalgene Labware, Nagle Nunc International, Rochester, NY, USA) equipped with standard cob bedding. Mice were group-housed with up to five same-sex siblings in plastic cages (dimensions 28cm x 16cm x 12cm, Nalgene Labware) on standard cob bedding.

2.1.1 Rats

Experiments in Chapters 3-5 were carried out on male Sprague-Dawley rats bred in-house (Bioscience Support Facility). In total, 84 rats were included in these experiments, ranging from 9-11 weeks in age (average weight: 373.7 ± 4.9g).

2.1.2 Mice

Chapter 6 used a transgenic mouse line (tg271Q-300) maintained on a C57BL/6 background. These mice were previously created to study how reduced glycine receptor function affects the startle response (Becker et al., 2002). This was done by incorporating a mutant human glycine receptor subunit transgene into the mouse genome (C57BL/6). The mutant subunit was generated by substituting a glutamine for an arginine at position 271 in the extracellular domain of the glycine receptor α1 subunit (Becker et al., 2002). The mutant glycine receptor subunit was then expressed throughout the brain and spinal cord of transgenic mice under the control of the neuron-specific elements of the Thy-1
promoter (Becker et al., 2002). This results in a 70% reduction in glycinergic transmission (Becker et al., 2002). For unknown reasons, an even larger reduction in GABA<sub>A</sub>-receptor-mediated inhibitory transmission is also seen (90% reduction). Thus, the expression of the mutant human glycine receptor α1 subunit in these transgenic mice results in an overall functional downregulation of postsynaptic inhibition (Becker et al., 2002).

The founder line of transgenic mice was donated to our lab by Dr. Hans Weiher (Institut für Diabetesforschung, Munich, Germany). Mice were bred in-house (Bioscience Support Facility) and the colony was maintained by breeding wildtype females with heterozygous transgene carrier males, as this was found to be the most successful breeding protocol. Animals were phenotyped visually, as transgenic animals display an instantly apparent phenotype when handled. Firstly, when picked up by the tail, transgenic mice display a hind feet clench behaviour (Figure 2.1), which is very distinct from the response of wildtype mice (Figure 2.1). Furthermore, transgenic mice develop handling-induced tremors. Together, these overt behaviours allow for visual phenotyping of transgenics and their wildtype littermates (Becker et al., 2002). A subset of animals were genotyped for confirmation of the visual phenotyping using PCR (35 cycles, 55°C annealing temperature) with glycine receptor α1 specific primers designed based on the endogenous murine receptor (5’-TGCAAAACCCACAAGAACA-3’ and 5’-TGGCATTGTAAGGTTGTA-3’; common to both wildtype and transgenic animals) and mutant human receptor (5’-TATTCCCAGCTGCTTG-3’; 5’-CGCCCCTTGACTGATGCCA-3’; only present in transgenic mice; mutant primer sequences provided by Dr. Weiher). These experiments used 23 male transgenic mice (22.8 ± 0.3g) and 23 male wildtype littermates (29.9 ± 0.6g) between 16-24 weeks in age.

2.2 SURGICAL PROCEDURES
Aseptic surgery was carried out to instrument animals for sleep-wake studies. All preoperative and postoperative surgical protocols conformed to the guidelines on survival rodent surgery set by the Division of Comparative Medicine (University of Toronto,
Fig. 2.1. Phenotypic characteristic of wildtype vs transgenic mice. When a wildtype mouse is picked up by the tail, the forelimbs are held downwards and the hindlimbs spread apart (A). Conversely, transgenic mice (B) display hind feet clenching when picked up by the tail.
Toronto, Canada). Surgeries were performed in an isolated surgical room. All surgical instruments were sterilized prior to use.

2.2.1 Instrumentation of rats

The rats used in Chapters 3-5 were surgically instrumented with electroencephalographic (EEG) and masseter and neck electromyographic (EMG) electrodes to record muscle tone across the sleep-wake cycle. A microdialysis probe was also implanted into all rats: probes were placed in the left trigeminal motor nucleus for manipulation of motoneurons with candidate drugs during sleep and wake (Figure 2.2). The procedures detailed below are the same as those outlined in published works from our laboratory (Brooks and Peever, 2008a; Burgess et al., 2008).

To implant electrodes and a microdialysis probe, sterile surgery was performed under anesthesia induced with intraperitoneal (i.p.) ketamine (85mg/kg) and xylazine (15mg/kg), and maintained with additional anesthesia given by inhalation (isoflurane, 0.5-2%). Effective depth of anesthesia was determined by the abolishment of the pedal withdrawal and blink reflexes. Body temperature was monitored with a rectal probe (CWE Inc., Ardmore, PA, USA) and maintained at 37 ± 1ºC.

Three insulated, multi-stranded stainless steel wire EMG electrodes (Cooner Wire, Chatsworth, CA, USA) were implanted into the left and right masseter muscles. The wires were tunneled subcutaneously to an incision along the dorsal surface of the cranium. Three EMG electrodes were also inserted into the neck muscle. Animals were positioned into a stereotaxic apparatus (KOPF, Tujunda, CA, USA) and four stainless steel screws (JI Morris Co., Southbridge, MA, USA), attached to insulated 34 gauge wire (Cooner Wire) were implanted in the skull for recording cortical EEG; their coordinates were: 2mm rostral and 2mm to the left and right of bregma, and 3mm caudal and 2mm to the left and right of bregma. An additional reference electrode was also secured onto the skull; its position was: 9.4mm caudal and 0.5mm lateral to bregma.

To implant a microdialysis probe into the left trigeminal motor nucleus a ~2mm burr hole was made at 9.4 mm caudal and 1.8 mm lateral to bregma (Paxinos and Watson, 1998). A microdialysis guide probe (CMA Inc., St. Laurent, QC, Canada) was
Fig. 2.2. Schematic representation of a surgically instrumented rat. Rats are implanted with EEG and EMG electrodes into the left and right masseter as well as the neck muscle. All electrodes are connected to a headplug so that sleep-wake state (via EEG) and the corresponding muscle activity (via EMG) can be determined. A microdialysis probe is implanted into the left trigeminal motor nucleus, which innervates the left masseter muscle, allowing for the application of aCSF or candidate drugs onto trigeminal motoneurons across the sleep-wake cycle. A close-up of the microdialysis probe is shown: the tip of the probe has a semi-permeable membrane that is 250µm in diameter and 1mm in length. As left trigeminal motoneurons are pharmacologically manipulated via reverse microdialysis, left masseter muscle activity is monitored and compared to baseline conditions. Right masseter and neck muscles serve as additional controls. (Adapted from Popesko et al. 1992)
then lowered 8.2mm below the skull surface by stereotaxic manipulation. Dental cement (1234, Lang Dental, Wheeling, IL, USA) secured the probe in place and after the cement was dry, EEG and EMG electrodes were connected to pins (Allied electronics, Fort Worth, TX, USA) and inserted into a custom-made head-plug (Allied electronics) that was affixed to the skull with dental cement.

After surgery rats were given a subcutaneous injection of 0.03 mg/kg buprenorphin and kept warmed by a heating pad. Food and water were available \textit{ad libitum}. They were also given a dietary supplement (i.e., Nutri-Cal) and soft food for the following 2 days. Rats recovered for at least 7-10 days before experimental testing began.

\textbf{2.2.2 Instrumentation of mice}

Mice used in Chapters 6 were surgically instrumented with EEG and masseter, hindlimb and neck EMG electrodes to record muscle tone across the sleep-wake cycle (Figure 2.3).

To instrument mice, animals were anesthetized using isoflurane (1-2%), positioned into a stereotaxic apparatus (KOPF), and implanted with EEG and EMG electrodes. EEG recordings were obtained using four stainless steel micro-screws (1mm anterior ±1.5mm lateral to bregma; 3mm posterior ±1.5mm lateral to bregma). EMG electrodes were made from multistranded stainless steel wires (AS131, Cooner Wire), which were sutured onto masseter, hindlimb and neck muscles. All electrodes were attached to a micro-strip connector (CLP-105-02-L-D, Electrosonic, Toronto, ON, Canada), which was affixed onto the animals head with dental cement (Ketac-cem, 3M, London, ON, Canada).

After surgery, mice were given ketoprofen (3mg/kg) and 5% dextrose in 0.9% saline. Mice were individually housed in a sound-attenuated and ventilated chamber on a 12:12 light-dark cycle (110 lux; lights on 7:00, lights off 19:00) for 10 days post surgery. Food and water were available \textit{ad libitum}. They were also given a dietary supplement (i.e., Nutri-Cal) and soft food for the 2 days following surgery.
Fig. 2.3. Schematic representation of a surgically instrumented mouse. Mice are implanted with EEG and EMG electrodes into the masseter, hindlimb and neck muscles. All electrodes are connected to a headplug so that sleep-wake state (via EEG) and the corresponding muscle activity (via EMG) can be determined. (Adapted from Popesko et al. 1992).
2.3 EXPERIMENTAL PROCEDURES AND SLEEP RECORDING

2.3.1 Recording environment

During experiments, animals were housed in Ratum® system (BAS, Inc., West Lafayette, IN, USA), which is a movement-responsive caging system eliminating the need for a commutator or liquid swivel. This caging system was housed inside a sound-attenuated, ventilated, and illuminated (lights on: 110 lux) chamber.

2.3.2 Data acquisition

EEG and EMG activities were recorded by attaching a lightweight cable to the plug on the animal’s head, which was connected to a Super-Z head-stage amplifier and BMA-400 AC/DC Bioamplifier, (CWE Inc.). The EEG was amplified 1000 times and band-pass filtered between 1 and 100 Hz. EMG signals were amplified between 500-1000 times and band-pass filtered between 30 Hz and 30 kHz (Chapters 3-5) and 30 Hz and 500 Hz (Chapter 6). All electrophysiological signals were digitized (Spike 2 Software, 1401 Interface, CED Inc., Cambridge, UK) at 500 Hz (Chapters 3-5) and at 1000 Hz (Chapter 6) and monitored and stored on a computer. In mice (Chapter 6) infrared video recordings were captured (Sony DCR-HC28) and synchronized with the electrophysiological recordings.

2.3.3 Microdialysis probe

In Chapters 3-5, rats were instrumented with a microdialysis probe for the pharmacological manipulation of trigeminal motoneurons with candidate drugs. The microdialysis probe (34KDa cut-off; membrane length and diameter: 1mm by 250 µM, CMA Inc.) was placed into the left trigeminal motor nucleus. The microdialysis probe was connected to teflon tubing (inside diameter=0.12mm; Eicom, Japan), which was connected to a 1mL gastight syringe via a liquid switch (BAS Inc.). The probe was continually perfused with filtered (0.2µm Nylon, Fisher Scientific, Ottawa, ON, Canada) artificial cerebral spinal fluid (aCSF: 125mM NaCl, 5mM KCl, 1.25mM KH$_2$PO$_4$, 24mM NaHCO$_3$, 2.5mM CaCl$_2$, 1.25mM MgSO$_2$, 20mM D-glucose) at a flow rate of 2µl/min using a syringe pump (BAS Inc.).
2.3.4 Sleep recording in rats

For Chapters 3-5, each experiment took 2 days to complete. On the first day at 8:00-10:00h, rats were placed into a plastic bowl (MD-1514 Rodent Bowl Kit; Bas, Inc.) in the sleep recording chamber and given at least one hour to habituate before they were connected to the electrical tether. They were then given a minimum of 3 hours to habituate to this before recordings began. Baseline recordings (without the microdialysis probe in place) were established on day 1 of experiments, between 13:00-16:00h. The microdialysis probe was inserted at 17:00h and aCSF perfused throughout the night. Probes were inserted the night before experiments began because previous studies demonstrate that probe insertion induces spontaneous neurotransmitter release and local neuronal activation (Di Chiara, 1990; Kodama et al., 1998).

On the second day of experimentation, perfusion of candidate drugs began at 8:00 – 9:00h. Drug treatments were randomized. Each drug was applied onto the trigeminal nucleus for 2-4 h; this allowed sufficient time for the animal to transition through at least 3 complete sleep cycles (i.e., wake to NREM to REM sleep). An aCSF washout period of at least 2 hours followed every drug treatment.

2.3.5 Sleep recording in mice

For the mice used in Chapter 6, animals were transferred to a round plexi-glass cage (diameter: 20cm) inside a sound-attenuated, ventilated, and illuminated (lights on: 110 lux) chamber at the start of each experiment. Each mouse was given 24 hours to habituate to this new environment. Animals were then tethered, using a lightweight tether attached to the Ratern® system (BAS, Inc.). Mice were given a further 24 hours to habituate to the recording tether and then 24 hours of undisturbed EEG and EMG recordings synchronized with infrared video recordings were obtained.

2.4 DATA ANALYSIS

2.4.1 Behavioural state

I classified 4 behavioural states. Active wake (AW) was characterized by high frequency, low voltage EEG signals coupled with high levels of EMG activity (i.e., chewing,
grooming, drinking). Quiet wake (QW) was characterized by high frequency, low voltage EEG signals but in the absence of overt motor activity. NREM sleep was characterized by high amplitude, low frequency EEG signals and minimal EMG activity. REM sleep was characterized by low amplitude, high frequency theta-like EEG activity and REM atonia interspersed by periodic muscle twitches. Sleep states were visually identified and analyzed in 5-sec epochs using the Sleepscore v1.01 script (CED Inc.).

In Chapter 6, because transgenic mice displayed excessive motor activation in REM sleep, further steps were taken to ensure the accuracy of behavioural state identification. REM sleep was identified using the EEG and EMG criteria described above, with the additional aid of video footage. As in wildtype mice, episodes of identified REM sleep always followed periods of NREM sleep. Furthermore, post hoc analyses of REM sleep characteristics were performed to confirm the identification of this behavioural state. These included comparisons of REM sleep characteristics (ie. percentage of total time spent in REM sleep and EEG spectral profile) between wildtype and transgenic mice as well as contrasting differences in waking and REM sleep in transgenic animals (ie. length of waking vs. REM bouts and EEG spectral profiles of each state). Similar criteria were used to identify REM sleep in the REM sleep without atonia cat lesion model (Henley and Morrison, 1974).

2.4.2 EMG analysis

Raw EMG signals were full-wave rectified, integrated and quantified in arbitrary units (a.u.). Average EMG activity was quantified in 5s epochs for each behavioural state. For the additional analysis of REM sleep (see section 2.4.3), EMG activity was further quantified in 10ms epochs. When candidate drugs were applied onto the left trigeminal motor pool (Chapters 3-5), EMG data were not analyzed for the first 30 minutes of perfusion because the latency from the syringe pump to the microdialysis probe is ~15 minutes. When pharmacological manipulations were made, at least three episodes of each behavioural state (i.e., AW, QW, NREM and REM) were analyzed for each experimental condition. In each animal, the average EMG activity was calculated for each behavioural state for each manipulation performed.
2.4.3 EMG Analysis in REM sleep

EMG activity in REM sleep consists of two motor components: the stereotypical periods of motor atonia occur during tonic REM sleep and the occasional muscle twitches that punctuate the background atonia occur during phasic REM sleep (i.e., during rapid eye-movements) (Aserinsky and Kleitman, 1953; Jouvet, 1967). Because the main aim of this thesis was to determine the role for inhibition in modulating motor activity in REM sleep, a method for identifying and quantifying the phasic (i.e., muscle twitches) and tonic (REM atonia) periods of REM sleep was developed.

To do this, all REM episodes were identified and the EMG activity in each episode was quantified in 10ms epochs. To differentiate between tonic and phasic periods of EMG activity, the first 5 seconds of the REM episode in which no phasic muscle twitches occurred was identified. In almost all REM periods, this occurred during the first 5 seconds of the REM episode, as muscle twitches are conspicuously absent during this time. After identifying the first 5s of non-phasic activity, the 99th percentile of EMG activity occurring during this 5s was determined. Since EMG activity was quantified in 10ms epochs, this meant ranking the values of EMG activity that occurred in all 500 epochs (ie. 10ms epochs over 5s) and determining the value at which 99% of these 500 values fell below. This calculated value was then used as the threshold to separate tonic and phasic activity. The muscle twitches that define phasic REM sleep were classified as motor events that exceeded this threshold value (ie. exceeded the 99th percentile); conversely, tonic REM activity (ie. REM atonia) was classified as any period in which muscle activity was equal to or less than the threshold value (ie. less than the 99th percentile). In every animal, the threshold value was calculated for each REM period and REM atonia and muscle twitches were quantified for each REM episode during baseline and for each pharmacological manipulation performed.

2.5 VERIFICATION OF MICRODIALYSIS PROBE LOCATION

2.5.1 Chemical stimulation of motoneurons

In Chapters 3-5, rats were instrumented with microdialysis probes to pharmacologically manipulate trigeminal motoneurons across the sleep-wake cycle. Two procedures were
used to demonstrate that microdialysis probes were both functional and located in the left trigeminal motor pool. At the end of each experiment, 0.1mM AMPA, a potent glutamatergic AMPA receptor agonist (α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid, FW: 186.17; Tocris, Ellisville, MO), was perfused into the left trigeminal motor pool. AMPA induced a rapid and potent increase in basal levels of left masseter muscle tone without affecting either the right masseter or neck EMG activity. This result verified that trigeminal motoneurons were viable and able to respond to glutamatergic activation, that microdialysis probes were functional at the end of each experiment and that probes were located in the trigeminal motor nucleus.

2.5.2 Histology

Post-mortem histological analysis was also used to demonstrate that microdialysis probes were physically located in the left trigeminal nucleus. Under deep anaesthesia (ketamine: 85mg/kg and xylazine: 15mg/kg, i.p.) rats were decapitated, their brains removed and placed in chilled 4% paraformaldehyde (in 0.1M PBS) for 24 hours. Brains were cryoprotected in 30% sucrose (in 0.1M PBS) for 48 hours; they were then frozen in dry-ice and transversely sectioned in 40μm slices using a microtome (Leica, Richmond Hill, ON, Canada). Brain sections were mounted, dried and stained with Neutral Red. Tissue sections were viewed using a light microscope (Olympus, Center Valley, PA, USA) and the location of probe lesion tracts were plotted on standardized brain maps (Paxinos and Watson, 1998).

2.6 STATISTICAL ANALYSES

Statistical analyses specific to each experiment are outlined in the Methods sections of Chapters 3-6.

2.7 EXPERIMENTAL PROTOCOLS

Experimental protocols, drug preparations and data analyses specific to each experiment are outlined in the Methods sections of Chapters 3-6.
CHAPTER THREE: GLYCINERGIC AND GABA\textsubscript{A}-MEDIATED INHIBITION OF SOMATIC MOTONEURONS DOES NOT MEDIATE REM SLEEP MOTOR ATONIA

This chapter has been adapted from a published manuscript in the Journal of Neuroscience (Brooks and Peever, 2008a)

3.0 SUMMARY

A hallmark of REM sleep is a potent suppression of postural muscle tone. Motor control in REM sleep is unique because it is characterized by flurries of intermittent muscle twitches that punctuate muscle atonia. Because somatic motoneurons are bombarded by strychnine-sensitive inhibitory post-synaptic potentials during REM sleep, it is assumed that glycinergic inhibition underlies REM atonia. However, it has never been determined whether glycinergic inhibition of motoneurons is indeed responsible for triggering the loss of postural muscle tone during REM sleep. Therefore, I used reverse-microdialysis coupled with polysomnography to determine if glycinergic and/or GABA\textsubscript{A}-mediated neurotransmission at the trigeminal motor pool regulates masseter muscle atonia during REM sleep in rats. By antagonizing glycine and GABA\textsubscript{A} receptors on trigeminal motoneurons, I unmasked a tonic glycinergic/GABA\textsubscript{A}ergic drive at the trigeminal motor pool during waking and NREM sleep. Blockade of this drive potently increased masseter muscle tone during both waking and NREM sleep. This glycinergic/GABA\textsubscript{A}ergic drive was immediately switched-off and converted into a phasic drive during REM sleep. Blockade of this phasic drive potently provoked muscle twitch activity in REM sleep. It did not, however, prevent or reverse REM atonia. Muscle atonia in REM even persisted when glycine and GABA\textsubscript{A} receptors were simultaneously antagonized and trigeminal motoneurons were directly activated by glutamatergic excitation, indicating that a powerful, yet unidentified, inhibitory mechanism overrides motoneuron excitation during REM sleep. My data refute the prevailing hypothesis that REM atonia is caused by glycinergic inhibition. The inhibitory mechanism mediating REM atonia therefore requires re-evaluation.
3.1 INTRODUCTION

A defining feature of mammalian rapid eye-movement (REM) sleep is a potent suppression of postural muscle tone. Compared to waking and non-rapid eye-movement (NREM) sleep, motor control in REM sleep is unique because it is characterized by flurries of periodic muscle twitches that punctuate REM sleep atonia. Determining the neural mechanisms mediating muscle atonia is of major clinical importance because abnormal motor control during REM sleep underlies some of the major sleep disorders, including REM sleep behaviour disorder (RBD), narcolepsy, obstructive sleep apnea (OSA) and bruxism.

There is considerable controversy concerning the neural mechanisms generating muscle atonia in REM sleep. The prevailing hypothesis is that glycinergic inhibition of somatic motoneurons is responsible for loss of postural muscle tone in REM sleep (Chase and Morales, 2005). This hypothesis stems from observations that trigeminal and lumbar motoneurons are hyperpolarized by large amplitude inhibitory postsynaptic potentials (IPSPs) that are reduced, but not eliminated by antagonism of glycine receptors (Chase et al., 1989; Soja et al., 1987a).

Studies of respiratory motor control in sleep, however, have shown that neither glycinergic nor GABAergic inhibition at the hypoglossal motor pool mediates the typical suppression of inspiratory genioglossus muscle activity during natural or carbachol-induced REM sleep (Kubin et al., 1993; Morrison et al., 2003a). Although inspiratory muscle suppression in REM sleep is not caused by glycinergic/GABAergic inhibition of respiratory motoneurons, it has never been determined whether the REM-specific IPSPs recorded from non-respiratory motoneurons (e.g., trigeminal) are in fact responsible for triggering REM atonia in postural muscles.

I aimed to determine whether masseter muscle atonia during REM sleep is caused by glycinergic and/or GABAergic inhibition of trigeminal motoneurons in freely-behaving rats. The trigeminal-masseteric motor system was used because trigeminal motoneurons innervate masseter muscles, which are postural muscles that are affected by, and contribute to, the pathogenesis of several sleep disorders including RBD, OSA, cataplexy/narcolepsy and bruxism (Guilleminault et al., 1974; Horner, 1996; Kato et al., 2003; Schenck and Mahowald, 2002). Importantly, the glycinergic and GABAergic
control of trigeminal motoneurons has also been well-documented during natural REM (Chase et al., 1989; Soja et al., 1987a).

I used reverse-microdialysis coupled with polysomnography to determine whether changes in glycinergic and GABA$_A$-mediated neurotransmission at the trigeminal motor pool are responsible for loss of masseter muscle tone during REM sleep. First, I identified the presence of a tonic glycinergic and GABAergic tone at the trigeminal motor pool during waking and NREM sleep by antagonizing glycine and GABA$_A$ receptors on trigeminal motoneurons. Then, I demonstrated that this tonic inhibitory tone is transformed into a phasic inhibitory drive in REM sleep. Blockade of glycinergic and GABA$_A$-mediated inhibition at the trigeminal motor pool during REM sleep triggered a potent increase in periodic muscle twitches; it did not, however, prevent REM atonia. My data therefore confirm the presence of a phasic inhibitory drive during REM sleep but do not support the prevailing hypothesis that glycinergic inhibition of motoneurons is responsible for postural muscle atonia in REM sleep.

3.2 METHODS

3.2.1 Animals

Studies were performed using 19 male Sprague Dawley rats (average weight: 342 ± 10g). Animals were surgically instrumented and recorded by the methods described in Chapter 2, sections 2.2-2.4.

3.2.2 Drug preparation

Strychnine (strychnine hydrochloride, FW: 370.9; Sigma-Aldrich, Oakville, ON) a glycine receptor antagonist; bicuculline ((-)bicuculline methochloride, FW: 444.87; Tocris, Ellisville, MO) a GABA$_A$ receptor antagonist; glycine (FW: 75.07; Tocris), and muscimol (MW: 121.31; Tocris) a GABA$_A$ receptor agonist were prepared in aCSF. Each drug was made immediately prior to each experiment. The AMPA receptor agonist (α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid, FW: 186.17; Tocris) was made in advance and stored in stock solutions at -20ºC. All drugs were vortexed and filtered (0.22µm PVDF, Fisher Scientific) before use.
3.2.3 Experimental protocols

3.2.3.1 Study 1 – Is glycinergic inhibition of somatic motoneurons responsible for REM atonia?
To answer this question I antagonized glycine receptors by perfusing 0.1mM strychnine into the left trigeminal motor pool while monitoring left masseter muscle EMG activity during sleep and waking. I used this concentration of strychnine because previous in-vitro and in-vivo studies show that it antagonizes glycinergic neurotransmission on somatic motoneurons (Jonas et al., 1998; Morrison et al., 2002; Song and Huang, 1990).

3.2.3.2 Study 2 - Is GABA_A-mediated inhibition of motoneurons responsible for REM atonia?
I antagonized GABA_A receptors by applying 0.1mM bicuculline into the left trigeminal motor pool while monitoring left masseter muscle EMG activity across the sleep cycle. This dosage of bicuculline was previously shown to antagonize GABA_A-mediated neurotransmission on somatic motoneurons in-vitro and at the hypoglossal motor pool in-vivo (Jonas et al., 1998; Liu et al., 2003; Pagnotta et al., 2005).

3.2.3.3 Study 3 – Does REM atonia require concurrent glycinergic and GABA_A-mediated inhibition of motoneurons?
Because GABA and glycine are co-released onto motoneurons (Jonas et al., 1998; O'Brien and Berger, 1999) and because there is an increase in the release of both GABA and glycine in both cranial and spinal motor pools during pontine-induced REM sleep (Kodama et al., 2003), I simultaneously antagonized both glycine and GABA_A receptors by perfusing 0.1mM strychnine and 0.1mM bicuculline onto the trigeminal motor pool during sleep-wake behaviours.

3.2.3.4 Study 4 – Is REM atonia mediated by increased inhibition and reduced excitation of motoneurons?
I hypothesize that motor atonia during REM sleep is mediated by concomitant inhibition and disfacilitation (i.e., reduced excitation) of motoneurons during REM sleep. To test this hypothesis I antagonized both glycine and GABA_A receptors (using 0.1mM strychnine and bicuculline) while simultaneously activating trigeminal motoneurons with
0.1mM AMPA. This dose of AMPA provokes a robust increase in muscle tone during waking and NREM sleep when applied to the trigeminal or hypoglossal motor pools in rats (Aoki et al., 2006; Burgess et al., 2008).

3.2.3.5 Study 5 - Demonstration that doses of strychnine and bicuculline antagonize glycine and GABA\textsubscript{A} receptors.
I microdialyzed 1mM of glycine and 1µM of muscimol (GABA\textsubscript{A} receptor agonist) into the left trigeminal motor pool before and while simultaneously applying 0.1mM strychnine and 0.1mM bicuculline. I used these doses of glycine and GABA\textsubscript{A} receptor agonists because they suppress genioglossus muscle EMG activity when applied to the hypoglossal motor pool in anesthetized rats (Liu et al., 2003; Morrison et al., 2002). All manipulations were made during waking when masseter muscle tone was maximal so the inhibitory effects of glycine and muscimol would induce the greatest degree of suppression. After a steady-state suppression of masseter tone was observed, I began perfusing glycine/muscimol and strychnine/bicuculline.

3.2.4 Data analysis

3.2.4.1 Behavioural state and EMG analysis
Behavioural state and EMG analysis was performed as described in Chapter 2, section 2.4.

3.2.4.2 EEG spectral analysis
Spectral analysis was performed using EEG Band Detect v1.06 in Spike 2. The EEG was windowed using a Hamming function and subjected to a fast Fourier transform to yield the power spectrum. The power within four frequency bands was recorded as absolute power and as a percentage of the total power of the signal that was calculated over each 5-sec epoch. The band limits used were delta (\(\delta\)): 0.48-4 Hz; theta (\(\theta\)): 4.25-8 Hz; alpha (\(\alpha\)): 8.25-15 Hz; beta (\(\beta\)): 15.25-35 Hz.

3.2.5 Verification of microdialysis probe location
Microdialysis probe locations were verified as described in Chapter 2, section 2.5.
3.2.6 Statistical analyses

All statistical analyses used Sigmastat (SPSS Inc., Chicago, IL) and applied a critical two-tailed alpha value of P<0.05. Differences in basal EMG tone during waking, NREM, and tonic REM sleep under baseline (i.e., aCSF) and drug treatments (i.e., 0.1mM strychnine or 0.1mM bicuculline or 0.1mM strychnine/bicuculline or 0.1mM strychnine/bicuculline/AMPA) was determined using analysis of variance with repeated measures (RM-ANOVA) and post hoc comparisons were performed using a Student-Newman-Keuls (SNK) test. Comparisons between treatments for frequency, amplitude and duration of muscle twitches per REM episode were made using RM ANOVA (i.e., drug treatments versus aCSF) and post hoc comparisons were performed using a SNK test. All data are expressed as mean ± standard error of the mean (SEM).

3.3 RESULTS

3.3.1 Inserting microdialysis probes into the trigeminal motor pool transiently increased masseter tone but had no effect on the sleep-wake cycle

Inserting a microdialysis probe into the left motor pool caused a transient yet robust activation of the left masseter muscle (paired t-test, p=0.011) without affecting the activity of either right masseter (paired t-test, p=0.261) or neck muscle (paired t-test, p=0.637) activities. This finding demonstrates that probe insertion only activates motoneurons in the left trigeminal motor pool. I also found that microdialysis of 0.1mM AMPA into the left trigeminal motor pool at the end of each experiment increased left masseter muscle activity (paired t-test, p=0.035; Figure 3.1A) without altering levels of either the right masseter (paired t-test, p=0.563; Figure 3.1A) or neck muscle EMG activity (paired t-test, p=0.219). This procedure verified that probes were located within the left trigeminal nucleus and that microdialysis probes were functional. It also shows that motoneurons were viable and able to respond to changes in excitatory neurotransmission.

In all 19 rats, I used post-mortem histology to confirm that microdialysis probes were either within or immediately adjacent to the left trigeminal motor pool (Figure 3.1B,C). I also found that placing a probe and perfusing glycineric and GABAergic
Fig. 3.1. Microdialysis probes were located in the left trigeminal motor pool. A. Group data showing that AMPA perfusion increased LM but not RM tone, indicating the probe was located in the left motor pool. Above is a representative trace showing left (LM) and right masseter (RM) EMG tone before and during AMPA perfusion into the left trigeminal nucleus. The AMPA effect on LM tone is indicated by an arrow. B. Panel (a) is an example of a lesion site left by a microdialysis probe in the left trigeminal motor pool. Panels (b) and (c) are sections immediately flanking the rostral and caudal borders of the trigeminal nucleus; no lesion was found in these areas, demonstrating that the probe was located exclusively within the motor pool. Scale bars represent 500µm. C. Black filled circles represent the location of probe lesions in the left trigeminal nuclei in the 19 rats used in this study. Note: * indicates a significant change (p<0.05) in activity from baseline levels; a.u., arbitrary units. All values are mean ± SEM.
antagonists into the trigeminal nucleus had no effect on the amount of time spent in each sleep-wake state when baseline and antagonist treatment (i.e., strychnine and bicuculline) were compared (RM-ANOVA, p=0.374). There was also no change in the ratio of high to low frequencies in EEG powers (i.e. %β / %δ) before and after antagonist treatment (RM-ANOVA, p=0.872), indicating that these interventions did not affect sleep-wake regulation.

**3.3.2 Masseter muscle atonia in REM sleep is not mediated by glycinergic inhibition of trigeminal motoneurons**

If a functional glycinergic drive underlies loss of muscle tone in REM sleep, then antagonism of glycine receptors on motoneurons should reverse REM sleep atonia. In 11 rats I perfused 0.1mM strychnine into the left trigeminal motor pool while monitoring masseter EMG activity during sleep and waking. Strychnine perfusion had a potent stimulatory effect on basal levels of masseter tone during both QW and NREM sleep. Compared to left masseter EMG activity under baseline conditions, antagonism of glycine receptors increased basal masseter tone during QW by 51 ± 8% (p<0.05 post hoc test) and during NREM sleep by 29 ± 4% (p<0.05 post hoc test; Figure 3.2A,B). This stimulatory effect on masseter tone was tonically sustained throughout periods of QW and NREM sleep, demonstrating the presence of a tonic glycinergic drive during these states. I also found that strychnine increased left masseter tone by the same magnitude at the beginning (43 ± 15% above baseline; paired t-test, p=0.013) and end (41 ± 14% above baseline; paired t-test, p=0.012) of the 2.3 ± 0.1h perfusion period (paired t-test, p=0.899; Figure 3.3), indicating that glycine receptor antagonism was not subject to time-dependent changes.

Although I identified a tonic glycinergic drive at the trigeminal motor pool during both waking and NREM sleep, I could not confirm the presence of such drive during tonic REM sleep. Compared to baseline conditions, strychnine perfusion had no effect on basal levels of masseter tone during tonic REM sleep (p=0.958 post hoc test; Figure 3.2A,B). Indeed, I demonstrate that the tonic glycinergic drive present in NREM sleep is rapidly lost upon entrance into REM sleep, but is reinstated during post-REM
Fig. 3.2. There is an endogenous glycineergic drive at the trigeminal motor pool in waking and NREM sleep but not during tonic REM sleep. A. Raw EMG and EEG traces from 1 rat showing that strychnine perfusion into the left trigeminal motor pool increased left masseter muscle (LM) tone during waking and NREM sleep; this intervention did not affect the atonia of tonic REM sleep. However, it did increase the left masseter muscle twitch amplitude during phasic REM. B. Group data (n=11) demonstrating that strychnine increased LM tone in quiet waking (QW) and NREM sleep, without changing levels of motor tone during tonic REM sleep. Although this intervention consistently increased LM tone in alert waking (AW) it was not statistically significant. C. Group data (n=101 REM periods) demonstrating that strychnine perfusion into the left trigeminal motor pool increased left masseter muscle twitch amplitude during phasic REM sleep, without significantly effecting either muscle twitch frequency or duration. Note: * indicates p<0.05; a.u., arbitrary units; #/s, number muscle twitches/second. All values are mean ± SEM.
Fig. 3.3. The magnitude of masseter muscle activation by glycinergic and GABAergic antagonists were unaffected by time. Group data (n=11) demonstrating that strychnine, bicuculline or strychnine/bicuculline perfusion into the left trigeminal motor pool increased left masseter EMG tone by the same magnitude at the beginning (white bars) and end (gray bars) of respective drug application periods. This finding demonstrates there was no time-dependent changes in antagonist efficacy. All measurements were taken during NREM sleep and values are mean ± SEM.
waking (Figure 3.4B-D). I analyzed levels of masseter EMG activity in the 30-second periods immediately preceding (i.e., NREM) and following (i.e., post-REM waking) each REM episode. In the 101 REM periods analyzed (mean REM duration: 80 ± 11s), I found that strychnine increased left masseter tone above baseline levels in all pre-REM (p<0.001 post hoc test) and post-REM (p<0.001 post hoc test) periods. However, there was rapid and complete loss of strychnine’s stimulatory effects during periods of tonic REM sleep (p=0.860 post hoc test; i.e., not different from baseline levels), which resulted in the persistence of masseter atonia despite continued glycine receptor antagonism (Figure 3.4B-D).

Because motoneurons are inhibited by glycinergic inputs during the muscle twitches of phasic REM sleep (Chase and Morales, 1983) I also determined whether strychnine affected muscle twitches. Antagonism of glycine receptors significantly increased the amplitude of muscle twitches by 27 ± 9% above baseline levels during phasic REM sleep (p=0.018). However, neither twitch duration (p=0.455) nor frequency (p=0.219) were affected by strychnine perfusion (Figure 3.2C).

### 3.3.3 GABA<sub>A</sub>-mediated inhibition of trigeminal motoneurons does not cause masseter atonia in REM sleep

Although I identified a phasic glycinergic drive during REM sleep, it is not responsible for mediating REM atonia. Since GABA is the most common inhibitory neurotransmitter in the brain (Bloom and Iversen, 1971) and since motoneurons are influenced by GABAergic processes in REM sleep (Soja et al., 1987a), I hypothesized that the motor atonia of REM sleep may be mediated by GABAergic inhibition. Therefore, I antagonized GABA<sub>A</sub> receptors on trigeminal motoneurons using 0.1mM bicuculline while monitoring masseter EMG activity during sleep and waking (n=11). Bicuculline perfusion into the left trigeminal nucleus induced a robust facilitation of masseter tone during both waking and NREM sleep. Compared to left masseter EMG activity under baseline conditions, antagonism of GABA<sub>A</sub> receptors increased basal masseter tone in AW by 106 ± 30% (p=0.009 post hoc test), in QW by 183 ± 30% (p<0.05 post hoc test) during both waking and NREM sleep.
**Fig. 3.4.** The tonic glycinergic drive present at the trigeminal motor pool in NREM sleep is abruptly switched-off in REM sleep.  

**A.** Typical EMG and EEG traces showing how left masseter muscle (LM) activity changes during the transitions into and out of REM sleep during baseline (i.e., aCSF at the trigeminal nucleus).  

**B.** Another trace from the same rat showing that strychnine perfusion increases LM activity during the NREM and waking periods preceding and following REM onset (compared to traces in A), but that masseter atonia persists even though glycine receptors are blocked.  

**C.** A typical raw trace showing the abrupt loss of masseter tone upon entrance into REM sleep despite strychnine perfusion at the trigeminal nucleus.  

**D.** Group data showing that compared to baseline, strychnine perfusion significantly increased LM activity in NREM and post-REM waking (QW), but not during REM sleep. Note: * indicates p<0.05; a.u., arbitrary units. All values are mean ± SEM.
This stimulatory effect on masseter tone was tonically sustained throughout individual periods of waking and NREM sleep and its stimulatory effects were maintained at constant levels across the 2.7 ± 0.2h perfusion period (paired t-test, p=0.657; Figure 3.3). These findings not only identify a tonic GABAergic tone at the trigeminal nucleus during waking and NREM sleep, but that 0.1mM bicuculline antagonized GABAergic inhibition.

Bicuculline perfusion had no effect on basal levels of masseter tone during tonic REM sleep (p=0.958; Figure 3.5A,B). I found that the stimulatory effects of bicuculline during NREM sleep were rapidly lost upon entrance into REM sleep (Figure 3.6C). In the 107 REM periods analyzed (mean REM duration: 74 ± 11s), I found that bicuculline increased masseter tone (relative to baseline levels) in all pre-REM (p<0.001 post hoc test) and post-REM (p<0.001 post hoc test) periods, however, there was a rapid and complete loss of bicuculline’s stimulatory effects during each REM episode (p=0.622 post hoc test; Figure 3.6B,D).

Unlike strychnine perfusion, which facilitated muscle twitch activity during REM sleep, I found that bicuculline had no significant effect (compared to aCSF baseline) on the amplitude (p=0.271), duration (p=0.651) or frequency (p=0.219) of masseter muscle twitches during REM sleep (Figure 3.5C).

3.3.4 Co-antagonism of glycine and GABA\textsubscript{A} receptors on trigeminal motoneurons does not reverse the masseter atonia in REM sleep

I found that neither glycine nor GABA\textsubscript{A} receptor antagonism reversed masseter muscle atonia during REM sleep. However, because GABA and glycine are co-released onto motoneurons (Jonas et al., 1998; O'Brien and Berger, 1999) and there is an increase in the release of both GABA and glycine onto motoneurons during pontine-induced REM sleep (Kodama et al., 2003), I hypothesized that both transmitters may be required to induce REM atonia. Therefore, I simultaneously antagonized glycine and GABA\textsubscript{A} receptors in the left trigeminal motor pool by perfusing 0.1mM strychnine and 0.1mM bicuculline (n=11). Co-antagonism of glycine and GABA\textsubscript{A} receptors resulted in a potent activation of masseter tone during both waking and NREM sleep. Perfusion of strychnine and bicuculline increased masseter tone in AW by 215 ± 35% (p<0.001 post hoc test), in QW
Fig. 3.5. Trigeminal motoneurons are inhibited by a tonic GABAergic drive during waking and NREM sleep but not REM sleep. A. Typical EMG and EEG traces showing how left masseter muscle (LM) activity changes during the transitions into and out of REM sleep during baseline (i.e., aCSF at the trigeminal nucleus). B. Another trace from the same rat showing that strychnine perfusion increases LM activity during the NREM and waking periods preceding and following REM onset (compared to traces in A), but that masseter atonia persists even though glycine receptors are blocked. C. A typical raw trace showing the abrupt loss of masseter tone upon entrance into REM sleep despite strychnine perfusion at the trigeminal nucleus. D. Group data showing that compared to baseline, strychnine perfusion significantly increased LM activity in NREM and post-REM waking (QW), but not during REM sleep. Note: * indicates p<0.05; a.u., arbitrary units. All values are mean ± SEM.
Fig. 3.6. Masseter muscle atonia in REM sleep is not mediated by GABAergic inhibition of trigeminal motoneurons. A. Raw traces demonstrating how left masseter muscle (LM) activity changes during the transition into and out of REM sleep during baseline (i.e., aCSF). B. An example trace from the same rat showing that bicuculline perfusion at the left trigeminal motor pool increases LM activity during the NREM and waking periods preceding and following REM onset (compared to baseline), but that masseter atonia is unaffected by blocking GABA_A receptors. C. A representative trace showing the abrupt loss of masseter tone upon entrance into REM sleep despite bicuculline perfusion at the trigeminal nucleus. D. Group data showing that compared to baseline, bicuculline perfusion significantly increased LM activity in NREM and post-REM waking (QW), but not during REM sleep. Note: * indicates p<0.05; a.u., arbitrary units. All values are mean ± SEM.
by 502 ± 95% (p<0.05 post hoc test) and during NREM sleep by 643 ± 121% (p<0.05 post hoc test; Figure 3.7A,B). This stimulatory effect was tonically sustained throughout individual episodes of waking and NREM sleep, and its stimulatory effects were maintained at constant levels across the 2.6 ± 0.1 hour perfusion period. Compared to baseline, left masseter tone increased during the first NREM period by 500 ± 130% (paired t-test; p=0.002) and during the last NREM period by 491 ± 170% (paired t-test; p=0.016); there was no difference in the magnitude of these responses (paired t-test; p=0.930; Figure 3.3).

Although combined glycine and GABA\textsubscript{A} receptor antagonism had potent excitatory effects on basal masseter tone during waking and NREM sleep, this stimulatory effect was immediately lost upon entrance into REM sleep, but immediately regained during post-REM waking. Dual strychnine/bicuculline perfusion tonically increased basal masseter tone (relative to baseline levels) during all pre-REM (p<0.001 post hoc test) and post-REM (p<0.001 post hoc test) periods; there was, however, rapid and complete loss of this stimulatory effect during tonic REM sleep (p=0.588 post hoc test; Figure 3.8B-D).

Although co-antagonism of glycine and GABA\textsubscript{A} receptors could not reverse REM sleep motor atonia, it significantly potentiated muscle twitch activity during phasic REM sleep (Figure 3.7C). Strychnine and bicuculline perfusion potently increased the mean duration of individual muscle twitches by 52 ± 2.7% (p=0.036); muscle twitch amplitude (p=0.330) and frequency (p=0.219), however, were unaffected.

3.3.5 REM atonia persists even when glycine and GABA\textsubscript{A} receptors are antagonized and trigeminal motoneurons are directly activated by AMPA

Because masseter muscle atonia during REM sleep could not be reversed by blockade of glycine and GABA\textsubscript{A} receptors, I hypothesized that REM atonia may be mediated by a disfacilitation of excitatory inputs (Chan et al., 2006; Fenik et al., 2005b). I therefore antagonized both glycine and GABA\textsubscript{A} receptors (using 0.1mM strychnine and bicuculline) while simultaneously activating trigeminal motoneurons with a potent dose of AMPA (0.1mM; n=5).
Fig. 3.7. A tonic glycinergic/GABAergic drive at the trigeminal motor pool functions to suppress masseter tone in waking and NREM sleep, but not during tonic REM sleep. A. Typical EMG and EEG traces illustrating that strychnine/bicuculline perfusion into the left trigeminal motor pool increases left masseter muscle (LM) tone during waking and NREM sleep; however, has no effect on masseter atonia in tonic REM sleep despite provoking masseter muscle twitch activity in phasic REM. B. Group data (n=11) showing that strychnine/bicuculline perfusion increases LM tone in alert (AW) and quiet waking (QW) and in NREM sleep, without changing levels of motor tone during tonic REM sleep. C. Group data showing strychnine/bicuculline perfusion into the left trigeminal nucleus increases masseter muscle twitch duration in phasic REM sleep, without significantly affecting either twitch amplitude or frequency. Note: * indicates p<0.05; a.u., arbitrary units; #/s, number muscle twitches/second. All values are mean ± SEM.
Fig. 3.8. Masseter atonia in REM sleep is not mediated by concurrent glycinergic and GABAergic inhibition of trigeminal motoneurons. A. Traces showing how left masseter muscle (LM) activity changes during the transition into and out of REM sleep during baseline. B. A trace from the same rat illustrating that strychnine/bicuculline perfusion at the left trigeminal motor pool increases LM activity during the NREM and waking periods preceding and following REM onset, but that masseter atonia is unaffected by this intervention. C. An example showing the sudden loss of masseter tone upon entrance into REM sleep despite strychnine/bicuculline perfusion at the trigeminal nucleus. D. Group data showing that strychnine/bicuculline perfusion increases basal LM tone in NREM and post-REM waking (QW), but not during REM sleep. Note: * indicates p<0.05; a.u., arbitrary units. All values are mean ± SEM.
This intervention had powerful stimulatory effects on masseter tone during waking and NREM sleep, increasing left masseter EMG activity above baseline levels by 1539 ± 502 (p=0.003 post hoc test) and 983 ± 280% (p=0.031 post hoc test; Figure 3.9A), respectively. This effect was also significantly greater than the effect that combined strychnine and bicuculline perfusion (without AMPA) had on levels of masseter tone during NREM sleep (paired t-test; p<0.001). However, this stimulatory effect was immediately abolished at the transition from NREM sleep into REM sleep, with REM atonia persisting despite simultaneous antagonism of both glycine and GABA_A receptors and activation of AMPA receptors (n=11 REM periods; Figure 3.9B). Compared to baseline levels, perfusion of strychnine, bicuculline and AMPA into the left trigeminal motor pool did not prevent or reverse masseter atonia in REM sleep (p=0.795 post hoc test; Figure 3.9C).

3.3.6 Strychnine and bicuculline antagonize the effects of glycine and GABA_A receptor agonism at the trigeminal motor pool

Although previous in-vitro and in-vivo studies have demonstrated that 0.1mM strychnine and 0.1mM bicuculline antagonize glycine and GABA_A receptors (Jonas et al., 1998; Morrison et al., 2003a; Pagnotta et al., 2005; Song and Huang, 1990), I aimed to verify that these doses blocked glycinergic and GABAergic inhibition on trigeminal motoneurons. To do this, I exogenously applied 1mM glycine and 1µM muscimol into the trigeminal nucleus before and while simultaneously microdialyzing 0.1mM strychnine and bicuculline in 3 rats.

I found that glycine and muscimol perfusion induced a significant suppression of waking levels of left masseter muscle tone; reducing it to 58 ± 2% of baseline levels (p=0.025 post hoc test). This suppression was rapidly reversed when 0.1mM strychnine and bicuculline were concurrently perfused with glycine and muscimol (p=0.009 post hoc test; Figure 3.10). Not only did strychnine and bicuculline block the agonists’ inhibitory effects, it increased masseter tone above waking levels during baseline conditions (p=0.04 post hoc test; 39 ± 9%; Figure 3.10). This observation not only demonstrates that this concentration of antagonists effectively reversed glycine and GABA_A receptor agonism, but that it also antagonized the endogenous inhibitory drive present in waking.
Fig. 3.9. REM atonia persists even when glycine and GABA_\text{A} receptors are antagonized and trigeminal motoneurons are directly activated by AMPA. A. Group data showing that simultaneous strychnine/bicuculline and AMPA perfusion into the left trigeminal nucleus potently increases basal LM tone in NREM and post-REM waking (QW), but not during tonic REM sleep. B. A typical example trace showing the sudden loss of left masseter (LM) tone upon entrance into REM sleep despite glycine and GABA_\text{A} receptor antagonism and direct activation of trigeminal motoneurons by AMPA. Notice that basal LM tone in NREM is facilitated by this intervention but this response is rapidly lost in REM sleep. C. Left top trace shows LM activity during baseline and right top trace shows LM activity during strychnine/bicuculline/AMPA perfusion. This intervention had no effect on atonia but did increase the duration of masseter twitches in phasic REM (see Figure 6 C). Group data (n=11 REM periods) showing masseter atonia is unaffected by strychnine/bicuculline/AMPA perfusion. Note: * indicates p<0.05; a.u., arbitrary units. All values are mean ± SEM.
Fig. 3.10. Strychnine and bicuculline antagonize the effects of glycine and GABA$_A$ receptor agonism at the trigeminal motor pool. Group data (n=3) showing that agonism of glycine and GABA$_A$ receptors by glycine and muscimol application (light gray bar) significantly reduces LM activity to $58 \pm 2\%$ of baseline levels (ie., aCSF; white bar). This suppression was reversed when strychnine and bicuculline were concurrently perfused with glycine and muscimol (dark gray bars). Note: * indicates $p<0.05$; a.u., arbitrary units. All values are mean ± SEM.
3.4 DISCUSSION

3.4.1 A tonic glycinergic and GABAergic drive at the trigeminal motor pool suppresses masseter motor tone in waking

Intracellular studies demonstrate that trigeminal motoneurons are hyperpolarized by IPSPs in waking cats (Chase and Morales, 2005). I confirm the presence of an endogenous glycinergic and GABAergic tone that contributes to levels of trigeminal motoneuron excitability and masseter muscle activity during waking. I show that antagonism of glycine and GABA receptors on trigeminal motoneurons results in a sustained increase in basal masseter tone throughout wakefulness, indicating that a tonic inhibitory drive functions to suppress waking muscle tone. A similar glycinergic/GABAergic tone at the hypoglossal motor pool has been shown to modulate inspiratory genioglossus muscle activity in waking rats (Morrison et al., 2003a).

A waking inhibitory tone may function to gate the glutamatergic and monoaminergic drives that activate somatic motoneurons during wakefulness (Chan et al., 2006; Mir et al., 2006). Stiff-person syndrome and hyperekplexia are neurological disorders caused by impaired GABAergic and glycinergic function (Gilbert et al., 2006; Shiang et al., 1993). A major symptom of these disorders is muscle hypertonia in waking (Kurczynski, 1983; Shiang et al., 1993). I identify a wake-related glycinergic/GABAergic tone at the trigeminal nucleus, that when blocked, produces muscle hypertonia. Together these observations support the notion that a waking inhibitory tone functions to dampen muscle activity and the inability to transduce this natural inhibitory drive (i.e., glycine and/or GABA receptors mutations) underlies the muscle rigidity of stiff-person syndrome and hyperekplexia.

3.4.2 Somatic motoneurons are inhibited by a tonic glycinergic and GABAergic drive during NREM sleep

Antagonism of glycine and GABA receptors at the trigeminal motor pool potently increased masseter muscle tone throughout NREM sleep, thus unmasking the presence of a tonic inhibition during this state. Inhibitory tone is maximal during NREM sleep since blocking it resulted in a 643% increase in masseter tone compared to the 215% and 502%
increase evoked in active and quiet waking, respectively. I suggest that a progressively strengthened glycinergic/GABAergic tone at the trigeminal motor pool functions to state-dependently suppress masseter tone from active waking into NREM sleep. This perspective is congruent with intracellular recordings in cats demonstrating that trigeminal and lumbar motoneurons are progressively hyperpolarized from active wake into NREM sleep (Chase et al., 1980; Glenn et al., 1978).

Basal muscle tone is not only suppressed in NREM sleep by a pervasive inhibitory tone, it is also suppressed by withdrawal of glutamatergic and noradrenergic excitation of motoneurons (Burgess et al., 2008; Chan et al., 2006; Mir et al., 2006). Therefore, both passive disfacilitation and active inhibition of motoneurons are responsible for suppressing basal muscle tone in NREM sleep.

3.4.3 A phasic inhibitory drive functions to oppose muscle twitches during REM sleep

I demonstrate that the functional glycinergic and GABA\(_A\)-mediated drive present at the trigeminal nucleus in waking and NREM sleep is immediately switched-off and converted to a phasic inhibitory drive during REM sleep. Antagonism of this drive potently facilitated muscle twitch activity without affecting REM sleep muscle atonia. This is in striking contrast to the tonic inhibitory drive of waking and NREM sleep, which when blocked resulted in a sustained increase in basal masseter tone. The phasic inhibitory drive of REM sleep appears to be mediated primarily by glycine because strychnine application onto trigeminal motoneurons potentiated muscle twitches in REM sleep, whereas bicuculline had no effect. These findings mirror observations from intracellular studies showing that motoneurons are maximally hyperpolarized by glycinergic IPSPs during periods of REM sleep with muscle twitches (Lopez-Rodriguez et al., 1992; Lopez-Rodriguez et al., 1990). During REM sleep, trigeminal and spinal motoneurons are concurrently inhibited and excited by glycinergic and glutamatergic inputs (Burgess et al., 2008; Chase and Morales, 1982, 1983; Lopez-Rodriguez et al., 1990; Morales and Chase, 1981; Soja et al., 1995), therefore I suggest that a phasic inhibitory drive during REM sleep functions to oppose the glutamatergic inputs that trigger muscle twitches.
Dysfunction of the REM-related inhibitory drive may explain the primary symptom of REM sleep behaviour disorder (RBD), a neurological condition of unknown origin whose presenting clinical symptom is excessive phasic muscle activity during REM sleep (Schenck et al., 1988). It may also explain why point-mutations in the α1 subunit of the glycine receptor, which causes hyperekplexia, also leads to myoclonic twitching in sleep (de Groen and Kamphuisen, 1978). These data suggest that the phasic inhibitory drive during REM sleep functions to oppose muscle twitch activity and loss of this drive could underlie the excessive myoclonic activity reported in RBD and hyperekplexia.

3.4.4 Glycinergic and GABA\textsubscript{A}-mediated inhibition of somatic motoneurons is not responsible for mediating REM sleep atonia

Our most fundamental observation is that REM sleep atonia could not be reversed by either glycine or GABA\textsubscript{A} receptor antagonism. This finding contradicts the prevailing hypothesis that REM atonia is mediated by glycinergic inhibition of motoneurons (Chase and Morales, 2005). Chase and colleagues established this hypothesis because they found that lumbar and trigeminal motoneurons are hyperpolarized by large amplitude IPSPs that are reduced (but not eliminated) by antagonism of glycine receptors (Chase et al., 1989; Soja et al., 1987a). Although I found that strychnine at the trigeminal motor pool unmasked a tonic inhibitory drive and increased basal masseter tone during waking and NREM sleep, it had no effect on REM atonia. Antagonism of GABA\textsubscript{A} receptors alone or together with glycine receptors also had no effect on REM sleep muscle atonia despite causing robust increases in muscle activity during waking and NREM.

One concern is whether REM atonia could not be reversed because glycine and GABA\textsubscript{A} receptors were not completely antagonized. However, I used strychnine and bicuculline at concentrations that were previously shown to eliminate glycine- and GABA\textsubscript{A}-mediated inhibition of motoneurons (Jonas et al., 1998; Morrison et al., 2003a; Pagnotta et al., 2005; Song and Huang, 1990). I also demonstrate that dosages of these antagonists blocked the suppressive effects that high doses of glycine and muscimol at the trigeminal motor pool had on masseter muscle tone (Figure 3.10).
The most concrete demonstration that glycine and GABA<sub>A</sub> receptors were antagonized, however, stems from the fact that strychnine and bicuculline provoked masseter twitch activity in REM sleep. Because IPSPs that hyperpolarize trigeminal motoneurons are at their maximal frequency and amplitude during periods of REM sleep when muscle twitches occur (Pedroarena et al., 1994) and because I show that antagonists potently increased muscle twitches, I conclude that motoneurons were indeed antagonized. Therefore, the persistence of muscle atonia during strychnine and bicuculline application indicates that REM atonia is not mediated by glycinergic or GABA<sub>A</sub>-mediated inhibition.

This conclusion is congruent with the findings that suppression of respiratory-related genioglossus muscle activity during either natural or carbachol-induced REM sleep could not be reversed by antagonism of glycinergic or GABA<sub>A</sub>-mediated inhibition at the hypoglossal motor nucleus in rats (Kubin et al., 1993; Morrison et al., 2003a). I conclude that glycinergic and GABA<sub>A</sub>-mediated inhibition of motoneurons is not the mechanism mediating the loss of postural muscle tone during REM sleep.

3.4.5 What is the root mechanism responsible for REM atonia?

Although motoneurons are hyperpolarized during REM sleep (Chase et al., 1980; Glenn and Dement, 1981a; Glenn et al., 1978; Morales and Chase, 1978; Nakamura et al., 1978) and glycine and GABA are released at motor pools during REM-like atonia (Kodama et al., 2003), I demonstrate that glycinergic and GABA<sub>A</sub>-mediated inhibition of trigeminal motoneurons does not mediate REM sleep atonia. Multiple lines of evidence indicate that muscle atonia in REM may result from disfacilitation of noradrenergic inputs onto motoneurons (Aston-Jones and Bloom, 1981; Fenik et al., 2005b; Lai et al., 2001a). However, glutamatergic, noradrenergic and serotonergic activation of motoneurons during REM sleep fails to reverse muscle atonia (Burgess et al., 2008; Chan et al., 2006; Jelev et al., 2001; Mir et al., 2006). The current study demonstrates that loss of postural muscle tone in REM sleep persists even when glycine and GABA<sub>A</sub> receptors are antagonized and motoneurons are directly activated by glutamatergic excitation, indicating that a powerful, yet unidentified, inhibitory mechanism overrides motoneuron excitation during REM sleep.
Although REM atonia is not triggered by either glycine- or GABA$_A$-mediated inhibition, it is possible that GABA$_B$ receptors could be involved. Previous studies demonstrate that activation of GABA$_B$ receptors on hypoglossal motoneurons suppresses their activity *in-vitro* (O'Brien and Berger, 1999) and reduces genioglossus motor tone in anesthetized rats (Okabe et al., 1994). However, it is unknown whether GABA$_B$ receptors are endogenously active during natural sleep-wake motor behaviours.

Another possible source of motoneuron inhibition during REM sleep may be from cholinergic neurons in the pedunculopontine tegmental nucleus. Neurons in this region are maximally active during REM sleep (el Mansari et al., 1989; Steriade et al., 1990) and they project to and inhibit motoneurons (Bellingham and Berger, 1996; Liu et al., 2005). The role that cholinergic neurons play in triggering REM atonia has not been determined but clearly merits consideration.

In summary, I conclude that REM atonia is induced by inhibition of somatic motoneurons, however, neither glycinergic nor GABA$_A$-mediated inhibition are responsible. The biochemical substrate mediating REM atonia remains unknown and therefore requires identification.
CHAPTER FOUR: ROLE FOR GABA\textsubscript{B}-MEDIATED SUPPRESSION OF MOTONEURONS DURING REM SLEEP

4.0 SUMMARY

Skeletal muscle tone is potently suppressed during REM sleep. I recently found that REM sleep atonia could not be prevented in masseter muscles by blockade of glycine and GABA\textsubscript{A} receptors at the trigeminal motor pool. REM atonia persisted even when glycine- and GABA\textsubscript{A}-mediated inhibition was blocked and high doses of AMPA were simultaneously applied. Accordingly, I concluded that REM atonia is triggered by an unidentified inhibitory mechanism(s). Although GABA\textsubscript{B} receptors are present on somatic motoneurons and postsynaptically inhibit them, the role of these receptors in mediating REM atonia is unknown. The aim of this study was to determine if GABA\textsubscript{B} receptors play a role in suppressing muscle tone during REM sleep. By antagonizing GABA\textsubscript{B} receptors on trigeminal motoneurons in rats, I found that masseter tone was increased during waking and NREM sleep, but REM atonia was not affected. However, when GABA\textsubscript{B} receptors as well as both GABA\textsubscript{A} and glycine receptors were simultaneously blocked, this not only increased masseter muscle tone during waking and NREM sleep, it also triggered a robust increase in basal muscle tone during REM sleep. Therefore, I show that an endogenous GABA\textsubscript{B} drive inhibits motoneurons and suppresses masseter tone during both waking and sleep. While GABA\textsubscript{B}-mediated inhibition itself does not trigger REM sleep atonia, blockade of GABA\textsubscript{B} as well as GABA\textsubscript{A} and glycine receptors is capable overriding REM atonia, indicating that GABA\textsubscript{B} receptors play a role in mediating this motor phenomenon.

4.1 INTRODUCTION

Skeletal muscle tone is potently suppressed in REM sleep, however the mechanisms mediating this suppression are not known. Disturbances of muscle tone during REM sleep underlie many of the major sleep disorders, including REM sleep behaviour disorder, periodic limb movement disorder, obstructive sleep apnea, and bruxism. It is imperative to understand the fundamental neurochemical mechanisms that mediate
muscle tone during sleep if effective pharmacological treatments for these disorders are to be found.

Investigations into the mechanisms underlying REM atonia have recently generated some debate (Berger, 2008; Brooks and Peever, 2008b; Chase, 2008; Funk, 2008; Kubin, 2008; Soja, 2008). During REM sleep, motoneurons are subjected to a barrage of inhibitory postsynaptic potentials (IPSPs); by hyperpolarizing motoneurons, these IPSPs are thought to be responsible for REM atonia (Chase and Morales, 2005). Antagonism of glycine receptors has been shown to, at least in part, block these IPSPs and thus a role for glycine in mediating REM atonia has long been hypothesized (Chase et al., 1989; Soja et al., 1991). Supporting this notion is the observation that glycine, as well as GABA, is released onto motoneurons during REM sleep (Kodama et al., 2003). Based on these observations, a role for inhibitory amino acids, GABA and particularly glycine, in mediating REM atonia has traditionally been accepted. More recent studies, however, have brought the contributions of this active inhibition of motoneurons in the regulation of REM atonia into question (Brooks and Peever, 2008a; Kubin et al., 1993; Morrison et al., 2003a). Blockade of glycine and GABA_A receptors at two different motor pools in naturally sleeping, freely behaving animals does not prevent REM atonia (Brooks and Peever, 2008a; Morrison et al., 2003a) as the traditional intracellular studies would have predicted (Chase et al., 1989; Soja et al., 1991). Even when glycine and GABA_A receptors are blocked and glutamate receptors are activated, REM atonia still persists (Brooks and Peever, 2008a). These observations suggest a yet unidentified mechanism is responsible for generating REM atonia.

In the CNS, GABA activates two pharmacologically distinct receptor systems, the ionotropic GABA_A receptor and the metabotropic GABA_B receptor (Bormann, 1988). While the release of GABA at the motor pool during REM sleep has been demonstrated (Kodama et al., 2003), only the contribution of GABA_A receptors has been considered (Brooks and Peever, 2008a; Chase et al., 1989; Kubin et al., 1993; Morrison et al., 2003b). Like GABA_A and glycine receptors, GABA_B receptors are present on motoneurons (Margeta-Mitrovic et al., 1999) and postsynaptically inhibit them (Haji and Takeda, 1993; Lalley, 1986). Therefore, one possible candidate in the control of REM atonia is GABAergic inhibition via GABA_B receptors.
The aim of this study was to determine if GABA\textsubscript{B} receptors play a role in suppressing muscle tone during REM sleep. Here I demonstrate that GABA\textsubscript{B} receptors are involved in mediating REM atonia but do so by acting in concert with glycinergic and GABA\textsubscript{A}-mediated inhibition. Like blockade of glycine and GABA\textsubscript{A} receptors (Brooks and Peever, 2008a), blockade of GABA\textsubscript{B} receptors alone had no effect on REM atonia. When GABA\textsubscript{B} receptors were blocked simultaneously with GABA\textsubscript{A} and glycine receptors, however, a partial reversal of REM atonia was observed. Therefore, this study identifies a novel mechanism by which GABAergic and glycinergic inhibition interact to suppress muscle tone in REM sleep.

4.2 METHODS

4.2.1 Animals
Studies were performed using 50 male Sprague Dawley rats (average weight: 391.0 ± 5.9g). Animals were surgically instrumented and recorded by the methods described in Chapter 2, sections 2.2-2.4.

4.2.2 Drug preparation
CGP52432 (FW: 420.27; Tocris, Ellisville, MO) a GABA\textsubscript{B} receptor antagonist, strychnine (strychnine hydrochloride, FW: 370.9; Sigma-Aldrich, Oakville, ON) a glycine receptor antagonist, bicuculline ((-)-bicuculline methochloride, FW: 435.87; Tocris, Ellisville, MO) a GABA\textsubscript{A} receptor antagonist, and baclofen ((R)-baclofen, FW: 213.66; Tocris, Ellisville, MO) a GABA\textsubscript{B} receptor agonist, were prepared in aCSF. Each drug was made immediately prior to each experiment. The AMPA receptor agonist (\(\alpha\)-amino-3-hydroxy-5-methylisoxazole-4-propionic acid, FW: 186.17; Tocris, Ellisville, MO) was made in advance and stored in stock solutions at -20\(^\circ\)C. All drugs were vortexed and filtered (0.22\(\mu\)m PVDF, Fisher Scientific) before use.
4.2.3 Experimental protocols

4.2.3.1 Study 1: Demonstration that GABA\textsubscript{B} receptors are present and functional on trigeminal motoneurons.
I agonized GABA\textsubscript{B} receptors by perfusing 0.5mM baclofen into the left trigeminal motor pool while monitoring left masseter muscle EMG activity. The effects on motor output were determined during waking when masseter muscle tone was maximal so the inhibitory effects of baclofen would induce the greatest degree of suppression. This concentration of baclofen was used because it is in the mid-range of concentrations used to agonize GABA\textsubscript{B} receptors in previous \textit{in vitro} and \textit{in vivo} studies (Matsuki et al., 2009; Okabe et al., 1994; Ouyang et al., 2007).

4.2.3.2 Study 2: Identification of an endogenous GABA\textsubscript{B}-mediated inhibitory drive onto motoneurons across the sleep-wake cycle.
I antagonized GABA\textsubscript{B} receptors by applying CGP52432 at increasing concentrations to establish a dose response curve. I perfused 0.01mM, 0.05mM, 0.1mM and 0.2mM CGP52432 into the trigeminal motor pool and determined the resulting effects on basal muscle tone. These concentrations were chosen because they encompass those used to successfully antagonize GABA\textsubscript{B} receptors in previous \textit{in vitro} and \textit{in vivo} studies (Chery and De Koninck, 2000; Fedele et al., 1997; Kawahara et al., 1999; Westerink et al., 1996).

4.2.3.3 Study 3: Investigation of a role for GABA\textsubscript{B}-mediated inhibition, alone or in combination with GABA\textsubscript{A} and glycine-mediated inhibition, in regulating REM atonia.
Because trigeminal motoneurons possess GABA\textsubscript{A}, GABA\textsubscript{B}, and glycine receptors (Margeta-Mitrovic et al., 1999; Yang et al., 1997), and GABA and glycine are coreleased onto motoneurons (Jonas et al., 1998; O'Brien and Berger, 1999), I hypothesized that REM atonia may require the concurrent activation of all three receptor types. Supporting this notion is the demonstration that activation of GABA\textsubscript{B} receptors can modulate the function of GABA\textsubscript{A} and glycine receptors (Hahner et al., 1991; O'Brien et al., 2004). Accordingly, I simultaneously antagonized GABA\textsubscript{B} receptors with GABA\textsubscript{A} and glycine receptors (via 0.2mM CGP52432 and 0.1mM bicuculline and strychnine) on trigeminal motoneurons and determined the resulting effects on REM motor tone. These results
were compared to baseline levels of activity during REM sleep as well as those resulting from the antagonism of just GABA\textsubscript{B} receptors (via 0.2mM CGP52432) and just GABA\textsubscript{A} and glycine receptors (via 0.1mM bicuculline and strychnine). The concentration of CGP52432 was chosen based on the dose response results from Study #2 above, and the concentrations of bicuculline and strychnine have been shown previously to successfully antagonize GABA\textsubscript{A} and glycine-mediated neurotransmission (Brooks and Peever, 2008a; Liu et al., 2003; Morrison et al., 2003a).

4.2.3.4 Study 4: Confirmation of results using microinjection.

To verify the results observed in the reverse microdialysis experiments described above, I microinjected CGP52432 with and without bicuculline and strychnine at three times the concentrations used in Study #3 (ie. 0.6mM CGP52432, 0.3mM bicuculline and strychnine). Microinjections of aCSF were used as the control condition. To administer injections, the semi-permeable tip of a microdialysis probe was removed and the probe, teflon tubing and Hamilton syringe were filled with the candidate drug or aCSF. During the transition into REM sleep, 0.2uL of solution was injected over a 15s period into the trigeminal motor pool and the resulting effect on REM atonia was determined. Although the majority of REM periods were not interrupted by this protocol, if the animal was roused during the injection or if the ensuing REM period was less than 30s in length, it was excluded from the analysis. This procedure allowed us to confirm that concentration was not a factor in any lack of effect seen nor was the spatial or temporal resolution of the reverse microdialysis procedure influencing the results.

4.2.4 Data analysis

4.2.4.1 Behavioural state and EMG analysis

Behavioural state and EMG analysis was performed as described in Chapter 2, section 2.4.

4.2.4.2 EEG spectral analysis

EEG spectral analysis was calculated using fast Fourier transformation of each 5s epoch, yielding a power spectra profile within four frequency bands. The band limits used were delta (\( \delta \)): 0.48-4 Hz; theta (\( \Theta \)): 4.25-8 Hz; alpha (\( \alpha \)): 8.25-15 Hz; beta (\( \beta \)): 15.25-35 Hz. A
mean EEG spectrum profile was obtained for each epoch and then, to minimize non-specific differences in absolute power between individuals, EEG power in each frequency bin was expressed as a percentage of the total EEG power in the epoch. The spectral profiles of each behavioural state were then compared between treatments.

### 4.2.5 Verification of microdialysis probe location

Microdialysis probe locations were verified as described in Chapter 2, section 2.5.

### 4.2.6 Statistical analyses

All statistical analyses used Sigmastat (SPSS Inc., Chicago, IL) and applied a critical two-tailed alpha value of P<0.05. Comparisons made between baseline and drug treatments were determined using either analysis of variance with repeated measures (RM-ANOVA) or paired t-tests. Student-Newman-Keuls (SNK) test was used for post hoc comparisons. Comparisons for the microinjection experiments (ie. drug treatments vs. aCSF) were made using t-tests. All data are expressed as mean ± standard error of the mean (SEM).

### 4.3 RESULTS

#### 4.3.1 Agonism of GABA<sub>B</sub> receptors reduces muscle tone in waking

Before determining what role, if any, GABA<sub>B</sub> receptors play in mediating REM sleep atonia, I wanted to confirm that GABA<sub>B</sub> receptors are present and functional on trigeminal motoneurons. To do this, left masseter muscle activity was recorded in five rats during waking under baseline and when 0.5mM baclofen, a GABA<sub>B</sub> receptor agonist, was perfused into the left trigeminal motor nucleus.

Application of 0.5mM baclofen onto left trigeminal motoneurons potently reduced left masseter muscle tone by 78.3 ± 5.3% in active waking (paired t-test, p=0.017; Figure 4.1). Therefore, GABA<sub>B</sub> receptors are present and functional on trigeminal motoneurons, as agonism of GABA<sub>B</sub> receptors at the trigeminal motor nucleus results in a reduction in motor output at the masseter muscle.
Fig. 4.1. Agonism of GABA<sub>B</sub> receptors reduces masseter EMG activity.
Representative EEG and EMG traces of left masseter (LM) and right masseter (RM) activity during active wake under baseline (left top trace) and when 0.5 mM baclofen (right top trace) was perfused into the left trigeminal motor pool. Group data (n=5) showing that perfusion of a GABA<sub>B</sub> receptor agonist into the left trigeminal motor nucleus reduced LM activity. RM activity was unaffected by this intervention (p=0.273; data not shown). * indicates p<0.05; a.u., arbitrary units. All values are mean ± SEM.
4.3.2 Antagonism of GABA<sub>B</sub> receptors increases muscle tone in waking and NREM sleep but not REM sleep

To determine if there is an endogenous GABA<sub>B</sub>-mediated inhibitory drive onto motoneurons during sleep and waking, left masseter activity was recorded across the sleep-wake cycle in six rats under baseline and when CGP52432, a GABA<sub>B</sub> receptor antagonist, was perfused onto the left trigeminal motor nucleus. To establish a dose response, CGP52432 was applied at concentrations ranging from 0.01mM to 0.2mM.

Blockade of GABA<sub>B</sub> receptors on left trigeminal motoneurons increased left masseter EMG activity above baseline levels in a dose-dependent manner in quiet wake (RM-ANOVA, p=0.008) and NREM sleep (RM-ANOVA, p=0.004) but not REM sleep (RM-ANOVA, p=0.234) or active wake (RM-ANOVA, p=0.131) (Figure 4.2). These data indicate that an endogenous GABA<sub>B</sub>-mediated drive inhibits motoneurons during both waking and NREM sleep, but not REM sleep.

4.3.3 Co-antagonism of GABA<sub>B</sub> receptors with GABA<sub>A</sub> and glycine receptors partially reverses REM atonia

I next investigated if the mechanism mediating REM atonia involves an interaction between GABA<sub>B</sub> receptors with GABA<sub>A</sub> and glycine receptors. I antagonized GABA<sub>B</sub> receptors both alone and in combination with GABA<sub>A</sub> and glycine receptors and determined the effects on muscle activity during REM sleep. To do this, left masseter EMG activity was recorded in 14 rats under baseline and when a) 0.2mM CGP52432, b) 0.1mM bicuculline & strychnine, and c) 0.2mM CGP52432, and 0.1mM bicuculline & strychnine were perfused into the left trigeminal motor nucleus.

Blockade of GABA<sub>B</sub> receptors alone had no effect on REM sleep atonia (p=0.528 post hoc test; Figure 4.3), nor did blockade of GABA<sub>A</sub> and glycine receptors (p=0.294 post hoc test; Figure 4.3), as previously reported (Brooks and Peever, 2008a; Morrison et al., 2003a). Simultaneous blockade of GABA<sub>B</sub> receptors with GABA<sub>A</sub> and glycine receptors, however, resulted in a robust increase in basal muscle tone during REM sleep (p=0.004 post hoc test; Figure 4.3). While this increase in basal muscle tone was not sustained throughout the entire REM period (Figure 4.4), the total amount of REM sleep
Fig. 4.2. Antagonism of GABA<sub>B</sub> receptors increases masseter EMG activity. Group data (n=6) showing that CGP52432 perfusion at the trigeminal nucleus dose-dependently increased EMG activity in waking and NREM sleep but not REM sleep. * indicates p<0.05; a.u., arbitrary units. All values are mean ± SEM.
Fig. 4.3. Simultaneous antagonism of GABA<sub>B</sub> receptors with GABA<sub>A</sub> and glycine receptors potently increases basal muscle tone during REM sleep. **A**, Typical EMG and EEG raw traces illustrating that perfusion of CGP52432 with bicuculline and strychnine into the left trigeminal motor pool can increase tonic levels of muscle activity during REM sleep, whereas perfusion of just CGP52432 or just bicuculline and strychnine does not. **B**, Group data (n=14) showing that only simultaneous perfusion of CGP52432, bicuculline and strychnine increases tonic REM activity above baseline levels. * indicates p<0.05; a.u., arbitrary units. All values are mean ± SEM.
Fig. 4.4. Co-antagonism of GABA<sub>A</sub>, GABA<sub>B</sub> and glycine receptors causes sustained increases in basal muscle tone during REM sleep. EEG and EMG raw traces of a complete REM period under baseline (A) and when CGP52432, bicuculline and strychnine (B) were simultaneously perfused into the trigeminal motor pool. Although combined GABA<sub>A</sub>, GABA<sub>B</sub> and glycine receptor antagonism did not completely abolish REM atonia, basal muscle tone was increased for sustained periods of time when all three receptor types were blocked.
a tonia was significantly reduced from baseline levels (61 ± 6.5% vs. 91 ± 1.3%; p<0.001, post hoc test).

It is important to note that the differential effects on muscle tone were REM-specific, as blockade of GABA<sub>A</sub> and glycine receptors, with and without the concurrent blockade of GABA<sub>B</sub> receptors, resulted in a potent activation of muscle tone in waking (p<0.001, post hoc test), and NREM sleep (p<0.05, post hoc test); REM atonia, however, was only affected when all three receptor types were blocked simultaneously (Figure 4.5). Taken together, these findings suggest that the mechanism underlying REM atonia involves, at least in part, an interaction between GABA<sub>B</sub> receptors with GABA<sub>A</sub> and glycine receptors.

Simultaneous blockade of GABA<sub>A</sub>, GABA<sub>B</sub> and glycine receptors also augmented phasic REM activity (Figure 4.6). When all three receptor types were blocked, there was an increase in the duration (p=0.002, post hoc test), frequency (p<0.001, post hoc test), and amplitude (p=0.010, post hoc test) of phasic muscle twitches. Blockade of GABA<sub>A</sub> and glycine receptors increased the duration (p=0.007, post hoc test) and frequency (p=0.001, post hoc test) of twitches while having no effect on the amplitude (p=0.250, post hoc test). Antagonism of GABA<sub>B</sub> receptors alone had no effect on phasic muscle twitch activity (duration: p=0.613; frequency: p=0.280; amplitude: p=0.198; post hoc tests).

4.3.4 Pharmacological manipulations sufficiently antagonize receptors

Because REM atonia could not be completely reversed by blockade of GABA<sub>A</sub>, GABA<sub>B</sub> and glycine receptors, I conducted an additional set of experiments in which I microinjected CGP52432, bicuculline and strychnine at concentrations that were three times that used in the microdialysis experiments. Either 0.3mM bicuculline and strychnine (n=12) or 0.6mM CGP52432 with 0.3mM bicuculline and strychnine (n=13) was microinjected into the trigeminal motor nucleus at the onset of REM sleep and the effects on REM atonia were determined. These results were compared to aCSF microinjections (n=6).

Consistent with my microdialysis results, microinjection of 0.3mM bicuculline and strychnine had no significant effect on tonic levels of masseter activity (t-test,
Fig. 4.5. Blockade of GABA_B receptors with GABA_A and glycine receptors increases muscle tone across the sleep-wake cycle. A, Group data (n=14) showing that compared to baseline, bicuculline and strychnine perfusion increased left masseter activity in NREM and post-REM waking (quiet wake), but not during REM sleep. B, In addition to increasing left masseter activity in NREM and post-REM waking (quiet wake), co-perfusion of CGP52432, bicuculline and strychnine also increases left masseter tone in REM sleep. * indicates p<0.05; a.u., arbitrary units. All values are mean ± SEM.
Fig. 4.6. Antagonism of GABA_A and glycine receptors, with or without the co-antagonism of GABA_B receptors, increases phasic twitch activity during REM sleep. Group data showing that perfusion of bicuculline and strychnine with and without CGP52432, increases phasic twitch frequency (A) and duration (B), while phasic twitch amplitude is only increased when all three receptor types are blocked (C). CGP52432 alone has no effect on phasic REM activity. * indicates p<0.05; a.u., arbitrary units. All values are mean ± SEM.
This finding rules out the possibility that the lack of effect on REM atonia seen with bicuculline and strychnine was due to incomplete receptor saturation or desensitization of the receptors over the perfusion period. Similarly, microinjection of 0.3mM CGP52432 with 0.6mM bicuculline and strychnine increased tonic levels of activity (t-test, p=0.003; Figure 4.7), as seen in the microdialysis experiments, however, periods of atonia still persisted. This is again consistent with the assertion that incomplete receptor antagonism was not a factor in my results. Taken together, these data confirm my findings that the mechanism mediating REM atonia involves, but only in part, an interaction between GABA_B receptors with GABA_A and glycine receptors.

4.3.5 Experimental interventions specific to motor output of the left trigeminal motor nucleus

Finally, I used a series of controls to ensure that the pharmacological manipulations used in these studies were specific to the motor output of the left trigeminal motor nucleus. First I confirmed that insertion of the microdialysis probe into the left trigeminal motor nucleus caused a transient yet robust activation of the left masseter muscle (paired t-test, p=0.039; Figure 4.8A), while having no effect on the activity of the right masseter (paired t-test, p=0.887; Figure 4.8A) or the neck muscle (paired t-test, p=0.275). This demonstrates that probe insertion only activates motoneurons in the left trigeminal motor pool. At the end of each experiment, 0.1mM AMPA was perfused into the left trigeminal motor nucleus which induced a specific activation of left masseter activity (paired t-test, p=0.018; Figure 4.8B), while right masseter (paired t-test, p=0.321; Figure 4.8B) and neck (paired t-test, p=0.968) activity were again unaffected. This further demonstrates that probes were located in the left trigeminal nucleus and verified that microdialysis probes were still functional and trigeminal motoneurons were still viable and able to respond to excitatory stimuli at the end of each experiment. Finally, post-mortem histology was used to confirmed probe placement within or adjacent to the trigeminal motor nucleus in all rats (Figure 4.8C,D).

Insertion of a probe and perfusion of GABA_A, GABA_B, and glycine receptor antagonists into the trigeminal nucleus did not affect the amount of time spent in REM

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Fig. 4.7. Microinjection of antagonists confirms that blockade of GABA$_A$ and glycine receptors with GABA$_B$ receptors, but not alone, increases tonic REM activity. Group data showing that bicuculline and strychnine (n=12) still have no effect on REM atonia even when microinjected into the trigeminal motor nucleus at a higher concentration than that used in the microdialysis experiments. As in the microdialysis experiments, microinjection of higher concentrations of CGP52432, bicuculline and strychnine (n=13) during REM sleep results in an increase in tonic levels of activity. * indicates p<0.05; a.u., arbitrary units. All values are mean ± SEM.
Fig. 4.8. Microdialysis probes were located in the left trigeminal motor pool. A (top), A typical EEG and EMG raw trace showing that insertion of a microdialysis probe into the left trigeminal motor pool induced a transient activation of left masseter (LM) tone, while right masseter (RM) tone was unaffected. A (bottom), Group data showing that probe insertion increased LM but not RM EMG activity. B (top), EEG and EMG representative trace showing that LM, but not RM, tone was increased when AMPA was perfused into the left trigeminal motor pool. B (bottom), Group data showing that AMPA perfusion increased LM but not RM EMG tone. C, Example of a lesion site left by a microdialysis probe in the left trigeminal motor pool. Scale bar, 500µm. D, Black filled circles (left side of each diagram) represent the location of microdialysis probe lesions in the trigeminal motor pool and grey filled circles (right side of each diagram) represent the location of microinjection probe lesions in the trigeminal motor pool. * indicates p<0.05; a.u., arbitrary units. All values are mean ± SEM.
sleep (RM ANOVA, p>0.05), nor was there a change in EEG spectral power (RM ANOVA, p=0.232) with any of the treatments, indicating that these interventions did not affect REM sleep regulation. In addition, the stimulatory effects of the antagonists used were maintained at constant levels across the perfusion period for each of the interventions (RM ANOVA: CGP52432: p=0.424; bicuculline and strychnine: p=0.518; CGP52432, bicuculline and strychnine: p=0.791), thus there were no time-dependent changes in antagonist efficacy.

4.4 DISCUSSION

The data presented here support the assertion that REM atonia is not mediated by a single biochemical pathway (Brooks and Peever, 2008b). After confirming that GABA$_B$ receptors are both present and functional on trigeminal motoneurons and identifying an endogenous GABA$_B$-mediated drive in waking and NREM sleep, I demonstrated a novel mechanism of REM atonia in which GABA$_B$ receptors, but only when acting in concert with GABA$_A$ and glycine receptors, have a role in regulating basal motor tone during REM sleep. While these results do not completely elucidate the underlying cause of REM atonia, they do further expand our knowledge of the complicated set of mechanisms responsible for this phenomenon.

4.4.1 Presence of an endogenous GABA$_B$-mediated inhibitory tone in waking and NREM sleep

Intracellular studies in cats reveal the occurrence of IPSPs onto motoneurons during both waking and NREM sleep (Chase and Morales, 2005). I previously confirmed the presence of a glycinergetic and GABA$_A$-mediated inhibitory drive that contributes to levels of trigeminal motoneuron excitability and masseter muscle activity in these states in the rat (Brooks and Peever, 2008a). A comparable state-specific inhibitory drive has also shown at the hypoglossal motor nucleus (Morrison et al., 2003a; Morrison et al., 2003b). In the current study, I demonstrate a GABA$_B$-mediated tone in waking and NREM sleep: antagonism of GABA$_B$ receptors at the trigeminal motor pool significantly increased masseter muscle activity in both quiet waking and NREM sleep. This is in agreement with intracellular recordings in the cat demonstrating that motoneurons gradually
hyperpolarize during the transition from active waking into NREM sleep (Chase et al., 1980; Glenn and Dement, 1981b; Morales and Chase, 1978). Unlike the previously identified glycinergic/GABA\textsubscript{A}-mediated tone that progressively increases from active wake to quiet wake and is then maximal in NREM sleep (Brooks and Peever, 2008a), the GABA\textsubscript{B}-mediated inhibitory tone is absent in active wake and then of equal strength in quiet waking and NREM sleep, with blockade of GABA\textsubscript{B} receptors resulting in up to a 218% increase in muscle tone in both states. It therefore appears that GABA\textsubscript{B} receptors compliment the progressively increasing state-dependent glycinergic/GABA\textsubscript{A}-mediated control of motor suppression with a constant inhibitory tone that is present in quiet waking and NREM sleep. Taken together, these data strengthen the notion that basal muscle tone is suppressed from active waking into NREM sleep by a glycinergic/GABAergic inhibition of motoneurons together with a passive withdrawal of excitatory glutamatergic and noradrenergic inputs (Brooks and Peever, 2008a; Burgess et al., 2008; Chan et al., 2006; Mir et al., 2006).

4.4.2 Regulation of REM atonia by GABA\textsubscript{B} receptors with GABA\textsubscript{A} and glycine receptors

The endogenous GABA\textsubscript{B}-mediated tone identified in waking and NREM sleep is absent in REM sleep: blockade of GABA\textsubscript{B} receptors on trigeminal motoneurons had no effect on masseter muscle tone during REM sleep. Likewise, I have previously shown that neither glycine nor GABA\textsubscript{A} receptor antagonism has an effect on REM atonia (Brooks and Peever, 2008a). This was again replicated in this study and is supported by work at the hypoglossal motor pool (Kubin et al., 1993; Morrison et al., 2003a), indicating this is not a phenomenon specific to trigeminal motoneurons. From these findings, it appears that there is a negligible independent role for GABAergic or glycinergic inhibition in REM atonia.

There is evidence, however, to suggest that GABA\textsubscript{B} receptor activation can modulate the activity of GABA\textsubscript{A} and glycine receptors (Hahner et al., 1991; O'Brien et al., 2004). If true, this could reconcile the intracellular data suggesting the active inhibition of motoneurons is responsible for REM atonia (Chase and Morales, 2005) with more recent studies unable to corroborate this relationship (Brooks and Peever, 2008a;
Kubin et al., 1993; Morrison et al., 2003a). To investigate this possibility, I simultaneously antagonized GABA$_B$ receptors with GABA$_A$ and glycine receptors. Unlike when these receptors were antagonized individually, co-antagonism was successful in altering REM atonia: blockade of all three receptor types resulted in a robust increase in basal muscle tone. This supports a role for the active inhibition of motoneurons in REM atonia but suggests the mechanism is not as simple as previously thought.

Blockade of GABA$_A$ and glycine receptors had no effect on REM atonia, however it did result in an increase in muscle twitches. This is in agreement with previously published data indicating the presence of an inhibitory drive in REM sleep which acts to oppose excitatory phasic inputs (Brooks and Peever, 2008a; Chase and Morales, 1983). The increase in muscle twitch activity was maintained when GABA$_B$ receptors were co-antagonized with GABA$_A$ and glycine receptors, however was not seen when just GABA$_B$ receptors were antagonized. Therefore, the interaction between GABA$_A$, GABA$_B$ and glycine receptors is specific to the control of basal muscle tone in REM sleep, as activation of basal muscle activity is only seen when all three receptor types are blocked, whereas augmentation to muscle twitch activity is also seen when just GABA$_A$ and glycine receptors are blocked.

The synergistic effect on muscle tone when GABA$_B$ receptors were antagonized with GABA$_A$ and glycine receptors offers a novel mechanism by which REM atonia is mediated. This, however, does not completely explain the regulation of REM atonia. While atonia was altered, it was not entirely abolished. Periods of atonia still persisted, despite the powerful effects this intervention had on motor tone, implicating a yet unidentified mechanism in the control of motor tone during REM sleep.

### 4.4.3 Methodological considerations in the persistence of REM atonia

One potential concern in my inability to completely reverse REM atonia is that I did not completely antagonize receptors. To address this, I microinjected the antagonists directly into the motor pool at the onset of REM sleep at higher concentrations than those used in the microdialysis experiments. This not only addressed concentration concerns, but also confirmed that my inability to eliminate atonia was neither due to receptor desensitization
over the long microdialysis period nor an indiscriminate diffusion of drugs into surrounding areas. Despite increasing the concentration of antagonists and microinjectioning at the onset of REM sleep, REM atonia still persisted. This was true when GABA_A and glycine receptors were antagonized alone or in conjunction with GABA_B receptors. In fact, the increase in tonic activity that I saw with co-antagonism of GABA_A, GABA_B and glycine receptors was comparable to that occurring when one third of the concentration was perfused into the motor pool. Thus, the persistent periods of atonia remaining when all three receptor types were blocked was not due to incomplete receptor antagonism or receptor desensitization, but likely due to the presence of an additional, yet unidentified, mechanism at work.

It is important to note that regardless of the concentration of antagonists used or the method of drug delivery, REM atonia was only affected when GABA_A and glycine receptors were antagonized with GABA_B receptors. This confirms that a GABA_B-mediated mechanism, and not just a non-specific increase in overall inhibition, was responsible for the increases in tonic activity seen during REM sleep. Collectively, these data verify my findings that the mechanism mediating REM atonia involves, but is not exclusive to, an interaction between GABA_B receptors with GABA_A and glycine receptors.

4.4.4 Functional interaction of GABA_B receptors with GABA_A and glycine receptors

GABAergic inhibition is mediated via two distinct receptor types: ionotropic GABA_A receptors which, like the ionotropic glycine receptor, are responsible for fast inhibition, and metabotropic GABA_B receptors which mediate slow inhibitory events (Nicoll et al., 1990). Unlike the fast IPSPs generated by GABA_A and glycine receptors, GABA_B-mediated IPSPs have a delayed onset and a longer decay time (Bettler et al., 2004; Kim et al., 1997). This prolonged membrane hyperpolarization decreases the frequency at which information can be transmitted through the cell (Nicoll et al., 1990). Based on the kinetics of these two receptor types, it is conceivable that co-activation could result in the powerful, long-lasting inhibition that would be necessary for REM atonia. My results, however, indicate more than just a co-activation of receptors is occurring, as the combined effect on atonia that is seen when all three receptors are simultaneously
antagonized is not just a sum of the individual effects. This suggests a modulation of receptor function. The observation that GABA$_A$ and GABA$_B$ receptor agonists have increased efficacy when applied together (Li et al., 2010) supports the idea that cooperation between receptor types may be responsible for the potent suppression of muscle tone in REM sleep.

There are numerous examples in the literature demonstrating that a modulation of signaling can result from direct interactions between receptors (Balasubramanian et al., 2004; Fiorentini et al., 2003; Lee et al., 2002; Liu et al., 2000). Specifically, these studies demonstrate a regulation of ionotropic ligand-gated ion channels by metabotropic G protein-coupled receptors. Such a relationship has been well characterized in glutamate receptors. Metabotropic glutamate receptor agonists can potentiate the response of excitatory amino acids acting at ionotropic receptors in a number of different systems, including motoneurons (Bleakman et al., 1992; Cerne and Randic, 1992; Jones and Headley, 1995; Ugolini et al., 1997). A similar relationship between metabotropic GABA$_B$ and ionotropic GABA$_A$ and glycine receptors could explain the synergistic interaction between these receptor types that I observed during REM sleep. In support of this, GABA$_A$ and GABA$_B$ receptors, like ionotropic and metabotropic glutamate receptors (Perroy et al., 2008), have been shown to physically interact (Balasubramanian et al., 2004). In fact, there are several reports of GABA$_A$ and GABA$_B$ receptors influencing each other’s binding properties (Kardos et al., 1994; Kardos and Kovacs, 1991) and signaling activity (Barila et al., 1999; Kardos et al., 1994; Obrietan and van den Pol, 1998; Shibuya et al., 1997). While a similar physical interaction between GABA$_B$ and glycine receptors has not been characterized, studies have shown that activation of GABA$_B$ receptors can modulate the function of both GABA$_A$ and glycine receptors (Hahner et al., 1991; O'Brien et al., 2004). It is possible that the G protein mechanism activated by GABA$_B$ receptors (Bowery, 1993) alters second messenger systems thereby modifying GABA$_A$/glycine receptor function. Protein phosphorylation is a major mechanism of neurotransmitter receptor function (Huganir and Greengard, 1990; Raymond et al., 1993) and modulation of both GABA$_A$ and glycine receptor function by phosphorylation has been demonstrated (Swope et al., 1992). Such a functional relationship could explain the underlying cross-talk between these receptor types that I
hypothesize to be responsible for the effects on REM atonia seen when GABA_B receptors are antagonized with GABA_A and glycine receptors.

4.4.5 REM atonia mediated by postsynaptic GABAergic/glycinergic effects

In addition to hyperpolarizing postsynaptic membranes, GABA_B receptors also act presynaptically as both autoreceptors, controlling the release of GABA, as well as heteroreceptors, controlling the release of other transmitters, in particular glutamate (Bettler et al., 2004; Kerr and Ong, 1995). In fact, excitatory inputs onto trigeminal motoneurons are controlled by presynaptic GABA_B receptors (Min et al., 2002). As such, one alternative explanation for the increase in motor tone I observe during REM sleep is that by antagonizing GABA_B receptors, I am blocking their inhibitory presynaptic effect on excitatory transmitter release and therefore increasing the release of excitatory neurotransmitters. Increasing excitatory inputs onto motoneurons during REM sleep could conceivably cause an increase in basal muscle tone. This, however, is unlikely for two reasons. First, the antagonist used in this study has been shown to preferentially block postsynaptic as opposed to presynaptic GABA_B receptors (Pozza et al., 1999). Moreover, our laboratory has previously shown that increasing excitatory glutamatergic inputs onto motoneurons during REM sleep, both independently and when glycinergic and GABA_A-mediated inhibition are blocked, does not alter REM atonia (Brooks and Peever, 2008a; Burgess et al., 2008). Taken together, this supports the assertion that the increases in basal muscle tone that I observed in REM sleep involves postsynaptic inhibition mediated by GABA_B receptors with GABA_A and glycine receptors.

4.4.6 Conclusion

Although blockade of GABA_A, GABA_B and glycine receptors did not completely abolish REM sleep atonia, it did result in dramatic and prolonged increases in basal muscle tone. This is the first study to examine a role for GABA_B receptors, both alone and in conjunction with GABA_A and glycine receptors, in mediating REM atonia. The novel findings reported here give new insight into the biochemical substrates responsible for REM atonia. This study is important in that it not only points to a role for GABA_B-mediated inhibition in muscle control during REM sleep but also emphasizes that REM
atonia is not mediated by a single mechanism but is governed by a complicated collection of mechanisms that are not yet fully understood.
CHAPTER FIVE: INHIBITORY REGULATION OF THE TEMPORAL PATTERN OF PHASIC MUSCLE ACTIVITY DURING REM SLEEP

5.0 SUMMARY

Motor control in REM sleep is unique because it is characterized by intermittent muscle twitches that punctuate muscle atonia. Previously, I found a phasic inhibitory drive onto trigeminal motoneurons that functions to suppress muscle twitches during REM sleep. The aim of this study was to determine how this inhibition contributes to the temporal pattern of phasic twitch activity across REM sleep. To do this, I first quantified the pattern of masseter muscle activity across individual REM periods in rats. To examine the role for inhibition in regulating this pattern of activity, GABA\textsubscript{A} and glycine receptors were blocked on trigeminal motoneurons. I demonstrate that muscle activity is not uniformly distributed across REM periods, but gradually increases in frequency toward the end of REM episodes. When the endogenous inhibitory drive onto motoneurons is removed, there is a disproportionate increase in the frequency of twitches at the beginning of REM periods. REM atonia is unaffected by this intervention. Thus, inhibition appears to regulate the pattern of muscle twitch activity by suppressing twitches more strongly in the earlier stages of REM sleep. I conclude that glycine-ergic/GABA\textsubscript{A}-mediated inhibition of trigeminal motoneurons is differentially regulated across REM sleep.

5.1 INTRODUCTION

Rapid eye movement (REM) sleep is characterized by a complete suppression of skeletal muscle tone interspersed with phasic muscle twitches. Many studies have been undertaken to investigate the mechanisms underlying the atonia of REM sleep (Brooks and Peever, 2008a; Burgess et al., 2008; Chan et al., 2006; Chase et al., 1989; Fenik et al., 2005a; Jelev et al., 2001; Kodama et al., 2003; Kubin et al., 1993; Lai et al., 2001b; Morrison et al., 2003a; Morrison et al., 2003b; Soja et al., 1991), however understanding the pattern of muscle twitches that punctuate this atonia has been largely overlooked.

The neurophysiological activity of REM sleep is dynamic in nature but investigations into the regulation of muscle control during this state have traditionally
treated REM sleep as a homogenous state (Chase et al., 1989; Fenik et al., 2005a; Kodama et al., 2003; Kubin et al., 1993; Lai et al., 2001b; Soja et al., 1991). Even studies that do differentiate between REM atonia and phasic muscle twitches do not describe the distribution of each type of activity within the REM period (Brooks and Peever, 2008a; Burgess et al., 2008; Chan et al., 2006; Jelev et al., 2001; Morrison et al., 2003a; Morrison et al., 2003b). As such, little is known about the regulation of muscle activity over the course of REM sleep. A temporal pattern of activity has been reported in other REM sleep events such as rapid eye movements (Aserinsky, 1971; Salzarulo, 1972), ponto-geniculo-occipital (PGO) waves (Marks et al., 1980), middle ear muscle activity (Benson and Zarcone, 1979; Pessah and Roffwarg, 1972), and EEG power (Ferri et al., 2001; Takahara et al., 2006). Quantification of the intra-REM pattern of activity of these measures reveals a heterogeneous organization of events across REM sleep. This has given insight into the neural mechanisms responsible for these phenomena. Given that these other phasic events are not uniformly distributed across REM sleep, it seems plausible to hypothesize that phasic muscle twitches would not be either. In fact, an uneven distribution of phasic motor activity across REM sleep has been reported in the genioglossus muscle (Lu et al., 2005). Understanding the temporal organization of phasic muscle twitches in a REM period will provide insight into the nature of the influences acting on motoneurons during REM sleep and therefore should be investigated. Furthermore, this work could be of use to better understand how the temporal influences of REM sleep affect the motor dysregulation occurring in sleep disorders such as REM sleep behaviour disorder (RBD) obstructive sleep apnea (OSA).

In REM sleep, excitatory glutamatergic inputs onto motoneurons are responsible for the muscle twitches that break through the atonia of this state (Burgess et al., 2008; Soja et al., 1995). These excitatory inputs are opposed by inhibitory inputs (Chase and Morales, 1983; Glenn and Dement, 1985; Morales and Chase, 1981; Pedroarena et al., 1994): this coactivation of excitatory and inhibitory drives determines the amount of muscle twitch activity occurring across a REM period. By blocking glycine and GABA_A receptors on motoneurons, I and others have confirmed a role for inhibitory inputs in the suppression of muscle twitches in REM sleep (Brooks and Peever, 2008a; Morrison et al., 2003a). While this role for inhibition in the regulation of phasic twitch activity has been
established, how these inhibitory inputs influence the pattern of twitching has not been described.

The aim of this study was to gain insight into the temporal control of motoneuron excitability during REM sleep. To do this, I detailed the temporal organization of muscle activity across REM sleep. After establishing the intra-REM pattern of muscle activity, I examined the role for glycinergic and GABA\textsubscript{A}-mediated inhibition in regulating this pattern of activity. Here I demonstrate that motor tone is not uniformly controlled across the REM period. Furthermore, I identify a dynamic inhibitory drive in REM sleep that contributes to the regulation of this pattern of twitch activity. Together, these data illustrate the mechanism by which inhibitory inputs influence the distribution of muscle twitches across REM sleep.

5.2 METHODS

5.2.1 Animals

Studies were performed using 15 male Sprague Dawley rats (average weight: 351.1 ± 9.7g). Animals were surgically instrumented and recorded by the methods described in Chapter 2, sections 2.2-2.4.

5.2.2 Drug preparation

Strychnine (strychnine hydrochloride, FW: 370.9; Sigma-Aldrich, Oakville, ON) a glycine receptor antagonist, and bicuculline (\textemdash)-bicuculline methochloride, FW: 435.87; Tocris, Ellisville, MO) a GABA\textsubscript{A} receptor antagonist, were prepared in aCSF. Each drug was made immediately prior to each experiment. The AMPA receptor agonist (\textgreek{a}-amino-3-hydroxy-5-methylisoxazole-4-propionic acid, FW: 186.17; Tocris) was made in advance and stored in stock solutions at -20°C. All drugs were vortexed and filtered (0.22\mu m PVDF, Fisher Scientific) before use.
5.2.3 Experimental protocols

5.2.3.1 Study 1: Describe the temporal pattern of muscle activity across REM sleep
Many studies have investigated the mechanisms underlying REM atonia (Brooks and Peever, 2008a; Burgess et al., 2008; Chan et al., 2006; Chase et al., 1989; Fenik et al., 2005a; Jelev et al., 2001; Kubin et al., 1993; Lai et al., 2001b; Morrison et al., 2003a; Soja et al., 1991), however few have focused on understanding the pattern of muscle twitches that punctuate this atonia. To investigate the temporal distribution of muscle activity during REM sleep, I recorded masseter muscle activity in 15 rats across the sleep-wake cycle and identified all REM episodes occurring in the recording window. I then analyzed the patterns of masseter muscle activity across these REM periods.

5.2.3.2 Study 2: Investigate the role for glycinergic and GABA\textsubscript{A}-mediated inhibition in the temporal pattern of muscle activity across REM sleep
I and others have previously identified a glycinergic and GABA\textsubscript{A}-mediated control of phasic, but not tonic, REM activity (Brooks and Peever, 2008a; Morrison et al., 2003a). After establishing the temporal pattern of muscle activity across REM sleep that occurs during baseline conditions (Study #1), I investigated the role for glycinergic and GABA\textsubscript{A}-mediated inhibition of motoneurons in this pattern of activity. Accordingly, I simultaneously antagonized glycine and GABA\textsubscript{A} receptors (via 0.1mM strychnine and bicuculline) on trigeminal motoneurons and determined the resulting effects on REM motor tone. I then compared the pattern of masseter muscle activity occurring in REM sleep to that seen under baseline conditions. The concentrations of strychnine and bicuculline used have been shown previously to successfully antagonize glycine and GABA\textsubscript{A}-mediated neurotransmission (Brooks and Peever, 2008a; Liu et al., 2003; Morrison et al., 2003a).

5.2.4 Data analysis
Behavioural state and EMG analysis was performed as described in Chapter 2, section 2.4.

5.2.5 Verification of microdialysis probe location
Microdialysis probe locations were verified as described in Chapter 2, section 2.5.
5.2.6 Statistical analyses

All statistical analyses used Sigmastat (SPSS Inc., Chicago, IL) and applied a critical two-tailed alpha value of P<0.05. All comparisons made between baseline and the drug treatment were determined using analysis of variance with repeated measures (RM-ANOVA) and post hoc comparisons were performed using a Student-Newman-Keuls (SNK) test. Linear regressions determined the relationship between REM length and motor activity, with analysis of covariance (ANCOVA) being used to compare the effects of treatment on this relationship. All data are expressed as mean ± standard error of the mean (SEM).

5.3 RESULTS

The goal of this study was to gain insight into the temporal control of motoneurons during REM sleep. To do this, I examined the distribution of both tonic muscle tone and phasic twitch activity within individual REM periods to generate a detailed analysis of muscle activity during REM sleep. After establishing the temporal pattern of muscle activity occurring under baseline conditions, I investigated how this pattern of activity changed when inhibitory inputs were blocked at the motor pool.

5.3.1 Pattern of muscle activity across REM sleep

To investigate the temporal distribution of muscle activity during REM sleep, I recorded masseter muscle activity in 15 rats across the sleep-wake cycle and identified all REM episodes within a three hour recording period. Before exploring the pattern of twitch activity in these REM periods, I examined the relationship between the length of a REM episode with the amount of phasic twitch activity occurring in that episode.

5.3.1.1 REM duration as a predictor of phasic twitch activity

A total of 172 REM episodes were analyzed, with an average duration of 85.5 ± 7.2s. By grouping REM episodes by duration, I determined that there was an equal distribution of REM episode lengths in my sample (RM ANOVA, p=0.098; Figure 5.1): there was a trend for shorter REM episodes (ie. less than 30 seconds in duration); however this did not reach significance. Therefore, my data represent a broad range of REM periods,
**Fig. 5.1. Frequency of REM episode durations.** Group data (n=15) depicting the frequency distribution of different REM episode durations occurring during baseline recordings. There was no difference in the proportion of REM durations (p=0.098). All values are mean ± SEM.
allowing us to compare both patterns of motor activity within individual REM periods as well as how these patterns change with REM duration.

To establish the relationship between twitch activity and REM duration, the total number of twitches occurring in a REM period was plotted against the duration of the episode. I saw a positive correlation between the length of a REM episode and the number of twitches occurring in that episode (Linear regression, \(r=0.813, p<0.001\); Figure 5.2A). I also saw a positive relationship between the frequency of muscle twitches occurring in a REM period and the length of the episode (Linear regression, \(r=0.382, p<0.001\); Figure 5.2B). Taken together, these data indicate that REM duration is a good predictor of the total amount of phasic activity that will occur in an episode.

5.3.1.2 A temporal pattern of progressively increasing muscle activity across REM sleep
After establishing the relationship between REM duration and twitch activity, I next used several different measurements to investigate the pattern of muscle activity within individual REM periods, regardless of their length. To quantify the temporal pattern of activity, each of the 172 REM episodes were divided into quarters and the activity in each quarter was compared. This was done for both tonic muscle tone and phasic twitch activity.

Although subtle, there was a small but significant increase in basal muscle tone across the REM period, indicating that tonic levels of muscle activity increase progressively across REM sleep (RM ANOVA, \(p=0.004\); Fig 5.3A,B). A similar pattern of increasing phasic twitch activity was also found. The amount of phasic twitch activity gradually increased across individual REM episodes, with the frequency of twitch activity doubling from the first to the last quarter (RM ANOVA, \(p<0.001\); Figure 5.3A,C). In addition, there was a progressive increase in both the duration (RM ANOVA, \(p=0.004\); Figure 5.3A,D) and amplitude (RM ANOVA, \(p<0.001\); Figure 5.3A,E) of muscle twitches.

It is important to note that the progressive increase in twitch activity within a REM period was conserved across REM periods of differing lengths. When REM periods were grouped in 30s duration intervals, a gradual increase in twitch frequency within each episode was seen across all groups regardless of their length (RM ANOVA, \(p<0.05\); Figure 5.4).
Fig. 5.2. Muscle twitch activity increases with REM duration. The number (A: $r=0.813; p<0.001$) and frequency (B: $r=0.382, p<0.001$) of masseter muscle twitches occurring in a REM period are positively correlated with the length of the episode.
Fig. 5.3. Muscle activity increases progressively across REM sleep. A. Typical EEG and EMG trace of the masseter illustrating the temporal pattern of muscle activity across REM sleep. Group data showing how tonic muscle tone (B) and phasic twitch activity (C-E) change across individual REM periods. By dividing each REM period into quarters, gradually increasing amounts of both tonic and phasic muscle activity is revealed: basal muscle tone (B) and the frequency (C), duration (D) and amplitude (E) of phasic twitch activity all progressively increase across REM sleep. Different letters indicate statistically significant differences (p<0.05); a.u., arbitrary units. All values are mean ± SEM.
Fig. 5.4. REM duration does not affect the temporal pattern of increasing muscle activity across REM sleep. Group data showing that the pattern of progressively increasing twitch frequency across a REM period occurs regardless of the duration of the episode. REM periods were grouped by their duration in 30s intervals and the frequency of muscle twitches were plotted for each quarter of REM sleep, revealing that REM duration does not alter the pattern of increasing twitch activity across a REM period. All values are mean ± SEM.
Collectively, these data indicate that muscle activity is not uniformly regulated across REM sleep. Mechanisms underlying this pattern of activity should therefore follow a similar pattern of differential regulation across REM sleep.

5.3.2 Contributions of glycinergic/GABA$_A$-mediated inhibition to the pattern of muscle activity across REM sleep

I next investigated what role glycinergic/GABA$_A$-mediated inhibition of motoneurons has in the pattern of muscle activity I see across REM sleep. To do this, I recorded masseter muscle activity across the sleep-wake cycle while glycine and GABA$_A$ receptors were antagonized (via 0.1mM strychnine and bicuculline) on trigeminal motoneurons. I then compared the patterns of tonic and phasic masseter muscle activity in all REM periods occurring in the recording period to those seen under baseline conditions.

5.3.2.1 Glycinergic/GABA$_A$-mediated inhibitory control of phasic, but not tonic, REM motor activity

I identified a total of 107 episodes of REM sleep when strychnine and bicuculline were applied onto the motor pool. Of these, almost 40% of REM periods were less than 30 seconds in length (RM ANOVA, p<0.001; Figure 5.5), with all other REM lengths occurring in equal amounts. Thus, when inhibition was removed, short REM episodes were more common than longer episodes. The overall distribution of REM duration frequencies was not, however, different from baseline (RM ANOVA, p=0.375). In addition, the overall mean REM duration was not different between baseline and strychnine & bicuculline (paired t-test, 85.5 ± 7.2s vs. 71.2 ± 6.5s; p=0.168). Therefore, while there was a shift towards shorter REM periods, these data represent a diverse range of REM episodes to compare patterns of motor activity within the drug treatment as well as to compare with baseline levels of activity.

Overall, tonic REM activity was not affected by blockade of glycine and GABA$_A$ receptors (RM ANOVA, p=0.115), as previously reported (Brooks and Peever, 2008a; Morrison et al., 2003a). Phasic muscle activity, however, was increased after blockade of glycinergic and GABA$_A$-mediated inhibition (RM ANOVA, p=0.010). The increase in
Fig. 5.5. Frequency of REM episode durations during the drug treatment. Group data comparing the frequency of REM episode durations when glycine and GABA<sub>A</sub> receptors were blocked (via 0.1mM strychnine and bicuculline) on trigeminal motoneurons. During the pharmacological manipulation, there was a higher proportion of short (<30s) REM periods, with all other episode lengths occurring in equal amounts. Different letters indicate statistically significant differences (p<0.05). All values are mean ± SEM.
phasic activity was due to an increase in both twitch frequency (RM ANOVA, p=0.003) and duration (RM ANOVA, p=0.014), while the amplitude (RM ANOVA, p=0.171) of twitches did not change.

Removal of inhibition at the motor pool strengthened the positive linear relationship (ANCOVA, p<0.001) between the total number of twitches occurring in a REM episode and the duration of the episode (Linear regression, r=0.837, p<0.001; Figure 5.6A). The positive correlation between twitch frequency and REM duration seen under baseline conditions, however, no longer existed when inhibition was removed (Linear regression, r=0.0917, p=0.362; Figure 5.6B); this was due to a disproportionate increase in the frequency of twitches occurring in shorter REM periods, as indicated by the increase in the y-intercept after drug treatment (ANCOVA, p<0.001).

5.3.2.2 Glycinergic/GABA_A-mediated inhibitory regulation of the temporal pattern of phasic muscle activity across REM sleep

The disproportionate increase in the rate of muscle twitching in shorter REM episodes suggests that inhibition of motoneurons may have a stronger role in the earlier stages of a REM episode. If true, I would expect to see a change in the pattern of muscle twitch activity across a REM period when inhibition is removed. To investigate this, I divided all 107 REM episodes into quarters and the activity in each quarter was compared within the drug treatment as well as to baseline levels of activity. This was done for both tonic muscle tone and phasic twitch activity.

Blockade of glycine and GABA_A receptors had no effect on the temporal pattern of tonic muscle tone across REM sleep, as compared to baseline levels of activity (RM ANOVA, p=0.147; Figure 5.7A,B). Accordingly, the pattern of progressively increasing tonic activity observed under baseline conditions remained after blockade of glycine and GABA_A receptors (RM ANOVA, p=0.036; Fig 5.7B).

Unlike tonic muscle activity, there was a change in the temporal pattern of phasic twitch activity when glycine and GABA_A receptors were blocked. Antagonism of glycine and GABA_A receptors resulted in a disproportionate increase in the frequency of muscle twitches occurring in the first quarter of REM sleep (RM ANOVA, p=0.045; Figure 5.7A,C): the frequency of twitches increased by 110% in the first quarter compared to
Fig. 5.6. Muscle twitch activity increases with REM duration. A. The positive relationship between the number of masseter muscle twitches occurring in a REM episode and the length of the episode seen under baseline conditions (dashed line through open circles: $r=0.813; p<0.001$) is strengthened by blockade of glycine and GABA$_A$ receptors (via 0.1mM strychnine and bicuculline) on trigeminal motoneurons (solid line through triangles: $r=0.837; p<0.001$). B. Conversely, the relationship between the frequency of muscle twitches occurring in a REM period and the length of the episode ($r=0.382, p<0.001$) is lost after application of strychnine and bicuculline onto motoneurons ($r=0.0917, p=0.362$).
Fig. 5.7. Glycinergic/GABA<sub>A</sub>-mediated inhibition regulates the size and distribution of muscle twitches during REM sleep.

A. Representative EEG and EMG traces of masseter activity during REM sleep under baseline (top trace) and when 0.1mM strychnine and bicuculline (bottom trace) was perfused into the trigeminal motor pool. Group data comparing the effects of strychnine and bicuculline (solid line through triangles) on tonic muscle tone (B) and phasic muscle twitch activity (C-E) to that seen under baseline (dashed line through open circles). Blockade of glycine and GABA<sub>A</sub> receptors has no effect on tonic activity (B). Phasic muscle activity is, however, affected: the frequency of twitches disproportionately increased in the first quarter of REM (C). The duration of twitches also increased, however the pattern of progressively increasing activity remained intact (D). Twitch amplitude was not affected (E). * indicates a statistically significant difference between treatments (p<0.05); different letters indicate statistically significant differences within treatments (p<0.05); a.u., arbitrary units. All values are mean ± SEM.
28-36% in the remaining quarters. By disproportionately increasing twitch frequency at the beginning of REM sleep, blockade of glycine and GABA\textsubscript{A} receptors caused the pattern of progressively increasing twitch frequency across REM sleep to be lost (RM ANOVA, p=0.351; Figure 5.7C).

While removal of inhibition altered the pattern of increasing twitch frequency across REM sleep, the pattern of progressively increasing twitch size was preserved (RM ANOVA; duration: p=0.002; amplitude: p=0.005; Figure 5.7A,D,E). Therefore, blockade of glycine and GABA\textsubscript{A} receptors on motoneurons results in an overall increase in muscle twitch activity, but only affects the pattern of twitch frequency.

5.3.3 Verification of experimental interventions: specificity to motor output of the left trigeminal motor nucleus

To verify that my results were a true reflection of the manipulations at the motor pool and not due to unintended secondary effects, I performed a series of controls. First, through a variety of measures that have been described in detail in previously published reports (Brooks and Peever, 2008a; Burgess et al., 2008; Schwarz et al., 2008), I ensured that the pharmacological manipulation used in this study was specific to the motor output of the left trigeminal motor nucleus. I also performed post-mortem histology to confirm probe placement within or adjacent to the trigeminal motor nucleus in all rats (Figure 5.8). Only rats in which all control measures had been satisfied were included in the study.

5.4 DISCUSSION

Here I describe and quantify the temporal pattern of muscle activity across REM sleep. I show that both tonic and phasic muscle activity progressively increase over the course of a REM period. My findings further expand on this by describing a role for an inhibitory drive in the regulation of muscle activation across REM sleep. When glycinergic/GABA\textsubscript{A}-mediated inhibition was blocked at the motor pool, phasic muscle activity increased, but this increase was not uniform across the REM period: while there was an overall increase in twitch activity, it was more pronounced in the beginning stages
Fig. 5.8. Microdialysis probes were located in the left trigeminal motor pool. Black filled circles (left side of each diagram) represent the location of microdialysis probe lesions in the trigeminal motor pool. All probes were located within or adjacent to the motor nucleus.
of REM sleep. This suggests that the inhibitory drive in REM sleep contributes to the temporal pattern of motor control by suppressing twitch activity more strongly in the earlier stages of a REM period. Tonic muscle tone was unaffected by this intervention, as previously described (Brooks and Peever, 2008a; Morrison et al., 2003a), and is therefore not under the control of glycinergic/GABA\textsubscript{A}-mediated inhibition. In this study, by investigating the heterogeneous nature of REM sleep, I have developed a model of REM sleep motor control that helps to illustrate the complicated set of mechanisms that underlie muscle activity in this state.

5.4.1 Model of excitatory and inhibitory inputs during REM

It has been proposed that the muscle twitches of REM sleep are the result of excitatory glutamatergic inputs (Burgess et al., 2008; Soja et al., 1995) superimposed on a background of motoneuron inhibition (Chase and Morales, 1983; Glenn and Dement, 1985; Morales and Chase, 1981; Pedroarena et al., 1994). By examining the temporal organization of muscle activity across REM sleep, my data support this assertion and further suggest that the pattern of muscle twitches occurring across REM sleep is due to a progressive decrease in inhibition with a concomitant gradual increase in excitation (Figure 5.9).

Based on this model, I would predict that muscle twitch activity would increase with REM sleep duration: as REM sleep progresses, there would be gradually less inhibition and more excitation, resulting in more twitch activity (Figure 5.9A). This would result in longer REM episodes having more twitches as well as a higher rate of twitching. The converse would be true for shorter periods: I would predict that shorter REM periods would have fewer twitches, as shorter REM periods would be subjected to more inhibition and less excitation. Together, this would translate to a positive correlation between twitch activity and REM duration, as demonstrated by my data (see Figure 5.2).

This model of increasing excitatory and decreasing inhibitory inputs predicts a pattern of progressively increasing muscle twitch frequency across REM sleep. Twitches should also be smallest, both in duration and amplitude, in the earlier stages of REM sleep when inhibition is at its highest and excitation is at its lowest. Over time, as inhibition gradually decreases and excitation steadily increases, the occurrence and size
Fig. 5.9. Proposed model of inhibitory/excitatory inputs responsible for phasic muscle activity in REM sleep. Raw EEG and EMG traces of the masseter muscle during REM sleep under baseline (A) and when 0.1mM strychnine and bicuculline (B) was perfused into the trigeminal motor pool. Each raw trace is accompanied by a schematic depicting the hypothesized pattern of inhibitory and excitatory activity impinging on motoneurons during REM sleep under each treatment. It is proposed that the amount of inhibitory activity gradually decreases with time spent in REM sleep, while there is a progressive increase in excitatory activity. This model of activity is hypothesized to account for the temporal pattern of muscle twitch activity normally seen in REM sleep (A) as well as the changes in twitch activity that are seen when inhibition is removed (via strychnine and bicuculline: B).
of twitches should increase. My data reflect this predicted temporal pattern of twitch activity (see Figure 5.3). In addition, the pattern of increasing twitch activity should exist regardless of REM duration, as all REM periods would be subjected to this temporal pattern of control. This again is seen in my data (see Figure 5.4).

Together, these data support the predictions of my model of motor control in REM sleep. This proposed pattern of decreasing inhibitory inputs across REM sleep is further supported by the temporal relationship identified between PGO waves and inhibitory motor control. PGO waves reach a peak in frequency at the midpoint of REM sleep and then trail off near the end of the period (Marks et al., 1980). Both a reduction in phasic motor activity and the postsynaptic inhibition of motoneurons are correlated with the occurrence of PGO waves (Lopez-Rodriguez et al., 1992; Orem, 1980; Pivik et al., 1982). Therefore, it follows that as PGO waves progressively decrease in frequency across REM sleep, inhibitory inputs should as well. This notion is supported by my data and strengthens my proposed model of motor control across REM sleep.

5.4.2 Experimental confirmation of model: Blockade of inhibitory inputs

To further assess the validity of this model, predictions can be made on the effects that removal of inhibitory activity will have on motor output during REM sleep.

When inhibition is removed, the positive relationship between twitch activity and REM length should become stronger because the progressively increasing excitation would no longer be counteracted by the gradually decreasing inhibition (Figure 5.9B). The unopposed excitatory inputs, and therefore muscle twitches, would gradually increase with time spent in REM sleep, resulting in a stronger linear relationship between twitch activity and REM duration. Thus, as my data demonstrate, the rate of muscle twitching will be more constant across all REM periods, resulting in REM length becoming a better predictor of the total number of twitches that will occur in an episode (see Figure 5.6).

Based on this model, when inhibition is removed, I would not only predict that overall muscle twitch activity would increase, but also that the occurrence of twitches across the REM period would change. Removal of inhibitory influences should result in a disproportionate increase in the number of twitches in the beginning of REM sleep.
According to the model, excitation is more strongly opposed in the early stages of REM sleep as compared to the later stages. As a result, removal of inhibition would have a greater effect on motor output in the beginning of a REM episode: this would translate to a disproportionate increase in the rate of twitching in the earlier stages of REM sleep after blockade of inhibition, as seen in my data (see Figure 5.7A,C).

While blockade of glycine and GABA_A receptors will alter the occurrence of muscle twitches, the pattern of progressively increasing twitch size should remain intact. This is because excitation would still follow the same pattern of gradually increasing strength across REM sleep. Twitches should, however, be larger: the excitatory drive responsible for twitches will no longer be opposed by inhibition and therefore twitch activity will be of greater magnitude. My data again support this prediction (see Figure 5.7D,E).

Unlike phasic muscle activity, the temporal pattern of tonic activity was unaffected by blockade of glycine and GABA_A receptors. This confirms previous reports that REM atonia is not under the control of these inhibitory inputs (Brooks and Peever, 2008a; Morrison et al., 2003a).

Collectively, these data support proposed model of decreasing inhibition and increasing excitation across REM sleep (Figure 5.9). This model accounts for the temporal pattern of muscle twitch activity I see in REM sleep under baseline conditions as well as the changes in twitch activity that are observed when inhibition is removed. Together, with my proposed model, these data highlight the important role for glycinergic/GABA_A-mediated inhibition in the regulation of phasic muscle twitches as well as support the assertion that tonic and phasic motor tone are controlled by different mechanisms.

5.4.3 Dynamics of intra-REM regulation of events

Several studies have investigated the influences that modulate muscle control in REM sleep (Brooks and Peever, 2008a; Burgess et al., 2008; Chan et al., 2006; Chase et al., 1989; Fenik et al., 2005a; Jelev et al., 2001; Kodama et al., 2003; Kubin et al., 1993; Lai et al., 2001b; Morrison et al., 2003a; Morrison et al., 2003b; Soja et al., 1991), however the time course of these events has largely been ignored. It is assumed that the
mechanisms regulating REM motor control are constant throughout the state. Our data, however, indicate that motor control is not uniformly regulated across REM sleep. This is in agreement with other reports in which phasic bursts of muscle activity are shown to increase progressively over the course of a REM period (Lu et al., 2005; Orem, 1996; Perlis et al., 1995). Other REM events also show a dynamic pattern of regulation. For example, both rapid eye movements and PGO waves tend to increase in frequency, reaching a peak at the middle of the REM period and then decrease toward the end of the period (Aserinsky, 1971; Marks et al., 1980; Salzarulo, 1972). Two-thirds of all middle ear muscle activity, another REM phenomenon, is reported to occur in the first half of a REM period (Benson and Zarcone, 1979; Pessah and Roffwarg, 1972). Thus, REM sleep is not a homogenous state and therefore to fully understand the mechanisms that underlie the events of REM sleep, the dynamic nature of this state must be taken into account.

The presence of an inhibitory drive in REM sleep has been identified using a series of techniques (Brooks and Peever, 2008a; Chase et al., 1989; Kodama et al., 2003; Morrison et al., 2003a; Soja et al., 1991), however, until now, the temporal pattern of this drive has not been considered. This may explain some of the discrepancies in the literature: due to the dynamic organization of motor regulation that I identify here, the mechanisms detected will vary depending on when in the REM period measurements are made. For example, studies in which measurements are made after REM sleep has been established will differ from those which begin to record from the transition into REM sleep, as the mechanisms present in the early stages of a REM period will only be included in the latter. Similarly, summing activity across a REM period or excluding the phasic portions of an episode, both of which are commonly done, will also result in variation between studies. To elucidate the neurochemical mechanisms underlying REM motor control, all of REM sleep, including the time course of events, must be considered. As such, it is not surprising that a consensus in the literature has not been achieved (Brooks and Peever, 2008b).

5.4.4 Clinical implications and conclusion

The presence of an inhibitory drive in REM sleep has been previously identified (Chase et al., 1989; Kodama et al., 2003; Soja et al., 1991): blockade of inhibitory inputs at the
motor pool results in an increase in phasic muscle activity (Brooks and Peever, 2008a; Morrison et al., 2003a). In this study, I further our knowledge of this inhibitory drive by defining the temporal organization of inhibition across REM sleep. Inhibition not only opposes the excitatory inputs responsible for muscle twitches, but also shapes the dynamic organization of motor control in REM sleep. Together these opposing excitatory and inhibitory drives define the pattern of motor activity across REM sleep.

In addition to advancing our understanding of the complicated set of neurochemical mechanisms that underlie REM motor control, these data also highlight the importance of considering the microarchitecture of a state to fully understand the influences at work. Characterizing the temporal organization of REM sleep is not only of interest from a neurobiological perspective, but is also of clinical relevance. In many sleep disorders involving a dysregulation of motor control, the pathology occurs during the phasic portion of REM sleep. For example, in REM sleep behaviour disorder, the motor-behavioural episodes that define this disorder are more likely to occur during phasic periods of REM sleep (Manni et al., 2009). Relevant to obstructive sleep apnea, respiration is compromised during phasic REM sleep (Aserinsky, 1965), increasing the risk of apneic events in the transition from periods of tonic to phasic REM (George et al., 1987; Johnson and Remmers, 1984). By understanding the temporal organization of muscle activity in REM sleep, effective treatments targeting the influences that modulate REM motor control can be found.
6.0 SUMMARY

REM sleep behaviour disorder (RBD) is a neurological disorder characterized by excessive phasic muscle activity in REM sleep, which often leads to disturbed sleep and physical injury. While the cause of RBD is unknown, a strong association between RBD and neurodegenerative disorders has been found, with many patients going on to develop Parkinson’s disease or other synucleinopathies. There is evidence to suggest a dysregulation of inhibitory processes may underlie RBD. First, suppression of phasic muscle activity during REM sleep, which is lost in RBD, is caused by glycinergetic/GABAergic inhibition of skeletal motoneurons. RBD symptoms can be triggered by strokes that affect brainstem regions containing glycinergetic and GABAergic neurons. Lesions to similar areas in cats and rats also result in an RBD-like motor phenotype. Finally, drugs that enhance inhibitory function (ie. clonazepam and melatonin) are the most effective treatment for RBD patients. Together, this suggests that dysregulation of the endogenous inhibitory processes that normally suppress phasic muscle activation in REM sleep may underlie the exaggerated motor activity in RBD. I therefore hypothesize that transgenic mice with impaired inhibitory transmission will have excessive motor activity in REM sleep and therefore exhibit an RBD phenotype. To test this hypothesis, I used a transgenic mouse model in which both GABAergic and glycinergetic neurotransmission is severely downregulated. Using polysomnography, I demonstrate these mice display an RBD phenotype in sleep. Examination of sleep-wake architecture and cortical activation also reveal striking similarities between RBD patients and the transgenic mice. Finally, I show that these mice can be successfully treated with pharmacological agents commonly used to treat RBD. Together, these results indicate that impaired inhibitory neurotransmission may underlie RBD.
6.1 INTRODUCTION

Rapid eye movement (REM) sleep behaviour disorder (RBD) is a neurological disorder of unknown cause, characterized by complex motor activity during REM sleep usually associated with dream mentation (Olson et al., 2000; Schenck and Mahowald, 2002; Sforza et al., 1997). Patients show elaborate nocturnal motor behaviours, often with violent or injurious results to themselves or their bedpartner (Schenck and Mahowald, 2002). RBD is clinically diagnosed by polysomnography, a technique that employs simultaneous recording of electroencephalography (EEG) and electromyography (EMG). Polysomnographic recordings of RBD patients reveal a loss of normal skeletal muscle atonia during REM sleep accompanied by excessive phasic muscle twiching (American Academy of Sleep Medicine, 2001; Lapierre and Montplaisir, 1992; Mahowald and Schenck, 2005). In addition to a disruption of normal REM motor control, RBD is associated with abnormal movements during non-rapid eye movement (NREM) sleep, sleep disruptions and impairments of cortical activity (Fantini et al., 2003b; Fantini et al., 2002; Gagnon et al., 2004; Massicotte-Marquez et al., 2005; Massicotte-Marquez et al., 2008; Olson et al., 2000; Schenck et al., 1993).

While some cases of RBD may be idiopathic, there is a growing recognition of an association between RBD and neurodegenerative disorders, such as Parkinson’s disease, multiple systems atrophy, and dementia with Lewy bodies (Boeve et al., 2004; Boeve et al., 2001; Gagnon et al., 2002; Gagnon et al., 2006a; Olson et al., 2000; Schenck and Mahowald, 2002). In many cases, RBD precedes these other disorders by several years, suggesting that RBD may be an early marker in the development of neurodegenerative disease (Iranzo et al., 2006; Schenck et al., 1996, 2003). As such, an increased understanding of RBD is not only important in alleviating the debilitating idiopathic condition, but may also advance our knowledge of the mechanisms underlying the associated disorders.

Investigations into the underlying pathology of RBD have been hindered by the lack of a clinically relevant animal model. Multiple lesioning studies have been undertaken to elucidate the brain regions involved in mediating REM sleep atonia (Friedman and Jones, 1984; Hendricks et al., 1982; Holmes and Jones, 1994; Jouvet and Delorme, 1965; Lu et al., 2006; Sanford et al., 2001; Schenkel and Siegel, 1989; Shouse
These important works have identified specific brain areas that when lesioned produce REM sleep motor abnormalities similar to those seen in RBD and therefore are useful in identifying brain regions that may be affected in the disorder (Boeve et al., 2007; Schenck and Mahowald, 2002). However, to fully understand the pathology of RBD, an animal model should replicate as many aspects of the disease as possible, not just the behavioural manifestations in REM sleep. Therefore, to better understand RBD, an animal model that displays the motor abnormalities in both NREM and REM sleep as well as recapitulates other hallmark behavioural and physiological characteristics of the human condition is needed.

Although its cause is unknown, RBD is effectively treated with the benzodiazepine clonazepam (Lapierre and Montplaisir, 1992; Olson et al., 2000; Schenck et al., 1993; Sforza et al., 1997). The primary mechanism of clonazepam is to modulate GABAergic function via the benzodiazepine receptor, which leads to enhanced GABAergic inhibition (Choi et al., 1977; Costa and Guidotti, 1979; Macdonald and Barker, 1978). While clonazepam is the treatment of choice for RBD, the link between a disruption in inhibitory function and RBD has not been examined. There are, however, reports of GABAergic and glycineric disruptions in Parkinson’s disease (Demontis et al., 1982; Lloyd and Hornykiewicz, 1973; Lloyd et al., 1977; Nishino et al., 1988; Rinne et al., 1978); given the strong association between Parkinson’s disease and RBD (Schenck et al., 1996, 2003), a disruption of inhibitory processes may also be present in RBD. A role for inhibitory amino acids in mediating muscle activity in REM sleep has been established (Chase et al., 1989; Kodama et al., 2003): removal of this inhibitory drive results in excessive phasic activity in REM sleep, as described in RBD (Brooks and Peever, 2008a; Morrison et al., 2003a). Furthermore, abnormal motor activity in sleep has been reported in at least two CNS disorders in which glycineric or GABAergic function is impaired (ie. hyperekplexia and stiff person syndrome) (de Groen and Kamphuisen, 1978; Dinkel et al., 1998; Gastaut and Villeneu.A, 1967; Levy et al., 2005; Martinelli et al., 1996; Martinelli et al., 1978; Shiang et al., 1993; Solimena et al., 1990; Solimena et al., 1988). Taken together, this suggests a dysregulation of the endogenous inhibitory processes that normally suppress muscle activity in REM sleep could, at least in part, underlie the exaggerated motor activity in RBD.
To explore if a dysregulation of inhibitory processes may underlie the RBD phenotype, I used transgenic mice that express both a wildtype and mutant human alpha-1 subunit of the glycine receptor in the brain and spinal cord (Becker et al., 2002). By competing with the endogenous subtype, the mutant human subtype results in a 70% reduction in glycinergic transmission. Although the mechanism is unknown, expression of the mutant subunit also results in a 90% reduction in GABAergic transmission (Becker et al., 2002). Therefore, these transgenic mice have an overall disruption of postsynaptic inhibitory transmission. I examined the phenotype of these mice to investigate if GABAergic and glycinergic processes could play a role in the underlying pathology of RBD.

Here I report that a downregulation of glycinergic and GABAergic processes via a mutated glycine receptor subtype results in a phenotype remarkably similar to RBD. Using infrared video, I observed abnormal sleep behaviours in these transgenic mice that strongly resembled those seen in human RBD. Polysomnography confirmed and quantified my observations of an RBD-like phenotype. Examination of the sleep-wake architecture and cortical activation of these mice further strengthened the comparison between the transgenic mice and RBD patients. Finally, I successfully alleviated the motor phenotype of the transgenic mice with pharmacological agents commonly used to treat RBD. Taken together, my findings suggest that a dysregulation of inhibitory processes may, at least in part, underlie RBD.

6.2 METHODS

6.2.1 Animals

Adult male transgenic mice (Tg; 22.8 ± 0.3g; n=23) and their male wildtype littermates (Wt; 29.9 ± 0.6g; n=23) were surgically instrumented and recorded by the methods described in Chapter 2, sections 2.2-2.4.
6.2.2 Drug preparation and treatment

6.2.2.1 Clonazepam
Clonazepam, a benzodiazepine, is the standard agent used to treat RBD. It immediately alleviates the motor symptoms of RBD in upwards of 90% of patients (Lapierre and Montplaisir, 1992; Olson et al., 2000; Schenck et al., 1993; Sforza et al., 1997). To investigate the effects of clonazepam on the motor phenotype of this mouse model, a subset of Wt and Tg mice received i.p. injections of 0.3mg/kg clonazepam (Roche, Toronto, ON, Canada) dissolved in 0.9% saline. All injections were administered midway through the light period (1300h). The volume of drug given to each animal was determined prior to each injection based on the animal’s weight and this volume was topped up with saline such that each animal received 0.3mg/kg clonazepam in a 0.2mL bolus. EEG and EMG activity were quantified for the 3 hours following the injection. This activity was then compared within each animal to their own pre-treatment levels of activity.

6.2.2.2 Melatonin
Melatonin is a potential alternative to clonazepam in the treatment of RBD, as long-term melatonin treatment has been shown to be beneficial in alleviating RBD symptoms (Boeve et al., 2003; Kunz and Bes, 1997, 1999; Kunz and Mahlberg, 2010; Takeuchi et al., 2001). To chronically treat Tg mice, a melatonin (Sigma Aldrich, Oakville, ON, Canada) solution was prepared in ethanol and dissolved in the drinking water (12.5ug/ml tap water, in 0.066% ethanol) for 2-4 weeks. The water bottle containing melatonin was protected from light throughout the experiment. A fresh melatonin solution was prepared twice a week. Both Wt and Tg mice drank, on average, 4.1±0.28mL per 24hr, with 95% of water intake occurring during the dark phase. This is in line with what has previously been reported (Johnson et al., 2003). Although the volume of water-intake varied slightly among animals, melatonin was administered at approximately 2.0 mg/kg/day. A group of Wt and Tg mice were treated with melatonin for 2-4 weeks and then EEG and EMG activity was quantified over a 24 hour period. This activity was compared to their untreated counterparts.
6.2.3 Data analysis

6.2.3.1 Behavioural state and EMG analysis
Behavioural state and EMG analysis was performed as described in Chapter 2, section 2.4.

6.2.3.2 Sleep-wake architecture
The proportion of time spent in each sleep-wake state was calculated across a 24 hour period and compared between Wt and Tg mice. The number of state transitions (ie. arousals from NREM and REM sleep, NREM to REM transitions) was also quantified.

6.2.3.3 EEG spectral analysis
EEG spectral analysis was calculated in 1.0Hz bins using fast Fourier transformation of each 5s epoch, yielding a power spectra profile over a 0 to 16 Hz window. A mean EEG spectrum profile was obtained for each epoch and then, to minimize non-specific differences in absolute power between individuals, EEG power in each frequency bin was expressed as a percentage of the total EEG power in the epoch. The spectral profiles of each behavioural state were then compared between Wt and Tg mice.

6.2.4 Statistical analyses
All statistical analyses used Sigmastat (SPSS Inc., Chicago, IL) and applied a critical two-tailed alpha value of p<0.05. Differences in EMG activity between Wt and Tg mice were determined using t-tests. The proportion of time spent in each sleep-wake state and total EEG power in each state were compared between Wt and Tg mice using analysis of variance with repeated measures (RM-ANOVA) and post hoc comparisons were performed using a Student-Newman-Keuls (SNK) test. All data are expressed as mean ± standard error of the mean (SEM).

6.3 RESULTS

6.3.1 Tg mice have a motor phenotype that recapitulates RBD
RBD patients have abnormal motor control in sleep, consisting of excessive limb and body movements (Olson et al., 2000; Schenck and Mahowald, 2002; Sforza et al., 1997).
Using infrared video coupled with EEG to identify behavioural state, I observed a strikingly similar motor phenotype displayed by Tg mice. Clear behavioural manifestations of an RBD-like phenotype were present in all Tg mice. As in RBD, the most obvious motor abnormalities occurred in REM sleep. Tg mice displayed excessive movements during REM sleep, the like of which were never observed in Wt mice. While limb twitching and gross body and limb jerking occurred throughout each REM period, most movements appeared to be directed, complex locomotor events, with vigorous yet seemingly coordinated limb and head movements. These bursts of movement were interspersed with quiet periods, consisting of little to no activity.

Like RBD patients, abnormal motor control was also observed in NREM sleep, with Tg mice displaying myoclonic twitching throughout this state. Occasionally these jerks and twitches were restricted to one muscle group; however, most appear to be whole body twitches. Such twitches were never seen in Wt mice. Like the excessive motor activity in REM sleep, these myoclonic twitches were present in all episodes of NREM sleep.

Tg mice adopted an abnormal sleep posture, spending most REM periods lying on their side, whereas Wt mice slept in the stereotypical mouse sleeping posture (ie. curled into a ball). While this abnormal posture likely allowed Tg mice to remain asleep despite the vigorous movements occurring during REM sleep, an increase in awakenings from REM sleep was observed. Likewise, although Tg mice were able to sleep through much of the NREM myoclonic twitching, there did appear to be an increase in arousals from NREM sleep, particularly after some of the larger, whole body jerks. This agrees with observations in RBD patients, as a disruption of sleep continuity by sleep behaviours is one of the diagnostic criteria for RBD (American Academy of Sleep Medicine, 2001).

In addition to their sleep phenotype, I also characterized the waking behaviour of the Tg mice. Behaviourally, Tg mice looked like Wt mice during waking, displaying all of the normal waking activities (ie. eating, drinking, grooming, ambulating, burrowing/digging, etc). Tg mice did, however, have occasional jerks and jumps as well as spontaneous tremor episodes. In addition, these mice appeared to have a slightly slower, stiffer gait than Wt mice, likely due to hypertonic hindlimbs, although this was
only noticeable when animals were moving quickly. Subtle motor and gait slowing has also been reported in RBD (Postuma et al., 2006).

It is important to note that I do not believe that the abnormal motor behaviours observed in these mice are seizure activity. The mutation possessed by these Tg mice mimics the specific gene mutation occurring in hyperekplexia (Becker et al., 2002), a human disorder in which seizure is not a feature (Andermann et al., 1980; Suhren et al., 1966). Furthermore, seizure activity was not been reported in the original paper describing these mice (Becker et al., 2002), nor any subsequent papers utilizing these mice.

6.3.2 Polysomnographic confirmation of a RBD motor phenotype in Tg mice

I next confirmed and quantified the observed motor dysfunction during sleep and wake in Tg mice using polysomnography. A comparison of electrophysiological recordings of muscle activity from Tg mice with their Wt littermates confirmed that Tg mice have abnormalities in motor control during REM sleep. Tg mice had significantly elevated EMG activity during REM sleep in all muscles recorded (108% increase in masseter: t-test, p<0.001, Wt: n=16, Tg: n=14; 107% increase in hindlimb: t-test, p=0.021, Wt: n=3, Tg: n=4; 119% increase in neck: t-test, p=0.023, Wt: n=12, Tg: n=8 n=12; Figure 6.1). This increase in activity was due to an increase phasic twitching, as REM atonia remained intact in these animals (t-test, p=0.236; Figure 6.2A,B). Tg mice had an increase in the frequency (267% increase: t-test, p<0.001), duration (86% increase: t-test, p<0.001), and amplitude (65% increase: t-test, p<0.001) of phasic muscle twitches, with these larger, more frequent twitches occurring earlier in the REM period (46% decrease in latency to twitching from REM onset: t-test, p<0.001) (Figure 6.2A,C). This excessive phasic twitching during REM sleep is strikingly reminiscent of that seen in human RBD, in which increased phasic EMG activity is a hallmark of the disorder (American Academy of Sleep Medicine, 2001; Lapierre and Montplaisir, 1992; Mahowald and Schenck, 2005).

Because Tg mice display wake-like motor behaviour in REM sleep, I used post hoc analyses to verify that the behavioural state visually identified as REM sleep was in
Fig. 6.1. **EMG activity is elevated in Tg mice during REM sleep.** Typical raw EMG and EEG traces from a Wt (top left trace) and a Tg mouse (top right trace) during REM sleep. EMG activity of the neck, limb and masseter for each animal is shown. Tg mice have excessive activity in all muscles recorded during REM sleep, as compared to Wt mice. Group data for Wt (white bars) and Tg (black bars) mice demonstrating that Tg mice have significantly higher muscle activity in the masseter (Wt: n=16; Tg: n=14), limb (Wt: n=3; Tg: n=4), and neck (Wt: n=12; Tg: n=8) during REM sleep. * indicates p<0.05; a.u., arbitrary units. All values are mean ± SEM.
Fig. 6.2. Tg mice have excessive phasic muscle twitches during REM sleep. A. Raw traces from a Wt and a Tg mouse depicting masseter EMG activity during REM sleep. While REM atonia was not altered (B), phasic muscle twitch activity was significantly elevated in Tg mice (n=14; black bars) as compared to Wt mice (n=16; white bars) in all measurements made (C). * indicates p<0.001; a.u., arbitrary units. All values are mean ± SEM.
fact REM sleep and not wakefulness. First I compared REM sleep between Tg and Wt mice to demonstrate that this identified state is similar between the genotypes. Tg mice spent the same proportion of time across the 24 hour day in the state identified as REM sleep as Wt mice (t-test, p=0.670; Figure 6.3A). Furthermore, the distribution of EEG spectral power was similar between Wt and Tg during REM sleep, with theta being the predominant wave pattern, as expected for REM sleep (Figure 6.3B). Next I compared REM sleep with waking in Tg mice to examine differences between these two identified states. Bout length of waking episodes was significantly longer than those periods identified as REM sleep (paired t-test, p<0.001; Figure 6.3C). In addition, the distribution of EEG power in waking differed from that in REM sleep (RM ANOVA, p<0.001; Figure 6.3D), each reflecting the spectral profile of what would be expected for each state. Together, by comparing the similarities between Wt and Tg REM sleep as well as contrasting the differences between waking and REM sleep in Tg mice, these post hoc analyses validate my visual identification of REM sleep in Tg mice.

Like human RBD (Fantini et al., 2002; Olson et al., 2000; Schenck et al., 1993), Tg mice displayed motor hyperactivity during NREM sleep in the form of myoclonic twitches (Figure 6.4). Quantification of these twitches revealed that Tg mice have, on average, 174 ± 23 NREM-specific twitches over a 4 hour period (Figure 6.4 inset), and these twitches mostly occurred periodically, with almost 70% of twitches occurring less than 20s apart. Both the presence and periodicity of these twitches is in line with what has been reported in human RBD: 61% of patients experience periodic movements in NREM sleep every 15-30s (Schenck et al., 1993). Similar twitches were never observed in Wt mice (Figure 6.4 inset). Despite the presence of these myoclonic twitches only in Tg mice, overall basal EMG levels during NREM sleep were not different between Wt and Tg mice (t-tests: masseter: p=0.115; hindlimb: p=0.083; neck: p=0.071; Figure 6.4). Taken together, the excessive motor activity during REM sleep coupled with the periodic movements during NREM sleep, supports the notion that a dysregulation of inhibitory processes may underlie the irregular motor phenotype of human RBD.

Polysomnography confirmed the increases in muscle tone observed in Tg mice during waking, particularly in the hindlimbs. Muscle tone was increased by 68% in the masseter (t-test, p=0.002) and 118% in the hindlimb (t-test, p=0.023) during waking
**Fig. 6.3. Verification of state in Tg mice.** *Post-hoc* confirmation that the behavioral state visually identified as REM sleep in Tg mice was in fact REM sleep and not wakefulness. By comparing the amount of time Wt (n=19; white bar) and Tg (n=16; black bar) mice spent in REM sleep (A) as well as the EEG power distribution in REM sleep (B; Wt: n=15, dotted line; Tg: n=11, solid line), it is demonstrated that this identified state is similar between the genotypes. By comparing the length of waking bouts (white bar) with the length of REM bouts (black bar) in Tg mice (C) as well as the EEG power distribution in each of these states (D, wake: dotted line; REM: solid line), it is demonstrated that waking and REM sleep are different in Tg mice. * indicates p<0.001. All values are mean ± SEM.
Fig. 6.4. Tg mice have myoclonic twitches during NREM sleep. Typical raw EMG and EEG traces from a Wt (top left trace) and a Tg mouse (top right trace) during NREM sleep. EMG activity of the neck, limb and masseter for each animal is shown. While muscle tone is not elevated during this state, Tg mice do experience myoclonic twitches during NREM sleep, as seen here (inset). Myoclonic twitching is not seen in Wt mice. Group data for Wt (white bars) and Tg (black bars) mice for the masseter (Wt: n=16; Tg: n=14), limb (Wt: n=3; Tg: n=4), and neck (Wt: n=12; Tg: n=8) during NREM sleep. * indicates p<0.05; a.u., arbitrary units. All values are mean ± SEM.
There was not, however, a difference in EMG activity in the neck ($t$-test, $p=0.787$; Figure 6.5). RBD patients have subtle abnormalities in motor and gait speed, which have been suggested to be early markers of parkinsonism (Postuma et al., 2006). The measured increases in EMG tone during waking in Tg mice, together with the observed hypokinesia of gait, are therefore another clinically important characteristic shared by these Tg mice with RBD patients.

**6.3.3 Tg mice have increased amounts of quiet waking and more awakenings from sleep**

Next, I investigated whether disrupted glycinergic/GABAergic inhibition would produce the same changes in sleep-wake architecture in Tg mice as seen in RBD patients. To do this, the sleep-wake architecture of Wt (n=19) and Tg (n=16) mice were compared across a 24hr period. Like RBD patients (Schenck and Mahowald, 2002), sleep architecture, or the normal cycling between NREM and REM sleep, was preserved in the Tg mice (Figure 6.6A). Quantification of the number of arousals from sleep, however, confirmed my visual observation that Tg mice had more awakenings from both NREM (135% increase; $t$-test, $p=0.001$) and REM sleep (34% increase; $t$-test, $p=0.025$) (Figure 6.6A). Thus, like RBD patients (American Academy of Sleep Medicine, 2001; Schenck et al., 1993; Sforza et al., 1988), it appears that sleep behaviours may have disrupted sleep continuity in Tg mice.

Looking across the 24 hour period, sleep-wake amounts were altered in Tg mice (RM ANOVA, $p<0.001$). Tg mice spent significantly more time in waking ($63.9 \pm 1.4\%$ vs. $57.1 \pm 1.8\%$; $p<0.001$, *post hoc* test), at the expense of NREM sleep ($28.1 \pm 1.4\%$ vs. $35.7 \pm 1.6\%$; $p<0.001$, *post hoc* test), while REM sleep amounts were unaffected ($5.0 \pm 0.3\%$ vs. $5.8 \pm 0.2\%$; $p=0.658$, *post hoc* test) (Figure 6.6B). This difference in waking was due to Tg mice spending more time in quiet waking ($22.3 \pm 1.9\%$ vs. $10.3 \pm 0.6\%$; $p=0.001$, *post hoc* test), a state which includes the transition from wakefulness into NREM sleep (Figure 6.6B inset). A shift towards more light sleep has been reported in some RBD patients as well (Sforza et al., 1988).
Waking Phenotype

Fig. 6.5. Muscle tone is increased during waking in Tg mice. Typical raw EMG and EEG traces from a Wt (top left trace) and a Tg mouse (top right trace) during waking. EMG activity of the neck, limb and masseter for each animal is shown. Group data for Wt (white bars) and Tg (black bars) mice demonstrating that Tg mice have significantly higher muscle activity during waking in the masseter (Wt: n=16; Tg: n=14) and limb (Wt: n=3; Tg: n=4), but not the neck (Wt: n=12; Tg: n=8). * indicates p<0.05; a.u., arbitrary units. All values are mean ± SEM.
Fig. 6.6. Tg mice spend more time in quiet waking and have more arousals from sleep. **A.** Representative 24-h hypnograms from a Wt and a Tg mouse showing the distribution of wake (W), NREM (N) and REM (R) sleep for the two 12-h intervals making up the 24-h light-dark cycle. The white and black bars under each hypnogram indicate the light and dark periods, respectively. A zoomed in half-hour portion of the hypnogram (shaded, outlined region) is shown for both Wt and Tg mice, depicting the increased number of state transitions occurring in the Tg mouse as compared to the Wt mouse. **B.** The percentage of time that Wt (n=19; white bars) and Tg (n=16; black bars) mice spend in wake, NREM and REM sleep across a 24-h period. The increased time spent in waking by Tg mice is due to an increase in the amount of quiet waking (inset). * indicates p<0.01; a.u., arbitrary units. All values are mean ± SEM.
6.3.4 Tg mice have an altered EEG spectral profile

In addition to abnormal motor activity in sleep, RBD patients have impaired cortical activation. Comparisons of the EEG spectral profile of RBD patients with healthy controls reveal that RBD patients have more power in lower frequencies in both waking and sleep (Fantini et al., 2003b; Gagnon et al., 2004; Massicotte-Marquez et al., 2005; Massicotte-Marquez et al., 2008). This EEG slowing has also been reported in Parkinson’s disease and other neurodegenerative disorders (Barber et al., 2000; Briel et al., 1999; Coben et al., 1985; Montplaisir et al., 1997; Penttila et al., 1985; Prinz et al., 1982; Serizawa et al., 2008; Soikkeli et al., 1991). To further validate these Tg mice as a model of RBD, I investigated if these mice have abnormalities in their EEG spectral profiles. Overall, the distribution of spectral power was similar between Wt and Tg mice, each with spectral analysis profiles of waking, NREM and REM sleep characteristic of what would be expected in each state (Figure 6.7). However, as in human RBD (Fantini et al., 2003b; Gagnon et al., 2004; Massicotte-Marquez et al., 2005; Massicotte-Marquez et al., 2008), Tg mice had an overall slowing of EEG power across all states, with significantly more power in the lower ranges than Wt mice (RM ANOVA, p<0.01), particularly in waking and NREM sleep (Figure 6.7). These data further suggest that a dysregulation of normal inhibitory transmission may contribute to the clinical symptoms of RBD.

6.3.5 Successful treatment of RBD motor phenotype with clonazepam and melatonin

Finally, I wanted to investigate whether the RBD motor phenotype of Tg mice could be alleviated by the pharmacological agents used to treat human RBD. Clonazepam, a benzodiazepine, is the treatment of choice for RBD: it is immediately effective in alleviating RBD motor symptoms in upwards of 90% of patients (Lapierre and Montplaisir, 1992; Olson et al., 2000; Schenck et al., 1993; Sforza et al., 1997). To test the efficacy of this drug in Tg mice, I administered a single i.p. injection of clonazepam to both Wt and Tg mice and determined the effects on EMG activity. While having no effect on motor activity in Wt mice (paired t-tests, p>0.05, n=10), clonazepam significantly reduced EMG activity by 26 ± 4% in Tg mice during REM sleep (paired t-test, p=0.002, n=7; Figure 6.8). This is in line with the 37% reduction in REM sleep
Fig. 6.7. Tg mice have EEG slowing in waking and sleep. EEG spectral profiles for Wt (n=15; dotted line) and Tg (n=11; solid line) mice for (A) wake, (B) NREM, and (C) REM sleep. Tg mice have more power in the lower frequency ranges and less power in the higher frequency ranges, particularly in waking and NREM sleep, resulting in an overall EEG slowing. * indicates p<0.01; a.u., arbitrary units. All values are mean ± SEM.
motor activity that has been reported in RBD patients treated with clonazepam (Lapierre and Montplaisir, 1992). Furthermore, as in human RBD (Lapierre and Montplaisir, 1992), clonazepam also reduced the occurrence of NREM myoclonic twitches in Tg mice by 51 ± 15% (paired t-test, p=0.043; Figure 6.8B inset). Waking EMG activity was also reduced by clonazepam in Tg mice (31 ± 13%; paired t-test, p=0.048).

Melatonin is an alternative to clonazepam in the treatment of RBD. Studies have shown that long-term melatonin treatment clinically improves motor symptoms in RBD patients (Boeve et al., 2003; Kunz and Bes, 1997, 1999; Kunz and Mahlberg, 2010; Takeuchi et al., 2001). Treatment of Tg mice with melatonin over a 2-4 week period showed similar results: EMG activity was significantly reduced during REM sleep in animals treated with melatonin compared to their untreated counterparts (43% reduction; t-test, p=0.014, untreated: n=14, treated: n=7; Figure 6.9). Melatonin, however, had no effect on NREM myoclonic twitches in Tg mice (t-test, p=0.691; Figure 6.9B inset); likewise, there are no reports of an effect of melatonin on NREM twitches in RBD patients. While not significant, there was also a strong trend for reduced EMG activity during waking after melatonin treatment in Tg mice (35%; t-test, p=0.063). Muscle activity in Wt mice was not affected by melatonin treatment (t-tests, p>0.05, untreated: n=16, treated: n=3).

6.3.6 Melatonin, but not clonazepam, restores EEG spectral profile and sleep-wake amounts in Tg mice

In addition to the effects of clonazepam and melatonin on the motor phenotype of Tg mice, I were interested in how these agents would impact the non-motor symptoms of RBD shared by these Tg mice. This is an area that, to date, has received little to no attention in the human literature but as the non-motor aspects of RBD gain more interest, the pharmacological impact on these symptoms is an area that will need to be explored. Interestingly, clonazepam and melatonin had opposite effects on the EEG spectral profile in Tg mice: EEG slowing was further exacerbated after clonazepam treatment (RM ANOVA, p<0.05), whereas melatonin reversed the EEG slowing (p<0.05). In fact, melatonin completely abolished EEG slowing in waking such that spectral power was not
Fig. 6.8. Clonazepam effectively suppresses excessive EMG activity during REM sleep in Tg mice. A. Raw trace examples from a Wt and a Tg mouse depicting masseter EMG activity during REM sleep before (top traces) and after (bottom traces) treatment with clonazepam. B. Group data showing that compared to baseline levels (white bars), clonazepam treatment (black bars) significantly reduces masseter EMG activity during REM sleep in Tg mice (n=7) but has no effect on EMG activity in Wt mice (n=9). Clonazepam also reduces the number of NREM myoclonic twitches in Tg mice (inset). * indicates p<0.05; a.u., arbitrary units. All values are mean ± SEM.
Fig. 6.9. Melatonin significantly reduces EMG activity during REM sleep in Tg mice. A. Raw EMG and EEG traces from an untreated (top traces) and a melatonin-treated (bottom traces) Wt and Tg mouse during REM sleep. Melatonin, like clonazepam, reduces the excessive EMG activity of Tg mice during REM sleep. B. Group data showing that compared to their untreated counterparts (white bars), melatonin treatment (black bars) significantly reduces masseter EMG activity during REM sleep in Tg mice (n=7) but has no effect on EMG activity in Wt mice (n=3). Melatonin has no effect on the number of NREM myoclonic twitches in Tg mice (inset). * indicates p<0.05; a.u., arbitrary units. All values are mean ± SEM.
different from Wt animals (RM ANOVA, p=0.209). Furthermore, melatonin, but not clonazepam affected sleep-wake amounts (melatonin: RM ANOVA, p<0.001; clonazepam: RM ANOVA, p=0.261) by increasing the amount of NREM sleep (p=0.003, *post hoc* test) at the expense of waking (p<0.001, *post hoc* test) in Tg mice, such that the sleep-wake amounts were no longer different from Wt animals (ANOVA, p=0.753). Thus, melatonin restored both EEG power and sleep-wake amounts to untreated Wt levels, whereas clonazepam had a negative effect, if any, on these non-motor symptoms. A similar tendency for melatonin to increase sleep efficiency has been noted in RBD patients (Kunz and Bes, 1999).

In Wt mice, clonazepam caused an overall quickening of EEG power (RM ANOVA, p<0.001), which corresponds to what has been reported in healthy subjects (Borbely and Achermann, 1991; Borbely et al., 1983). Melatonin, as the literature predicts, had no effect on EEG power (RM ANOVA, p>0.05) (Fisher et al., 2008; Langebartels et al., 2001). While neither treatment affected overall sleep-wake amounts in Wt mice (clonazepam: RM ANOVA, p=0.466; melatonin: RM ANOVA, p=0.993), there was a trend for more quiet waking/NREM sleep with both drugs, as would be expected (Borbely et al., 1991; Fisher et al., 2008; Tobler et al., 2001).

### 6.4 DISCUSSION

In this study I describe a transgenic mouse with severely downregulated inhibitory function that has a phenotype remarkably similar to human RBD. Both behavioural and physiological features analogous to RBD were observed in these mice (Table 6.1). The most salient feature of this mouse model is the strikingly similar motor abnormalities in both NREM and REM sleep: Tg mice have myoclonic twitches in NREM followed by excessive, vigorous movements throughout REM sleep. Waking EMG and gait slowing similar to that reported in RBD patients are also observed in the Tg mice. In addition to these behavioural characteristics of RBD, Tg mice show comparable alterations to their sleep-wake cycle, increased number of sleep disruptions, as well as impairments of cortical activation (Table 6.1). Finally, Tg mice respond in a parallel fashion to the primary, as well as a secondary, pharmacological agent used to treat RBD. Taken together, this mouse model represents the first animal model of RBD that displays
Table 6.1. Tg mice recapitulate the key characteristics of RBD. A summary comparison of RBD symptoms with observations in the Tg mice.

<table>
<thead>
<tr>
<th>RBD</th>
<th>Tg Mice</th>
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<tr>
<td>Abnormal REM sleep behaviours</td>
<td>Abnormal gross motor behaviour in REM sleep reminiscent of those described in RBD</td>
</tr>
<tr>
<td>• Excessive limb or body jerking</td>
<td></td>
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<tr>
<td>• Complex movements</td>
<td></td>
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<tr>
<td>• Vigorous/violent movements</td>
<td></td>
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<tr>
<td>Elevated phasic EMG in REM sleep</td>
<td>Excessive phasic REM activity</td>
</tr>
<tr>
<td>Periodic/aperiodic movements in NREM sleep</td>
<td>Periodic myoclonic twitches in NREM sleep</td>
</tr>
<tr>
<td>Subtle motor and gait slowing</td>
<td>Increased waking EMG, hypokinesia of gait</td>
</tr>
<tr>
<td>Preservation of sleep architecture</td>
<td>Normal cycling between NREM and REM sleep</td>
</tr>
<tr>
<td>Sleep disrupted by sleep behaviours</td>
<td>Increased arousals from sleep</td>
</tr>
<tr>
<td>Shift towards light sleep (elevated stage 1 in some cases)</td>
<td>More time spent in ‘quiet waking’</td>
</tr>
<tr>
<td>Slowing of the EEG</td>
<td>Altered EEG power spectral profile</td>
</tr>
<tr>
<td>Effective treatment with clonazepam</td>
<td>Clonazepam effectively suppressed REM EMG activity by 26 ± 4%</td>
</tr>
<tr>
<td>Improvement of RBD symptoms with melatonin</td>
<td>Melatonin reduced excessive REM EMG activity by 43%</td>
</tr>
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multiple clinical hallmarks of the disorder. My study provides strong support for a loss of normal inhibitory function in RBD.

6.4.1 Can the RBD motor phenotype be explained by a dysregulation of inhibitory processes?

RBD is a complex disorder that can manifest under several different conditions. It can be idiopathic, associated with neurodegenerative disorders, be pharmacologically induced, or present after focal brain lesions (Boeve et al., 2004; Boeve et al., 2001; Gagnon et al., 2002; Gagnon et al., 2006a; Gagnon et al., 2006b; Kimura et al., 2000; Olson et al., 2000; Plazzi and Montagna, 2002; Provini et al., 2004; Schenck and Mahowald, 2002; Tippmann-Peikert et al., 2006; Winkelman and James, 2004; Zambelis et al., 2002). While the mechanisms responsible for RBD are not known, it is conceivable that a common pathology could be responsible for several of the manifestations of this disorder. The many motor similarities between RBD and these Tg mice (Table 6.1) suggest that decreased inhibitory processes may play a key role in the pathogenesis of this condition. There is evidence to support this notion. First, inhibitory amino acids have an established role in mediating REM sleep motor control (Chase et al., 1989; Kodama et al., 2003). When glycinergic and GABA\textsubscript{A}-mediated inhibition is disrupted at the level of the motoneuron, excessive phasic twitch activity similar to that described in RBD results (Brooks and Peever, 2008a; Morrison et al., 2003a). Upstream of motoneurons, lesions to inhibitory brain regions induce an RBD-like phenotype in both cats and rats (Holmes and Jones, 1994; Lu et al., 2006; Schenkel and Siegel, 1989). Lesions and, more recently, microstructural alterations in similar brain regions have been associated with RBD in humans (Kimura et al., 2000; Plazzi and Montagna, 2002; Provini et al., 2004; Tippmann-Peikert et al., 2006; Unger et al.; Zambelis et al., 2002). Furthermore, abnormal motor activity in sleep has been reported in at least two CNS disorders in which glycinergic or GABAergic function is impaired (ie. hyperekplexia and stiff person syndrome) (de Groen and Kamphuisen, 1978; Dinkel et al., 1998; Gastaut and Villeneu.A, 1967; Levy et al., 2005; Martinelli et al., 1996; Martinelli et al., 1978; Shiang et al., 1993; Solimena et al., 1990; Solimena et al., 1988). In fact, a recent case report describes the co-occurrence of RBD and hyperekplexia, the disorder these Tg mice were
created to study (Becker et al., 2002), after pontine lesions (Peter et al., 2008). Collectively, these observations argue for a role for inhibitory dysregulation in RBD.

6.4.2 Could a disruption of inhibitory circuitry lead to the sleep disturbances in RBD?

Inhibitory circuitry is fundamentally involved in both the initiation and maintenance of sleep (Jones, 2005). Sleep-active GABAergic neurons in the hypothalamic preoptic area are part of a sleep-wake switch that initiates and maintains sleep by increasing in activity during the transition from waking to NREM sleep and further increasing from light to deep NREM sleep (Gallopin et al., 2000; Sherin et al., 1998; Sherin et al., 1996; Steininger et al., 2001; Szymusiak et al., 1998). An abnormality in this system, such as a deficit in inhibitory function, would result in unstable sleep-wake states (McGinty and Szymusiak, 2005). This is supported by the fact that Tg mice, which have a disruption in inhibitory transmission, have both an increased number of awakenings and a greater amount of quiet waking. Like these Tg mice, RBD patients show an increase in sleep disruptions and a greater amount of light sleep (American Academy of Sleep Medicine, 2001; Schenck et al., 1993; Sforza et al., 1988). While some sleep disruptions are likely caused by excessive sleep behaviours, both RBD patients and the Tg mice are able to maintain sleep through many of these events (Schenck et al., 1993), indicating a non-motor mechanism is responsible. A disruption in the onset and maintenance of sleep due to a dysfunction in the inhibitory control of sleep-wake states could explain both the increased amounts of light sleep and sleep disruptions experienced by RBD patients and Tg mice. In support of this notion, the sleep of both RBD patients and Tg mice appears to be improved by melatonin (Kunz and Bes, 1999), a drug which enhances inhibitory function (Coloma and Niles, 1988; Niles et al., 1987; Stankov et al., 1992; Wang et al., 2003; Wu et al., 1999).

6.4.3 Are the abnormalities in cortical activation in RBD explained by inhibitory dysfunction?

Comparisons of the EEG spectral profile of RBD patients with healthy controls reveal that RBD patients, like the Tg mice, have more power in lower frequencies in both waking and sleep (Fantini et al., 2003b; Gagnon et al., 2004; Massicotte-Marquez et al., 2005; Massicotte-Marquez et al., 2008). While the presence of this EEG slowing in RBD
patients has been established, the neuronal substrates underlying this phenomenon are not known (Massicotte-Marquez et al., 2008). Sleep disruptions seen in both RBD patients and Tg mice could be responsible for these impairments of cortical activity, as changes in waking EEG power are indicative of sleepiness (Akerstedt and Gillberg, 1990; Cajochen et al., 1995). It is, however, more likely the result of neuronal deficits related to the pathology of the disorder; this is supported by the fact that EEG slowing has also been reported in Parkinson’s disease and other neurodegenerative disorders (Barber et al., 2000; Briel et al., 1999; Coben et al., 1985; Montplaisir et al., 1997; Penttila et al., 1985; Prinz et al., 1982; Serizawa et al., 2008; Soikkeli et al., 1991). Brain imaging studies show a reduction in cerebral blood flow and glucose metabolism in frontal, parietal and temporal cortical regions in RBD patients (Caselli et al., 2006; Mazza et al., 2006; Shirakawa et al., 2002). Some of the subcortical regions reported to be affected in RBD are involved in cortical activation (Boeve et al., 2007; Gagnon et al., 2006a; Jones, 2005). GABAergic neurons are thought to play an important role in regulating the neuronal substrates involved in cortical activation (Ford et al., 1995; Jones, 2005) and Tg mice, which show an analogous slowing of the EEG, have disrupted inhibitory processes. Taken together, this suggests that a disruption of the inhibitory modulation of subcortical-cortical neuronal networks could underlie the EEG abnormalities that are seen in RBD.

6.4.4 Does the pharmacology of RBD support an underlying inhibitory deficiency?

The notion of a deficit in inhibitory function underlying RBD fits with the pharmacology: RBD patients, like the Tg mice, are effectively treated with both clonazepam and melatonin (Boeve et al., 2003; Kunz and Bes, 1997, 1999; Kunz and Mahlberg, 2010; Lapierre and Montplaisir, 1992; Olson et al., 2000; Schenck et al., 1993; Sforza et al., 1997; Takeuchi et al., 2001). While these pharmacological treatments have slightly different reported effects in RBD (Kunz and Bes, 1997; Kunz and Mahlberg, 2010; Lapierre and Montplaisir, 1992; Takeuchi et al., 2001), and thus likely act on different aspects of the affected circuitry, both function to enhance GABAergic transmission (Choi et al., 1977; Coloma and Niles, 1988; Costa and Guidotti, 1979; Macdonald and Barker, 1978; Niles et al., 1987; Stankov et al., 1992; Wang et al., 2003; Wu et al., 1999). Clonazepam, a benzodiazepine, facilitates GABA-mediated inhibition by increasing the
affinity of the GABA receptor for its ligand (Costa and Guidotti, 1979; Polc and Haefely, 1976; Skerritt and Johnston, 1983); melatonin not only enhances GABA receptor binding (Coloma and Niles, 1988; Niles et al., 1987; Stankov et al., 1992), but also increases GABA turnover (Rosenstein and Cardinali, 1986; Xu et al., 1995). Each, by potentiating inhibitory function, successfully alleviates the abnormal REM sleep motor activity seen in RBD and in Tg mice. This is consistent with the notion that a disruption of inhibition could underlie the RBD phenotype.

The close association of RBD with Parkinson’s disease (Schenck et al., 1996, 2003) has led some to suggest that RBD may be a dopaminergic deficiency disorder (Eisensehr et al., 2000; Eisensehr et al., 2003; Matheson and Saper, 2003). Supporting this hypothesis are reports of a dysfunction in the dopaminergic nigrostriatal system of RBD patients (Albin et al., 2000; Eisensehr et al., 2000; Eisensehr et al., 2003) as well as the fact that some RBD-associated disorders, (ie. restless leg syndrome and periodic limb movements in sleep), are alleviated with dopamine agonists (Montplaisir et al., 1999). The treatment of RBD with dopaminergic agents, however, is unconvincing, having moderate if any therapeutic effect (Fantini et al., 2003a; Kumru et al., 2008; Schmidt et al., 2006). Furthermore, surgical interventions that alleviate parkinsonian dopaminergic motor symptoms (eg. high-frequency stimulation of the subthalamic nucleus) have no effect on RBD (Arnulf et al., 2000; Iranzo et al., 2002). Therefore, unlike medications like clonazepam or melatonin that potentiate inhibitory function, dopamine-enhancing treatments do not alleviate RBD. While the involvement of dopaminergic mechanisms in RBD cannot be ruled out, these observations are more consistent with other nondopaminergic systems, such as the inhibitory substrates proposed here, playing a central role in the pathology of RBD.

In addition to idiopathic and secondary RBD, REM sleep without atonia can be pharmacologically induced. Iatrogenic RBD is particularly prevalent with antidepressant use: tricyclic antidepressants, selective serotonin reuptake inhibitors, and monoamine oxidase inhibitors all have been shown to induce or aggravate the symptoms of RBD (Gagnon et al., 2006b; Mahowald and Schenck, 2005). Controlled, detailed studies have not been performed on these patients; however it is possible that medication-induced RBD involves inhibitory disruptions. The chemical induction of RBD by antidepressants
may result from indirect effects on the brainstem inhibitory circuitry that controls REM motor tone. For example, RBD has been reported in patients taking fluoxetine, a serotonergic antidepressant (Schenck et al., 1992; Winkelman and James, 2004); fluoxetine has inhibitory effects on the glycine receptor (Ye et al., 2008). Thus, the mechanisms underlying pharmacologically-induced RBD could involve a perturbation of inhibitory processes.

6.4.5 Does Parkinson’s disease pathology support a role for inhibitory dysfunction in RBD?

Degeneration of nondopaminergic nuclei in the lower brainstem has been implicated in the beginning stages of Parkinson’s disease pathology (Braak et al., 2003); involvement of inhibitory circuitry may be included in this early nondopaminergic degeneration. Since RBD is thought to be an early marker of Parkinson’s, the initial involvement of inhibitory circuitry in Parkinson’s disease pathology would fit with both my data, indicating that RBD results from a disruption of inhibitory processes, as well as the observation that RBD patients experience a progressive worsening of REM sleep motor symptoms with time (Iranzo et al., 2009). While an assessment of inhibitory processes has not been reported in RBD patients, there are numerous reports of reductions in both GABAergic and glycinergic function in Parkinson’s disease in support of this idea (Demontis et al., 1982; Lloyd and Hornykiewicz, 1973; Lloyd et al., 1977; Nishino et al., 1988; Rinne et al., 1978). Taken together, this suggests RBD may be one step in a progressive pathological process that involves the degeneration of inhibitory structures.

Interestingly, RBD is reported to be more prevalent in certain subtypes of Parkinson’s, possibly indicating different pathologies in different Parkinson’s disease subtypes (Kumru et al., 2007; Postuma et al., 2008). Several studies have suggested that different Parkinson’s disease subtypes may represent different neuropathological processes (Graham and Sagar, 1999; Jankovic et al., 1990; Roos et al., 1996; Zetusky et al., 1985), which could explain why RBD is present in some but not all cases of Parkinson’s (Gagnon et al., 2002). It may be that GABAergic/glycinergic regions are more affected in certain subtypes of Parkinson’s disease than in others, indicating multiple paths of neurodegeneration, one of which involves the disruption of normal
inhibitory processes and thus concomitant RBD. Future work focusing on the involvement of an inhibitory substrate may provide new insights into not only RBD but also the pathology of Parkinson’s disease subtypes and other RBD-related disorders.

6.4.6 Conclusion

Our results indicate that a disruption of inhibitory processes may contribute to the pathogenesis of RBD. Here, I describe for the first time an animal model of RBD displaying a phenotype that includes both behavioural and physiological manifestations strikingly similar to the human condition (Table 6.1). These mice provide strong evidence that decreased inhibitory processes can account for the symptoms of RBD and therefore suggest that a disruption of inhibitory circuitry may be involved in the etiology of RBD.

The hallmark characteristics of RBD shared by the Tg mice demonstrate the usefulness of these mice in elucidating the underlying cause of RBD. In addition to providing a causal mechanism of the disease, these Tg mice could serve as a functional substrate to investigate how the non-motor symptoms of RBD respond to pharmacological treatments. The contrasting effects of clonazepam and melatonin on both EEG slowing and sleep-wake amounts support the notion that these agents have differing modes of action in their treatment of the human condition (Boeve et al., 2003; Kunz and Bes, 1999). These novel observations in Tg mice demonstrate the usefulness of this mouse model in evaluating the complete pharmacology of RBD and highlight the need for further investigation into the non-motor effects of these agents to provide a more rounded picture of the efficacy of the pharmacological treatment of RBD.

The use of a mouse model that mimics the catalog of behavioural and physiological mechanisms seen in the human disorder opens new perspectives to dissect the pathogenesis and pharmacology of RBD as well as may provide insight into clinical relevance of RBD in associated disorders. The results reported here emphasize the potential role for inhibitory disruptions in RBD and dictate a need for further investigation into abnormalities in GABAergic/glycinergic processes that may occur in the human condition.
CHAPTER SEVEN: GENERAL DISCUSSION

7.1 OVERVIEW

The brain regions responsible for the regulation of REM sleep motor atonia have been well established (Siegel, 2005), however the neurochemical mechanism mediating this muscle tone suppression at the level of the motoneuron is less clear. The goal of this thesis was to further our understanding of the biochemical substrates responsible for REM sleep motor control, with a specific focus on the direct role that GABA and glycine play.

7.1.1 The role for $\text{GABA}_A$ and glycine receptors in REM atonia

Chapter 3 of this thesis examined the role of glycinergic and $\text{GABA}_A$-mediated inhibition of motoneurons in REM sleep motor control. Traditionally it has been hypothesized that glycine is responsible for the regulation of REM atonia (Chase and Morales, 2005), although more recently this has been brought into question (Fenik et al., 2005a; Kubin et al., 1993; Morrison et al., 2003a; Morrison et al., 2003b). In support of these more recent works, this thesis demonstrates that while blockade of $\text{GABA}_A$ and glycine receptors increases muscle tone in waking and NREM sleep, it does not affect REM atonia: REM-specific muscle tone suppression occurred in the absence of these inhibitory inputs. In fact, atonia persisted even when $\text{GABA}_A$ and glycine receptors were blocked and motoneurons were directly excited by a glutamatergic agonist. This rules out a role for glycinergic/$\text{GABA}_A$-mediated inhibition in the regulation of REM atonia and indicates a powerful, yet unidentified inhibitory mechanism is at work. While REM atonia is not affected by antagonism of $\text{GABA}_A$ and glycine receptors, phasic muscle activity is: blockade of $\text{GABA}_A$ and glycine receptors increased the amount of phasic muscle activity that occurred during REM sleep.

Taken together, Chapter 3 of this thesis demonstrates that REM atonia is not mediated by glycinergic or $\text{GABA}_A$-mediated inhibition. Instead, it appears that the previously identified inhibitory drive in REM sleep (Chandler et al., 1980; Glenn and Dement, 1981a, b; Morales and Chase, 1981) acts to oppose the glutamatergic inputs that trigger muscle twitches in this state (Burgess et al., 2008; Soja et al., 1995). These
findings are important because they challenge the long-held hypothesis that glycine is responsible for REM atonia (Chase and Morales, 2005), as well as suggest that the two components of REM sleep motor activity, REM atonia and phasic muscle twitches, are differentially regulated.

7.1.2 The role for GABA<sub>B</sub> receptors in REM atonia

As described above, REM atonia appears to be induced by an inhibition of motoneurons; neither glycinergic nor GABA<sub>A</sub>-mediated inhibition, however, is responsible. One potential candidate in the control of REM atonia that has not been previously investigated is GABA<sub>B</sub>-mediated inhibition. Chapter 4 examined the role of GABA<sub>B</sub>-mediated inhibition of motoneurons in REM sleep motor control.

Like antagonism of GABA<sub>A</sub> and glycine receptors, blockade of GABA<sub>B</sub> receptors increased muscle tone in waking and NREM sleep, but it did not affect REM atonia. Phasic twitch activity was also unaffected by this manipulation. Therefore, alone, GABA<sub>B</sub>-mediated inhibition is not responsible for muscle control in REM sleep. However, when GABA<sub>B</sub> receptors were antagonized in conjunction with GABA<sub>A</sub> and glycine receptors, there was a reversal of REM atonia. This synergistic effect resulted in a partial loss of motor suppression, suggesting that the mechanism underlying REM atonia involves, at least in part, an interaction between GABA<sub>B</sub> receptors with GABA<sub>A</sub> and glycine receptors. These findings demonstrate that GABA<sub>B</sub>-mediated inhibition, acting in concert with GABA<sub>A</sub> and glycine receptors, mediates a portion of this state-specific motor suppression. This is not, however, the exclusive factor responsible for REM atonia as periods of atonia still remained. Thus, in addition to offering a novel mechanism by which muscle activity is regulated in REM sleep, this work emphasizes that REM atonia is not mediated by a single mechanism as previously thought (Chase and Morales, 2005), but is governed by a complicated set of mechanisms that are not yet fully understood.

7.1.3 Inhibitory control of phasic muscle activity

Chapter 5 of this thesis examined the contribution of inhibition to the temporal control of phasic muscle activity. In this chapter, it was first demonstrated that muscle activity is not uniformly controlled across REM sleep: both tonic and phasic muscle activity progressively increase across a REM period. Therefore, motor control is not
homogeneous throughout REM sleep. Furthermore, the phasic inhibitory drive identified in Chapter 3 was found to contribute to the dynamic nature of phasic twitch activity. Blockade of GABA\textsubscript{A} and glycine receptors on motoneurons altered the pattern of phasic muscle activity across REM sleep: removal of this chloride-mediated inhibition resulted in an increase in phasic twitch activity that was disproportionately larger at the start of a REM episode. This suggests that inhibition is regulating the pattern of twitch activity by suppressing muscle twitches more strongly in the earlier stages of REM sleep. As expected, based on the findings of Chapter 3 and elsewhere (Morrison et al., 2003a), the pattern of tonic muscle activity was not affected by this intervention.

Examination of the patterns of phasic muscle activity that occur in REM sleep in the presence and absence of inhibitory inputs onto motoneurons lead to a hypothesis of progressively decreasing inhibitory activity and concomitant gradually increasing excitatory activity working together to shape the dynamic pattern of REM sleep motor activity. In addition to describing this temporal control of motor activity, these data also support the notion put forth in Chapter 3 of this thesis that tonic and phasic muscle activity are differentially regulated in REM sleep.

7.1.4 Clinical implications of inhibitory dysfunction: REM sleep behaviour disorder

The chloride-mediated control of phasic muscle activity that was revealed in Chapters 3-5 of this thesis lead to the hypothesis that a dysregulation of inhibitory motor control may underlie RBD, the idiopathic sleep disorder that is characterized by a loss of normal REM motor control (Schenck and Mahowald, 2002). Chapter 6 examined the phenotype of a genetic mouse model in which glycinergic and GABA\textsubscript{A}-mediated transmission is severely downregulated (Becker et al., 2002). These animals displayed a strikingly similar motor phenotype in sleep and waking to RBD patients, even responding in a parallel manner to both the primary and secondary pharmacological agents used to treat RBD. Remarkably, other non-motor cardinal symptoms of RBD were also observed in these animals, such as alterations to the sleep-wake cycle, increased number of sleep disruptions, and impairments in cortical activation. Thus, these mice have both behavioural and physiological features analogous to the human condition. This is the first animal model to be described that displays multiple clinical hallmarks of RBD, not just
the motor manifestation. This suggests that a loss of normal inhibitory function may play a causal role in the pathology of this disorder.

In addition to its relevance to RBD, this mouse model also confirmed the findings of Chapters 3-5 of this thesis, that chloride-mediated inhibition is not responsible for REM atonia: as seen with the pharmacological manipulations, REM atonia was intact in these animals. Furthermore, by recording from three different groups of muscles in the same animal, this chapter demonstrated that cranial and spinal motor pools are similarly influenced by chloride-mediated inhibition, as the motor disruptions observed in the masseter muscle were similar to those seen in both the hindlimb and neck muscles: REM atonia remained intact while phasic muscle activity was severely augmented in all muscle groups. Similar findings have been reported after the pharmacological removal of inhibition at the hypoglossal motor pool (Morrison et al., 2003a). Together, these data indicate a similar mechanism is responsible for the REM-specific control of motoneuron activity in these motor systems.

### 7.1.5 The role for GABA and glycine in REM sleep motor control

Together, this thesis detailed the involvement of inhibitory influences in REM sleep motor control. It was determined that glycinergic/GABA$_A$-mediated inhibition was only involved in mediating REM atonia when working together with GABA$_B$ receptors. Without this novel interaction, glycine and GABA do not regulate motor atonia in REM sleep. While chloride-mediated inhibition alone does not mediate REM atonia, it does have a key role in controlling phasic muscle activity in this state. This inhibitory control of phasic muscle activity is dynamic in nature, following a pattern of progressively decreasing activity across REM sleep: this pattern of inhibitory activity helps to shape the temporal pattern of muscle twitches that occurs in REM sleep. Finally, this thesis examined the clinical implications that global disruptions to the inhibitory system can have, leading to the hypothesis that a loss of normal inhibitory function may underlie the sleep disorder RBD.

Collectively, this thesis contributes to the understanding of the neurochemical control of motor activity in REM sleep by further defining the role of inhibition in REM sleep motor control. Not only was the inhibitory control of motor activity in REM sleep
characterized, this thesis also provides novel evidence implicating a dysregulation of inhibitory function in the pathology of RBD. Furthermore, this thesis highlights the complexity of the mechanisms underlying muscle activity in REM sleep.

7.2 LIMITATIONS OF THESIS

This thesis pharmacologically manipulated the inhibitory system at the motor pool and found that while phasic muscle activity was altered, REM atonia could not be completely abolished (Chapters 3-5). This finding is supported by the persistence of REM atonia in the transgenic mouse model of global inhibitory disruption (Chapter 6). Thus, while this thesis determined an important role for inhibition in the control of motoneurons during REM sleep, the complete mechanism underlying REM atonia remains unknown. This thesis added to the knowledge of how inhibition shapes motor output in REM sleep but the cause of muscle suppression in this state was not resolved and therefore requires further investigation.

The source of the inhibition that was shown to be involved in REM motor control was also not identified in this thesis. Others have hypothesized that inhibitory interneurons impinge on motoneurons to suppress motor activity during REM sleep (Lu et al., 2006), however, manipulations at the motor pool, as presented here, do not address the source of this inhibitory input. Furthermore, in the mouse model (Chapter 6), a global reduction in inhibition was shown to affect muscle tone in a similar manner in REM sleep as the pharmacological manipulations (Chapter 3) but the source of this effect is unknown: it may be at the motor pool, as seen in the pharmacological rat model, or it may be further upstream. This again is an important issue that was not addressed in this thesis but should be explored.

This thesis also did not examine the function of REM atonia: an unresolved question in the field of sleep research is why this motor phenomenon occurs. The importance of REM atonia is evidenced by the powerful suppression of motoneurons that occurs during REM sleep. The detrimental effects that a lack of motor control in sleep can have are seen in many sleep disorders, particularly RBD. Therefore it is clear that atonia is an essential component of REM sleep; the functional purpose of this motor suppression, however, is not clear. Whether the purpose of atonia is to prevent
inappropriate movements during sleep or if atonia serves a more global function is not
known. For example, while there is a strong association between RBD and
neurodegenerative disease (Boeve et al., 2004; Boeve et al., 2001; Gagnon et al., 2006a;
Iranzo et al., 2006; Schenck et al., 1996), it is not known if RBD is a biomarker of these
related disorders or if a dysfunction in REM motor control can contribute to the
development of these diseases. While outside the scope of this thesis, understanding the
purpose of REM atonia is an important and interesting question that warrants further
attention.

Finally, it should be noted that the work presented in this thesis was
predominately performed on the trigeminal-masseteric motor system and therefore the
mechanisms described here may not be representative of all motor systems. There is
evidence that some muscle groups are controlled by similar mechanisms in REM sleep:
pharmacological and genetic removal of chloride-mediated inhibition produces changes
in motor output in the genioglossus (Morrison et al., 2003a), hindlimb and neck (Chapter
6) that are comparable to those seen in the masseter muscle (Chapters 3 and 6). It is not
known, however, if the mechanisms common to these motor systems can be extrapolated
globally. As such, the REM-specific control of muscle activity described in this thesis
may not reflect a universal mechanism of REM sleep motor control. In fact, this is a
concern that applies to all studies of REM sleep motor control. It is only through the
comparison of many different motor systems that a comprehensive understanding of the
REM-specific control of muscles can be gained.

7.3 FUTURE DIRECTIONS

This thesis revealed a role for GABA\textsubscript{B}-mediated inhibition, together with GABA\textsubscript{A} and
glycine receptors, in the control of REM atonia. This is not, however, the exclusive factor
responsible for REM atonia. Periods of atonia still persisted after this powerful
mechanism was blocked at the motor pool. A role for passive disfacilitation, the state-
specific removal of excitatory inputs, has been put forth as a mechanism underlying REM
atonia (Chandler et al., 1980; Nakamura et al., 1978). No reports to date, however, have
been successful in reversing REM atonia by applying excitatory transmitters back onto
the motor pool during REM sleep (Burgess et al., 2008; Chan et al., 2006; Jelev et al.,
Only histamine, when applied into the hypoglossal motor nucleus, has been shown to consistently increase tonic muscle activity in REM sleep; however there is no endogenously active histaminergic drive at motoneurons (Bastedo et al., 2009). Therefore, no manipulation at the motor pool of physiologically relevant excitatory or inhibitory inputs has been successful in completely abolishing REM atonia, despite the extensive effort and breadth of transmitters studied (Burgess et al., 2008; Chan et al., 2006; Jelev et al., 2001; Mir et al., 2006; Morrison et al., 2003a; Morrison et al., 2003b; Yee and Peever, 2008). This indicates that new ways to examine the mechanisms underlying REM atonia are needed.

One possible factor precluding the elucidation of the mechanisms of REM atonia is that studies have been primarily focused on manipulating membrane receptors. Some work has started to address this gap in the literature (Aoki et al., 2006), however more needs to be done. Future work should focus on a means that can render a motoneuron inactive. Investigations should not be confined to the manipulation of extracellular transmitters or surface receptors, as has been previously done. The mechanism must be resistant to powerful excitatory inputs, such as the application of glutamatergic agonists, as the maintenance of REM atonia is resistant to such an input as demonstrated in Chapter 3 of this thesis and elsewhere (Burgess et al., 2008). Furthermore, the inactivation of motoneurons must be quickly reversible yet strictly regulated, changing the excitability of motoneurons in a state-specific manner.

One potential source of motoneuron inactivation in REM sleep is $K^+$ leak channels. Resting $K^+$ channels are major determinants of membrane potential and are important for neuromuscular function (Goldstein et al., 2001). $K^+$ leak currents exert control over cell excitability by setting the resting membrane potential. Increased $K^+$ leak currents would stabilize motoneurons at hyperpolarizing voltages (Goldstein et al., 2001), resulting in less excitable motoneurons and therefore REM atonia.

$K^+$-selective leak channels are found at high levels in motoneurons (Talley et al., 2001) and have been described as essential in the excitability of motor activity (Goldstein et al., 2001). While the activity of these channels in REM atonia has not been investigated, $K^+$ leak currents have been implicated in the regulation of sleep-wake states
(McCormick and Bal, 1997), demonstrating that these channels can operate in a state-specific manner. Furthermore, these channels are regulated by a diverse array of biological stimuli (Talley et al., 2003): this would allow for the dynamic modulation of membrane properties that would be required in a mechanism underlying REM atonia. Importantly, while their function can be affected by a wide range of agents, the activity of K\(^+\)-selective leak channels is strictly regulated (Goldstein et al., 2001). In addition to the time course of activation that these channels display, this is a key feature that a REM atonia-generating mechanism must have. Based on these characteristics, K\(^+\)-selective leak channels would be an interesting and novel next step in the investigation into the mechanisms underlying REM atonia.

**7.4 SUMMARY**

Collectively, the data presented in this thesis increase our understanding of how inhibition contributes to REM sleep motor control. This thesis focused primarily on the mechanisms at work at the level of the motoneuron (Figure 7.1), where it was demonstrated that glycinergic and GABAergic inhibition play an important role in mediating muscle activity in REM sleep, particularly in the regulation of phasic muscle twitches. The higher brain centers that impinge on motoneurons to control motor output during sleep were not directly investigated in this thesis but the data presented here does speak to the intricate control these centers have on motor activity during sleep. Furthermore, the work presented in this thesis provides evidence for a common mechanism underlying REM sleep motor control in distinct motor pools. Not only do the pharmacological manipulations at the trigeminal motor nucleus (Chapter 3) produce similar effects as those at the hypoglossal motor nucleus (Morrison et al., 2003a), but a global disruption to inhibition has similar effects at cranial and spinal motor pools, as indicated by the comparable alterations to masseter, hindlimb and neck muscle activity in Tg mice with impaired inhibitory function (Chapter 6). This indicates that while these motor systems may have differing functions, the GABAergic/glycinergic mechanisms acting upon these systems are similar.

Taken together, this thesis provides a detailed description of the involvement of inhibitory influences in REM sleep motor control. These results further our understanding of the neural modulation of motor activity in sleep as well as demonstrate the differential
control of REM sleep motor components. This thesis detailed the dynamic regulation of phasic muscle activity during REM sleep in addition to providing a causal mechanism in the pathology of RBD. Future investigation into the inactivation on motoneurons during REM sleep is needed to fully understand the complex mechanisms responsible for REM atonia.
Fig. 7.1. Schematic representation of motor control in sleep. Sleep centers impinge on motoneurons, inhibiting motoneuron output. This results in the sleep-dependent suppression of muscle activity that is seen in sleep. GABAergic/glycinergic inhibition contributes to motoneuron inactivation in REM sleep via GABA$_B$ receptors together with GABA$_A$ and glycine receptors. In addition to this interaction, an unidentified inhibitory mechanism underlies REM atonia. Phasic glycinergic/GABA$_A$-mediated inhibition opposes phasic excitatory inputs to regulate phasic muscle twitch activity across REM sleep.
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