PHARMACOGENETIC INHIBITION OF THE VENTRAL MEDIAL MEDULLA INFLUENCES REM SLEEP IN MICE

by

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

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Abstract

During REM sleep, the body’s muscles are forced into a state of paralysis. Hence, understanding how the brain controls muscle paralysis during REM sleep is important since its dysfunction can lead to REM sleep behavior disorder (RBD). The neural circuitry underlying the generation of muscle paralysis during REM sleep is hypothesized to be mediated by the GABA/Gly neurons of the ventral medial medulla (VMM). To test this hypothesis, I used a pharmacogenetic technique called Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) to inhibit presumed GABA/Gly neurons of the VMM. I found that inactivation of these neurons significantly increased muscle activity during REM sleep. An additional unexpected finding of this study was that inactivation of VMM neurons also decreased the frequency of REM sleep episodes. This sheds a light on the role that the VMM plays in controlling REM sleep paralysis as well as controlling REM sleep itself.
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List of Abbreviations

5-HT=serotonin
AAV=adeno-associated virus
A.U=arbitrary units
AW=active wake
Cre$^+$=cre-recombinase
CNO=clozapine-n-oxide
DREADDs=designer receptors exclusively activated by designer drugs
DRN=dorsal raphe nucleus
LC=locus coeruleus
dDpMe=dorsal deep mesencephalic reticular nucleus
DLPT=dorsolateral pontine tegmentum
EEG=electroencephalogram
EMG=electromyogram
GABA=gamma-aminobutyric acid
GiA=alpha gigantocellular reticular nucleus
GIRK=G-protein-coupled inwardly rectifying potassium channel
GiV=ventral gigantocellular reticular nucleus
Glu=glutamate
Gly=glycine
hr=hour
Hz=hertz
i.p=intraperitoneal
kg=kilogram
LC=locus coeruleus
M=molar
mg=milligram
min=minute
ml=milliliters
NA=noradrenaline
NMDA=N-methyl D-aspartate
NREM=non-rapid eye movement
ORF=open reading frame
PBS=phosphate buffered saline
PMnR=paramedian reticular area
PPT=pedunculopontine nucleus
PS=paradoxical sleep
QW=quiet wake
rPCRt=rostral parvocellular reticular formation
RN=red nucleus
RBD=rapid eye movement behavior disorder

REM=rapid eye movement

s=second

SEM=standard error of the mean

SOM=supraolivary medulla

SubC=subcoeruleus nucleus

µm=micrometers

vGAT=vesicular GABA transporter

vGlut2=vesicular glutamate transporter 2

vlPAG=ventrolateral periaqueductal gray

VMM=ventral medial medulla

W=waking
Section 1: Introduction

Section 1.1 - Overview

Sleep is an evolutionarily conserved process that occurs in most species and one unifying characteristic of this state across all species is that it is a period of quiescence (Schwarz and Bringmann, 2013). It is commonly thought that sleep is the brain’s way of simply “shutting off”. Contrary to belief, it is actually a period of time, in which the brain is active, even matching or exceeding levels of active wake (Siegel and others, 2005). This so called “slumbering state” is brought about by differential activation of a neural network that is distinct from the awaking brain. Much headway has been made into understanding the neural circuitry responsible for the generation of the various characteristics of sleep.

In a sleep laboratory, behavioral states can be broadly broken down into waking, non-rapid eye movement (NREM) sleep or rapid eye movement (REM) sleep. These behavioral states are defined by the overall electrical activity of the brain coupled with the overall electrical activity of the muscles. An electroencephalogram (EEG) measures overall electrical signals generated within the brain while an electromyogram (EMG) measures overall electrical activity generated within the muscle. A waking state is characterized by low amplitude, high frequency EEG coupled with an EMG with high muscle tone. NREM sleep has a characteristic EEG with high amplitude, low frequency waves in the range of the delta frequency of (1-4 Hz) coupled with an EMG with low muscle tone. Whilst, REM sleep has a characteristic EEG signature of low amplitude, high frequency (theta rich: 4-8 Hz) coupled with an EMG with a lack of muscle tone (muscle atonia) present with periodic muscle twitches (Brown et al., 2012) (Figure 1).
Figure 1: EEG and EMG representation of behavioral states in the mouse. Wakefulness is characterized by low amplitude/high frequency EEG and a concomitant EMG with high muscle tone. In NREM sleep, the EEG is characterized by high amplitude/low frequency waves dominant in the delta range of (1-4 Hz) and a concomitant EMG with low muscle tone. Finally, REM sleep is characterized by an EEG similar to that of wakefulness but being dominant in the theta frequency range of (4-8 Hz) and has a concomitant EMG with muscle atonia punctuated by phasic twitches. s=second

Ever since the discovery of REM sleep by Jouvet in cats over 60 years ago, it has generated much interest into understanding the neural circuitry responsible for the physiological events that occur during this stage of sleep (Jouvet, 1962). During REM sleep, a plethora of physiological phenomena occur, such as rapid eye movements (REMs), high cortical activity, irregular heart rate, irregular breathing, vaginal moistening in females, penile erections in males and so on (Brown et al, 2012). One of the most astounding features of REM sleep is muscle atonia (i.e. muscle paralysis) of postural muscles and due to the concomitant high cortical activity that occurs; this stage of sleep is classically referred to as “paradoxical sleep” (Siegel et al., 2005). This thesis will focus on REM sleep and in particular the neural circuits and its corresponding neurotransmitters responsible for generating the motor basis of REM sleep-i.e. REM sleep atonia.
Section 1.2-REM sleep generation

Over the many decades, many experimental paradigms have been performed to hone in on the area responsible for the generation of REM sleep. One of the first experiments to do this was a transection study in the cat where transection at the level of the midbrain separating the brainstem from the forebrain eliminated cortical signs of REM sleep (theta rich EEG), while preserving muscle atonia and rapid eye movements (REMs) (Jouvet, 1962). This elucidates to the fact that REM sleep is generated at the level of the brainstem.

Further lesion studies in the cat restricted the generator of REM sleep to the pontine region of the brainstem and more specifically to an area called the subcoeruleus (Sub-C) (Carli and Zanchetti, 1965). This small pontine region is known by various other names as well, including pontine inhibitory area, nucleus reticularis pontis oralis (dorsal division), peri-locus coeruleus alpha and sublaterodorsal tegmental nucleus. In this thesis, the term ‘subcoeruleus’ (Sub-C) will be used. Other studies performed in the rat, using pharmacological studies pinpointed the Sub-C as well as the generator of REM sleep. This was done by applying the pharmacological agent gabazine (GABA\textsubscript{A} antagonist) onto the Sub-C, which initiated REM sleep and also showed that this area was indeed highly active during the REM sleep bouts, due to increased levels of c-Fos (Boissard et al., 2002). C-Fos is a marker of neuronal activity (Dragunow and Faull, 1989).

The neurons of the Sub-C that are responsible for REM sleep generation may be glutamatergic in nature. One line of evidence has shown that neurons in the Sub-C have a marker exclusively found in glutamatergic neurons, the vesicular glutamate transporter 2 (vGLUT2) (Lu et al., 2006a). Another study, adding more concrete proof that glutamatergic neurons in the Sub-C are indeed responsible for REM sleep generation, is a REM sleep deprivation paradigm that
was carried out in rats. These rats were allowed to have REM sleep rebound after the REM sleep deprivation paradigm, and after were stained for c-Fos and vGLUT2. It was found that most of the c-Fos labeled neurons in the Sub-C also expressed vGLUT2. Hence, this evidence suggests that the c-Fos labeled neurons in the Sub-C generate REM sleep itself and are glutamatergic in nature (Clement et al., 2011).

The onset of REM sleep is due to disinhibition and simultaneous excitation of the Sub-C (Luppi et al., 2010). During waking and NREM sleep, neurons from the ventrolateral periaqueductal grey (vPAG) and the deep dorsal pontine mesencephalic reticular (dDpMe) nuclei inhibit the Sub-C, via GABAergic transmission (Sapin et al., 2009; Boissard et al., 2003). These neurons are considered REM-OFF neurons, as they stop firing during REM sleep and the cessation of firing of REM-OFF neurons is a prerequisite for the generation of REM sleep. The activity of the vPAG and dDpMe during waking and NREM sleep are hypothesized to be promoted by areas such as the dorsal raphe (DR) and the locus coeruleus (LC), which are serotonergic and noradrenergic in nature, respectively, but the evidence for such a connection yet remains to be elucidated (Luppi et al., 2010). Just as there is a tonic inhibitory drive onto the Sub-C, there is a tonic excitatory drive onto Sub-C neurons. A hypothesized structure for the tonic excitatory drive is the lateral and ventrolateral PAG. Since it is known that both these structures contain glutamatergic neurons and there are projections from both structures onto the Sub-C (Boissard et al., 2003; Beitz, 1990). It has also been shown that the application of kynurenic acid (glutamate antagonist) reverses the REM sleep-like state induced by bicuculline (GABA_A antagonist) (Boissard et al., 2002). Considering the evidence, when the Sub-C is disinhibited, the tonic glutamate excitatory input activates the Sub-C, hence generating REM sleep (Figure 2).
Figure 2: Simple overview demonstrating the neural circuitry responsible for silencing and activating the Sub-C. a) This model demonstrates that the Sub-C is inactivated when receiving GABAergic inputs from the vlPAG and dDPme nuclei, which are hypothesized to be stimulated by nuclei such as DRN and LC. b) This model demonstrates that the Sub-C is activated when it is disinhibited by the vlPAG and the dDPme nuclei and becomes activated by the tonic glutamatergic drive, hence causing REM sleep via its upstream projections to cause cortical activation and downstream projections to generate REM sleep atonia.
Section 1.3-REM sleep atonia

As an organism transitions from wake to NREM sleep and then to REM sleep, there is a concomitant drop in muscle tone, with REM sleep exhibiting muscle atonia. The lack of muscle tone in REM sleep is proposed to be generated via two mechanisms, disfacilitation (withdrawal of excitation) and active inhibition (Brooks and Peever, 2012). During waking, motoneurons receive monoaminergic (i.e. noradrenaline and serotonin) input that results in the motoneurons becoming more receptive to excitatory input (White and Barnes, 1991). It is also shown that nuclei such as the LC and the DR cease firing when an organism enters REM sleep (Jacobs, 1986; John et al., 2004). Also, it has been shown that withdrawal of neuromodulators such as serotonin and/or noradrenaline in the hypoglossal motor pool is implicated in the drop in muscle tone seen during carbachol induced REM sleep (Kubin et al., 1994; Fenik et al., 2005). Hence, these lines of evidence suggests that when an organism enters REM sleep, there is a withdrawal of excitatory neuromodulators (i.e. disfacilitation), which aids in the drop of muscle tone seen in REM sleep. The other factor as stated above is an active inhibition of motoneurons during REM sleep. The role of active inhibition is believed to be at play to prevent the reenactment of dreams (Brooks and Peever, 2012).

Much controversy has surrounded the subject of which neurotransmitter systems are indeed responsible for generating muscle atonia during REM sleep. It was initially hypothesized that glycnergic (Gly) inhibition is responsible for somatic motor inhibition during REM sleep (Chase et al., 1989). This long-standing hypothesis was challenged by sleep neurobiologists such as Brooks and Peever (2008) and it was shown that Gly inhibition alone was not sufficient to generate REM sleep atonia. In their experimental paradigm, in the freely behaving rat, they antagonized glycine receptors at the level of trigeminal motor pool and were not able to reverse muscle atonia during REM sleep. This led to question what other possible neurotransmitters
could be involved in generating REM sleep atonia. This led to *Brooks and Peever (2012)* to find that antagonizing GABA\textsubscript{A}/GABA\textsubscript{B}/Gly receptors at the level of the trigeminal motor pool in the rat caused a reversal of REM sleep atonia in the masseter muscle. This finding shows that a synergistic action of the metabotropic action of GABA\textsubscript{B} receptors and ionotropic actions of GABA\textsubscript{A} and Gly receptors at the level of the motor pool is required to generate REM sleep atonia, at least in the case of the trigeminal motor pool. Another alternate neurotransmitter system that has been implicated in active inhibition during REM sleep is the cholinergic system. It was shown that activation of M2 and M4 muscarinic receptors at the level of the hypoglossal motor pool in the rat was responsible for active inhibition during REM sleep (Grace et al, 2013). For the purposes of this thesis though, the GABA/Gly neurotransmitter system will be investigated in the generation of REM sleep atonia.

**Section 1.4-Source of active inhibition & REM sleep atonia**

Discerning the source of inhibition in the brain that results in REM sleep atonia has been a subject of considerable research. It was initially shown by *Maghoun and Rhines (1946)* that electrical stimulation of the medial reticular formation (MRF) in decerberated and anesthetized cats resulted in the suppression of muscle reflexes. This suggests that inhibitory areas in the brainstem might be responsible for suppressing muscle activity. Then the medial medulla was implicated in REM sleep atonia as a study done by *Schenkel and Siegel (1989)* showed that chemical lesions using kainic acid of the medial medulla in the freely behaving cat resulted in a phenotype of REM sleep without atonia.

Further refining of the source of active inhibition in REM sleep atonia has implicated the ventral portion of the medial medulla, also known as the ventral medial medulla (VMM). A study done by *Vetrivelean and others (2011)* showed that lesioning of the VMM in rats resulted
in a general increase in EMG activity during REM sleep. Another study done by Siegel and others (2010), in the decerebrate cat, showed that electrical stimulation of the VMM resulted in an increase in the neurotransmitters GABA and glycine and a decrease in the neurotransmitters norepinephrine and serotonin at the level of the ventral lumbar horn, i.e. the location of postural motoneurons. This suggests that GABA/Gly from the VMM might be involved in the hyperpolarization of motoneurons during REM sleep, i.e. the generation of REM sleep atonia. It has been suggested that glutamatergic projections from the Sub-C onto GABA/Gly neurons of the VMM are responsible for activating these neurons, which results in muscle atonia during REM sleep (Luppi et al., 2011) (Figure 3).

![Diagram](image)

**Figure 3:** Overview schematic that illustrates that activation of the Sub-C (i.e. generator of REM sleep) causes REM sleep atonia. When the Sub-C is activated (via disinhibition and excitation), its upstream projections ultimately lead to the EEG profile of rich theta activity, characteristic of REM sleep. Its downstream projections cause activation of GABA/Gly neurons in the VMM, via the neurotransmitter glutamate. These neurons in the VMM in turn project to and inhibit motor neurons, via the neurotransmitters GABA/Gly, hence causing REM sleep motor atonia.
There are several lines of evidence for the involvement of the VMM in hyperpolarization of spinal motoneurons during REM sleep atonia. In rats, it has been shown that more than 50% of the neurons of the VMM have direct GABA/glycine projections onto lumbar motoneurons (Holstege and Bongers, 1991). Furthermore, application of non-N-methyl D-aspartate (NMDA) (glutamate agonist) to the general area of the VMM in cats trigger REM-like sleep atonia (Lai and Siegel, 1991). Also, it has been shown that microinjection of bicuculline (a GABA_A antagonist) into the Sub-C induces REM sleep and increases levels of c-Fos (marker of neuronal activity) in the VMM, which suggests that the VMM is active during REM sleep (Boissard et al., 2003). These lines of evidence suggest that glutamate activates GABA/Gly neurons of the VMM, which then projects to and inhibits motoneurons, resulting in REM sleep atonia.

Section 1.5-Phasic twitches

In addition to the potent suppression of basal muscle tone in REM sleep, there are also phasic twitches that punctuate this muscle atonia. The appearance of these phasic twitches during REM sleep is thought to be caused by the release of glutamate at the level of the motor pool during this state (Burgess et al., 2008; Soja et al., 1995). Some proposed brain regions that are thought to mitigate these phasic twitches are areas such as the red nucleus (RN), dorsolateral pontine tegmentum (DLPT) area, pedunculopontine tegmental (PPT) nucleus, rostral parvocellular reticular formation (rPCRt ) and paramedian reticular area (PMnR) (Gassel et al., 1965; Karlsson et al., 2005; Shouse and Siegel, 1992; Anaclet et al., 2010). It has also been shown that these depolarizing events at the level of the motoneuron that manifests itself as phasic twitches is preceded by a hyperpolarizing shift in membrane potential (Chase and Morales, 1982, 1983; Morales and Chase, 1981). This shows that there is an increase in a phasic inhibitory drive in conjunction with a tonic inhibitory drive to minimize the magnitude of these phasic twitches.
This phasic inhibitory drive might just be the same inhibitory system (i.e. the VMM) that generates the muscle atonia or might be a completely different inhibitory system.

**Section 1.6-Clinical implications**

The importance of understanding how muscle atonia in REM sleep is generated is vital since its dysfunction can lead to a pathology known as REM sleep behavior disorder (RBD). RBD is a sleep disorder that is characterized by its most well-known characteristic, which is abnormal muscle activity during REM sleep. This abnormal muscle activity in REM sleep is either sustained or intermittent loss of muscle atonia during REM sleep and/or exaggerated physical movement that are usually violent in nature, suggesting violent dreams. This violent dream enactment results in injury to oneself or their bed partner (Krenzer et al., 2013, Peever, 2011). It is also shown that RBD is a high predictor of alpha-synucleinopathies (Boeve, 2010). Alpha-synucleinopathies are neurodegenerative disorders caused by accumulation of alpha-synuclein protein in neurons that leads to diseases such as Parkinson’s disease, dementia with Lewy bodies, and multiple system atrophy (Eller and Williams, 2011).

A potential cause of human RBD is a breakdown in the GABA/Gly transmission at the level of the motoneuron. This breakdown in this system is further implicated as a culprit in RBD because the symptoms of RBD are recapitulated in a mouse model of RBD studied by Brooks and Peever (2011). This RBD mouse model has a mutant glycine receptor that causes a reduction in glycine-mediated inhibition as well as a reduction in GABA_\text{A} receptor-mediated inhibition. These RBD mice exhibited myoclonic jerks during NREM sleep, fragmentation of NREM sleep and increased motor activity in REM sleep, which are all symptoms of human RBD. Also, the same drugs used to treat human RBD, such as clonazepam and melatonin were effective in treating RBD symptoms in the mouse. These drugs act to enhance GABAergic
function (Schenck and Mahowald, 2002). Considering all this evidence, it points to a GABA/Gly system (potentially originating from the VMM) for mediating REM sleep atonia and is the perpetrator in RBD when the system fails.

Section 1.7-DREADDs technology

Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) is a novel technique developed to temporarily inhibit or excite neurons. This technology works by using muscarinic receptors that have been mutated so it is unable to bind to its natural ligand acetylcholine and made so it can only be activated by an otherwise inert designer drug, called clozapine-N-oxide (CNO) (Nawaratne et al., 2008). The ability of DREADDs to either excite or inhibit neurons is dependent on the type of mutated muscarinic receptor present on the neuronal membrane, i.e. the excitatory DREADD (hM3Dq) or the inhibitory DREADD (hM4Di). For the purposes of this thesis, only the inhibitory DREADD will be discussed.

Expression of hM4Di on neuronal membranes and activation via CNO will induce the membrane potential of the neurons to hyperpolarize. This hyperpolarization is due to G protein-coupled inwardly rectifying potassium (GIRK) channels opening (Dong et al, 2010). In essence, this hyperpolarization can be considered as turning “OFF” the neurons (i.e. preventing the neurons from firing) temporarily; thereby allowing one to use a loss-of-function approach to determine the function of a particular brain area. The function of a distinct population of neurons within a brain region can also be determined by DREADDs via the use of Cre-dependent DREADDs that will only expresses in neurons that express the enzyme Cre-recombinase, which can be achieved through the use of transgenic mice.

The use of hM4Di is advantageous compared to conventional loss-of-function-approaches (i.e. lesioning) in understanding the specific function of a brain area. Conventional
lesioning removes the area of interest permanently from participating in the neural network; hence compensatory mechanisms by the brain can occur and confound results. An example of compensatory mechanisms by the brain can be witnessed when lesioning sleep-promoting regions of the brain, which leads to a reduction in sleep time initially but within a couple of days normal durations of sleep return (Gulia, 2012). Additionally, lesioning, in particular electrolytic lesions can destroy fibers of passage, which again can affect neural communication and produce confounding results. Since hM4Di hyperpolarizes GABA/Gly neurons of the VMM, it will essentially silence these neurons temporarily and would avoid all the confounding factors mentioned above, hence the reason why this technique will be utilized in this thesis.

**Section 1.8-Thesis objective, hypothesis and expected outcomes**

The objective of this thesis is to discern the role of GABA/Gly neurons in the VMM in the generation of REM sleep atonia. As stated earlier, it has been shown that the neurotransmitters GABA/Gly are involved in the generation of REM sleep atonia (Brooks and Peever, 2012). It has also been shown that GABA/Gly neurons from the VMM project heavily to spinal motoneurons and that stimulation of the VMM leads to an increase in GABA/Gly at the level of the motoneuron (Holstege and Bongers, 1991; Siegel and Lai, 2010). Hence, it could be the GABA/Gly neurons of the VMM that are involved in the generation of REM sleep atonia. Therefore, I hypothesize that inhibiting the GABA/Gly neurons of the VMM will lead to a general increase in muscle activity during REM sleep via the reversal of muscle atonia during REM sleep and/or an increase in phasic twitches (caused by a decrease in inhibition). To test this hypothesis, I will be using DREADDs, more specifically the inhibitory DREADD (hM4Di) to inhibit the GABA/Gly neurons of the VMM.

A viral vector (i.e. an adeno-associated virus (AAV)) coding for the Cre-dependent
inhibitory DREADD will be delivered via viral transfer into the VMM of VGAT-Cre-recombinase mice. This will ensure that hM4Di will only express in the GABA/Gly neurons of the VMM since these mice only express Cre-recombinase in GABA/Gly neurons. Once expression of hM4Di occurs in GABA/Gly neurons of the VMM, the activation of this hM4Di receptor by CNO will induce the membrane potential of the neurons to hyperpolarize. In essence, this hyperpolarization can be considered as shutting “OFF” the GABA/Gly neurons of the VMM and the expected result in an increase in muscle activity during REM sleep (Figure 4). This increase in muscle activity will either manifest itself as REM sleep without atonia (i.e. increase in basal muscle tone) or an increase in phasic twitches.
Figure 4: Overview demonstrating the expected outcome of shutting of the GABA/Gly neurons of the VMM with hM4Di in respect to REM sleep. Under normal circumstances, when the Sub-C is activated, it will project to and excite GABA/Gly neurons of the VMM via glutamatergic transmission, which will in turn cause the VMM to inhibit motoneurons via GABA/Gly transmission. When hM4Di is placed into the VMM and activated via CNO, it will inhibit the GABA/Gly neurons of the VMM and therefore the Sub-C cannot excite these neurons, hence the GABA/Gly neurons of the VMM cannot hyperpolarize the motoneurons during REM sleep, leading to an increase in muscle activity during REM sleep.
SECTION 2: METHODS

Section 2.01-Animals

Transgenic mice (procured from Jackson Laboratory) were used in which the enzyme Cre-recombinase was expressed in all GABA/Gly neurons by flanking the genetic code encoding Cre-recombinase next to the vesicular GABA transporter (VGAT) promoter (Vong et al., 2011), which is exclusively found in GABA/Gly neurons. Animals were genotyped using PCR (35 cycles, 55° annealing temperature) with Cre-recombinase primer (Cre forward: CAC GAC CAA GTG ACA GCA AT; Cre reverse: AGA GAC GGA AAT CCA TCG CT). Male VGAT Cre+ mice on an agouti background were used. Current results represent 11 transgenic mice with a weight of 24 ± 0.80 g. All procedures and experimental protocols were approved by the Animal Care Committee at the University of Toronto and complied with the Canadian Council on Animal Care.

Section 2.02-Viral transduction

To transduce an engineered M4 muscarinic receptor into all GABA/Gly neurons of the ventral medial medulla, the viral vector (AAV8-hSyn-DIO-hM4Di-mCherry) was used (Figure 5a). This virus was procured from University of North Carolina (UNC) vector core, which was designed initially by Dr. Bryan Roth and others (Armbruster et al., 2007). The presence of a double-floxed inverted open reading frame (DIO) in the viral vector only allowed the expression of the M4 muscarinic receptor and the m-Cherry protein in neurons that contained Cre-recombinase. In this experimental paradigm it was GABA/Gly neurons. The m-Cherry fluorophore allows for visual localization of the M4 muscarinic receptor since it is directly conjugated to the muscarinic receptor. For all control experiments, a viral vector only coding for mCherry was used: AAV8-hSyn-DIO-mCherry (Figure 5b).
Figure 5: Schematic representation of double-floxed Cre-dependent adeno-associated viral (AAV) vector a) Viral construct of hM4Di conjugated with m-Cherry fluorophore under human synapsin (hSyn) promoter and action of Cre recombinase orienting into correct open reading frame (ORF). B) Viral construct of m-Cherry fluorophore under hSyn promoter and action of Cre recombinase orienting into correct ORF.
Section 2.03-Viral surgery protocol

Prior to viral surgery all mice were given the drug dexamethasone (Vétoquinol, Lavaltrie, QC) at a dosage of (6.25 mg/kg) to aid in reducing the brain swelling associated with such an invasive surgery (Hortobágyi et al., 2000). A 28 gauge cannula attached to PE20 tubing that was in turn attached to a 50 µl Hamilton syringe which in turn was attached to a syringe driver (Harvard Apparatus) was back-loaded with mineral oil followed by a small air bubble and then the viral vector (~5 µl). Sterile surgery was performed on mice using the anesthetic isofluorane (Baxter, Mississauga, ON) in conjunction with oxygen at a concentration of 2-2.5%. The anesthetic was delivered via the use of an isofluorane vaporizer (T3IS0, Benson Medical Industries, Markham, ON) and a flow-meter system (Western Medical, West Lake, OH) at a rate of 0.8-1.0 L/min. The outflow of the anesthetic was filtered via an activated-carbon scavenging canister (A.M. Bickford INC, Wales Center, NY). The mouse’s body temperature was maintained at 37-38 °C during surgery using a heat pad and a mouse temperature probe (TC-1000; CWE, INC, Andromore, PA).

Mice were placed in a stereotaxic apparatus (David Kopf Instruments, Chatsworth, CA) and the skull was exposed via a midline incision. The skull was leveled in which bregma and lambda were in the same horizontal plane. A 1 mm hole was then drilled at 6.64 mm posterior to bregma. The viral vector at a volume of 75-100 nanoliters was then targeted to the coordinates AP: - 6.64; ML: ± 0.30; DV: 5.70, defined as the coordinates for the VMM in this thesis. The diffusion period for the viral vector before the cannula was withdrawn from the brain was ~8 minutes. The animal was then stitched up and given the analgesic ketoprofen at 3 mg/kg. Animals were housed in polyurethane cages at room temperature on a 12:12 light-dark cycle [light on 0700h, lights off 1900h] for 2 weeks to recover.
Section 2.04 - Surgical implantation of EEG/EMG

Sterile surgery for implantation of EEG/EMG electrodes was performed on mice under isofluorane anesthesia (2-2.5%) two weeks after viral surgery, as previously described in publications from Dr. Peever’s lab (Burgess and Peever, 2013). EEG implantation was performed using multistranded stainless steel wire (AS632, Cooner wire) tied to four stainless screws, which were implanted into the skull (1 mm anterior ±1.5 mm lateral to bregma; 3 mm posterior ±1.5 mm lateral to bregma). EMG electrodes, which were also multistranded stainless steel wire (AS632, Cooner Wire), were implanted onto the surface of the right masseter muscle and neck muscle. All EEG/EMG electrodes were attached to a microstrip connector (CLP-105-02-L-D, Electrosonic), which was attached to the mouse’s skull with dental cement (Ketac-cem-3M). After the surgery, the animals were given the analgesic ketoprofen (3 mg/kg). Animals were housed in polyurethane cages at room temperature on a 12:12 light-dark cycle [light on 0700h, lights off 1900h] and given 10 days to recover.

Section 2.05 - Data acquisition

EEG and EMG signals were recorded by affixing a tether to the headplug, which is situated on the mouse’s head. This tether was then attached to a Super-Z head-stage amplifier and BMA-400 AC/DC Bioamplifier (CWE). The EEG was amplified 20-50 times and bandpass filtered between 1-100 Hz. The EMG was amplified 50-100 times and bandpass filtered between 30-1000 Hz. All electrophysiological signals were digitized at 1000 Hz and synchronized with video recordings (1080p Lifecam HD, Microsoft, Seattle, WA). All data was stored for later analysis.
Section 2.06-Drug preparation/delivery

Clozapine-N-Oxide (CNO; Dr. Bryan Roth, University of California) was dissolved in saline + 0.05% dimethyl sulfoxide (DMSO) to a working concentration of 0.5 mg/ml. DMSO was used in the saline solution since it aided in dissolving the CNO into saline. A volume of 300 µl of CNO (0.5 mg/ml) was injected via intraperitoneal (i.p) route into each mouse at 0930 h and 1830h on CNO treatment days. An injection at 0930h was chosen because it was 2.5 hours after lights-ON and studies have shown that REM sleep propensity is high during this period of time in mice (Fulda et al., 2011; Luo et al., 2013). An injection at 1830h was chosen because it was just before the onset of the dark phase at 19:00h. Saline with 0.05% DMSO at a volume of 300 µl was injected via an i.p route into each mouse at 0930h and 1830h on saline treatment days. This treatment (saline + 0.05% DMSO) served as the baseline in which drug treatment would be compared to since it served as the vehicle in which the drug CNO was dissolved in and also is an inert treatment (i.e. should be no observable effect).

Section 2.07-Experimental paradigm

Animals were given 3 days to habituate to the tether. Following habituation, animals were then habituated to i.p injections for two days. After, each mouse received CNO (5mg/kg) or saline (300 µl of saline + 0.05% DMSO) at 0930h (inactive phase) and at 1830h (active phase) while simultaneously being recorded for EEG/EMG for a period of 24 hours. The experimental procedures and the treatment procedures that each group of mice received and its expected outcomes are outlined in Figure 6. All treatment paradigms were randomized for each mouse.
Figure 6: Flowchart demonstrating the treatment courses each mouse received in the experimental paradigm and its expected outcomes. Group 1 received the treatment of the AAV-mCherry into the VMM and serves as the control for viral transfection. There should be no change in muscle activity during REM sleep when Group 1 receives the vehicle (saline+DMSO) or CNO (5 mg/kg). Group 2 received hM4Di into the VMM and there is an expected increase in muscle activity during REM sleep only when the animal receives CNO (5 mg/kg).
Section 2.08- Confirmation of viral targeting to the VMM and immunohistochemistry

Once the experimental paradigm was complete, the mice were placed under deep anesthesia (100 mg/kg ketamine + 10 mg/kg xylazine) and perfused transcardinally with 0.1M PBS at a rate of 4 ml/min followed by 4% PFA at a rate of 4 ml/min. The brain was then excised and post-fixed for a period of 24 hrs in 4% PFA and then cryoprotected in 30% sucrose (in 0.1M PBS) for 48 hrs. Brains were then frozen in molds with Tissue-Tek optimal cutting temperature (O.C.T) embedding compound (Electron Microscopy Sciences, Hatfield, PA) and then coronally sectioned into 40 µm slices from the pons (AP: -5.00) to the spinal cord using a cryostat (Leica CM3050S) and placed in (4x3) well plates containing 0.1M PBS with 0.05% Azide. The tissue from one column from the well plate was then mounted on charged slides and dried and then cover-slipped with permafluro media (Lab vision corporation, California). Slides were initially visualized under a fluorescent microscope (BX50W1 Olympus Microscope, Carsen Group Inc., Markham, ON) for the fluorophore mCherry and were later imaged using an Olympus slide scanner (FSX100, Olympus, Center Valley, PA). The fluorophore mCherry allows for the localization of viral expression, which is excited at 587 nm and emits at 610 nm (Shaner et al., 2004). The VMM was deemed to be virally transfected if transfection occurs in the defined area of the VMM as illustrated below (Figure 7). The VMM was defined in this thesis by extrapolating the VMM targeted in previous studies in cats (Lai et al., 2010) into the equivalent area in mice as well as suggested areas involved in the generation of REM sleep atonia i.e. GiA and GiV (Luppi et al., 2013).
Figure 7: Coronal sections from the mouse brain atlas showing the extent of the defined VMM (shaded area) for this thesis. The VMM region included parts of the GiA, GiV, medullary gigantocellular reticular nucleus (Gi) and raphe obscurus.

Free floating immunohistochemistry was done to determine the specificity of the Cre-dependent hM4Di to GABA/Gly neurons of the VMM. Therefore, free floating tissue sections confirmed to express hM4Di in the VMM were run through three 0.1M phosphate-buffered saline (PBS) washes at room temperature; with each wash lasting 5 minutes in duration. Then tissues were incubated at room temperature for one hour in 10% goat serum (Gibco, New Zealand), 0.4% Triton X-100 (Sigma-Aldrich, St. Louis, Montana) and 0.1 M PBS. Afterwards, tissues were incubated for twenty-four hours at 4 °C in (1:500) rabbit anti-mouse VGAT primary antibody (Millipore, catalogue #: AB5062P, concentration: 1 mg/ml), 4% goat serum, 0.4% Triton X-100 and 0.1 M PBS. The tissues were then run at room temperature through three 0.1M PBS washes, with each wash lasting 5 minutes in duration. Following the washes, the tissues were then incubated for 1 hr at room temperature in 4% goat serum and 0.1M PBS. The tissues were then again incubated for 1 hr at room temperature in (1:500) goat anti-rabbit Alexa 488 secondary antibody (Life technologies, catalogue #: A11008, concentration: 2 mg/ml), 4% goat serum, 0.25% Triton X-100 and 0.1M PBS. Afterwards, the tissues were run through three
0.1M PBS washes at room temperature, with each wash lasting 5 minutes in duration. The tissue sections were then mounted on charged slides and dried for 24 hrs and then cover-slipped with permaflor media (Lab vision corporation, California). Slides were then visualized under a fluorescent microscope (BX50W1 Olympus Microscope, Carsen Group Inc., Markham, ON) for the fluorophore mCherry and Alexa 488. Colocalization of the fluorophore mCherry and Alexa 488 suggested that hM4Di localized to GABA/Gly neurons.

**Section 2.09-Data Analysis**

**Sleep/Wake States**

To determine the sleep/wake amounts of each mouse under each treatment (saline and CNO (5 mg/kg) treatment), sleep/wake states were assigned 30 mins post i.p injection during the light phase for a total time of two hours of the mouse’s EEG/EMG recording. Sleep scoring was done 30 minutes post-injection since it has been shown that CNO takes effect within 15 mins when administered via i.p route (Alexander et al., 2009) and that REM sleep rarely occurs within the first 30 minutes post i.p. injection (Burgess et al, 2010). To assign sleep/wake states, each recording of a mouse containing an EEG and EMG were segmented into 5 s epochs and in conjunction with videography was scored as: active wake (AW), quiet wake (QW), non-rapid eye movement (NREM) sleep and rapid eye movement (REM) sleep. An EEG with low amplitude, high frequency coupled with an EMG with overt muscle tone was characterized as AW. An EEG with low amplitude, high frequency coupled with an EMG with low muscle tone (no overt activity) was characterized as QW. NREM sleep was characterized by high amplitude, low frequency EEG coupled with an EMG with low muscle tone. REM sleep was characterized by low amplitude, high-frequency theta-rich EEG coupled with an EMG with muscle atonia.
punctuated by muscle twitches (Burgess, 2008). For the purposes of this experimental paradigm, AW and QW were grouped into one category known as wake (W).

EMG analysis (General muscle activity)

To determine the general muscle activity associated with each sleep/wake state in the first two hours, raw EMG signals were full wave rectified, integrated and expressed in arbitrary units (a.u) for the masseter and neck muscle. Average EMG activity was quantified in 5 s epochs for each behavioral state and then averaged over the two hours for each respective sleep/wake state. Then, general EMG values for each state were then normalized to general muscle activity of NREM sleep in saline treatment. Muscle activity for each sleep/wake state was also looked at in two 1-hr time bins as well as four 0.5-hr time bins (data not shown).

EMG analysis (REM sleep basal muscle tone and phasic twitches)

Motor activity in REM sleep can be broken down into two states: tonic REM sleep and phasic REM sleep. Tonic REM sleep is the stereotypical periods of motor atonia while phasic REM sleep is when twitches punctuate motor atonia of REM sleep (Brooks and Peever, 2011). The main aim of this thesis was to quantify levels of motor atonia (i.e. basal muscle tone), twitch frequency and the amplitude of phasic twitches as these are the variables that constitute general muscle activity in REM sleep.

To quantify the variables mentioned above, episodes of REM sleep lasting a minimal duration of 15 s or longer in the two-hour period for each treatment (saline and CNO (5 mg/kg) treatment) were chosen for analysis. The reason for this specific length of REM sleep episodes was because episodes are usually devoid of phasic twitches when episode duration is less than 15 s. The EMG activity of the chosen REM sleep episodes was then quantified into 10 ms bins,
using Spike2 software (CED, Cambridge, UK). Then the data was exported to an algorithm developed by Dr. Patricia Brooks (Peever lab, University of Toronto), where the 99.99999999th percentile of the first 500 bins (or first 5 s) was calculated (Brooks and Peever, 2008, 2011, 2012). The reason why the first 5 s were chosen to calculate the 99.99999999th percentile is because the first 5 s of a REM sleep episode is usually devoid of phasic twitches. In the rare case that twitches were present, then another 5 s period devoid of phasic twitches within the same REM sleep episode was chosen. This 99.999999999th percentile value served as the threshold in which the differentiation is made between tonic REM sleep and phasic REM sleep. Muscle twitches that define phasic REM sleep activity were classified as anything above the threshold value (i.e. greater than the 99.99999999th percentile) and tonic REM activity (i.e. muscle atonia) is considered anything below this threshold value. In every animal, the threshold value was calculated for each REM sleep episode for saline and for CNO (5 mg/kg) treatment. With the threshold value calculated for each REM sleep episode, basal muscle tone (i.e. level of muscle atonia), twitch frequency (twitches/s) and amplitude of twitches were quantified.

The next step was to average all the REM sleep episodes for each treatment in each mouse for all three variables (basal tone, twitch frequency and amplitude). In regards to twitch frequency, the average twitch frequency was derived by averaging the twitch frequency of all REM sleep episodes per treatment in each respective mouse. In regards to basal tone, the average basal tone of all REM sleep episodes under saline treatment for each mouse was calculated and then all REM sleep episodes of saline and CNO (5 mg/kg) treatment of that respective mouse was normalized to the average saline basal tone value calculated previously for REM sleep. Following this normalization of basal tone, the average basal tone was calculated of all REM sleep episodes per treatment for each mouse. In regards to amplitude of phasic twitches, the average amplitude was derived by averaging the amplitude of phasic
twitches of all REM sleep episodes per treatment in each mouse and then normalizing to the average saline basal tone value of REM sleep of that respective mouse.

Another part of the experimental paradigm that required investigation was if inhibition of the VMM would cause a RBD phenotype. This disorder is characterized by excessive muscle activity during REM sleep but this phenotype only occurs in some REM sleep episodes (Boeve, 2010). Therefore, REM sleep episodes were plotted against time for both saline and CNO (5 mg/kg) treatment for all three variables: basal tone, twitch frequency and amplitude of phasic twitches of all mice for each respective muscle. The average for each variable under saline treatment was calculated and then the respective percentage of all REM sleep episodes that exceed the average value for both saline and CNO (5 mg/kg) was calculated. If an RBD phenotype is present, then one should have a greater proportion of REM sleep episodes exceeding the average value with CNO (5 mg/kg) treatment compared to saline treatment.

**Videography**

Mice were observed via videography for both saline and CNO (5 mg/kg) treatments in order to determine if GABA/Gly inhibition of the VMM leads to any abnormal behavior; especially when they were in REM sleep in order to determine if they displayed a RBD phenotype.

**Section 2.10-Statistical Analysis**

Before statistical tests were run, normality of the data was assessed using a Kolmogorov-Smirnov test (with Dallal-Wilkinson-Lilliefors P value). The statistical tests used in this thesis were paired t-test (two-tailed), two proportion z-test (one tailed), Wilcoxon matched pairs test and a 2-way repeated measures (RM)-ANOVA (post-hoc: Bonferroni). The specific statistical tests used for each experiment are stated within the results section. Kolmogorov-Smirnov test
(with Dallal-Wilkinson-Lilliefors P value), paired t-test, and Wilcoxon matched pairs test and 2-way RM-ANOVA were performed electronically using Prism5 (GraphPad, La Jolla, CA). Data are presented as mean ± standard error of the mean.
SECTION 3: RESULTS

Section 3.1- AAV mediated expression of mCherry alone does not influence sleep or motor activity

It had to be determined if expression of a foreign inert protein in the GABA/Gly neurons of the VMM could alter sleep/wake amounts or alter motor activity in sleep in the presence of CNO. The rationale for this aspect of this experimental paradigm is that it provides confidence that it is truly the activation of the hM4Di receptor that is causing the observed phenotype, if any, when the mice are treated with CNO and not the expression of a foreign protein. Therefore, I expressed the fluorophore mCherry in GABA/Gly neurons of the VMM in conjunction with CNO (5 mg/kg) treatment to determine its effect specifically on sleep/wake amounts and its effect on general muscle activity in sleep. Since, mCherry fluorophore is an inert protein and CNO is an inert ligand, I do not expect to see any significant changes on sleep-wake amounts and on general muscle activity in sleep.

I also wanted to determine if CNO treatment in these mice changes their behaviour, therefore I observed mice using EEG/EMG recordings in conjunction with videography in both saline and CNO treatment. I found that administration of CNO to these mice did not change general behaviours. For example, mice continued to groom, wheel run and sleep normally.

A pivotal part of these experiments was to determine if GABA/Gly neurons of the VMM was appropriately transfected with the fluorophore m-Cherry. Therefore, after each behavioural experiment, mice were sacrificed and fluorescent microscopy was used to confirm the fluorophore mCherry in the VMM. I confirmed that the VMM was appropriately transfected via fluorescent imagining of mCherry (n=4) (Figure 8 a & b). Unfortunately I could not colocalize mCherry expression to GABA/Gly neurons since immunohistochemistry to stain for VGAT
(indicative of GABA/Gly neurons) was unsuccessful. Hence, when I state that I targeted GABA/Gly neurons of the VMM, it should be noted that it is presumed GABA/Gly neurons of the VMM.
Figure 8: Histological conformation of the fluorophore mCherry in GABA/Gly neurons in the region of the VMM. a) Histological maps outlining the distribution of mCherry in and around the VMM (n=4). b) Representative example of the fluorophore mCherry in the VMM. Bar= 500 µm.
Next, I needed to determine if mCherry expression with CNO administration affects sleep/wake amounts. I found that CNO administration did not affect overall sleep/wake amounts (two-way RM-ANOVA; n=4, p=0.402) (Figure 9).

![Figure 9: CNO administration to mice expressing mCherry in the GABA/Gly neurons of the VMM does not affect sleep/wake amounts. Group data for mice (n=4) does not show a statistically significant alteration in sleep/wake amounts with CNO treatment compared to saline treatment. W=wake; NREM=non-rapid eye movement sleep; REM=rapid eye movement sleep. All values are mean ± SEM.](image)

Despite there being no significant change in overall REM sleep amounts with CNO treatment, I also wanted to make sure that there was no change in the frequency of REM sleep episodes. Therefore, I grouped REM sleep episodes by their duration, by grouping episodes into one-minute time bins for both saline and CNO treatment. I found that there was no significant difference in the frequency of REM sleep episodes across all time bins when mice were treated with CNO (two-way RM-ANOVA; n=4; p=0.611) (Figure 10).
The frequency of REM sleep episodes is not altered with CNO treatment in mice expressing mCherry in GABA/Gly neurons of the VMM. Group data for mice (n=4) with the mCherry fluorophore in the GABA/Gly neurons of the VMM does not show a change in the frequency of REM sleep episodes of different durations when treated with CNO. All values are mean ± SEM.

The last thing I needed to do was determine if CNO administration would change muscle activity in sleep. I found that general muscle activity in NREM sleep does not change in the masseter muscle nor in the neck muscle with CNO treatment (saline vs. CNO; n=4; masseter muscle: paired t test, p=0.839; neck muscle: paired t test p=0.659) (Figure 11a). I also found that CNO treatment does not affect general muscle activity during REM sleep either (saline vs. CNO; n=4; masseter muscle: paired t test, p=0.343; n=4; neck muscle: paired t test, p=0.421) (Figure 11b). This evidence suggests that CNO is inert and has no effect on general muscle activity during NREM sleep and REM sleep.
a) NREM

![Bar chart showing general muscle activity in NREM sleep and REM sleep for masseter and neck muscles under saline and CNO conditions.](image)

**Figure 11**: CNO administration to mice expressing mCherry in GABA/Gly neurons of the VMM did not significantly alter general muscle activity in NREM sleep and REM sleep. Group data for the masseter muscle (n=4) and neck muscle (n=4) exhibited no change in general muscle activity during a) NREM sleep and b) REM sleep when CNO was administered. All values are mean ± SEM.

Next I show that CNO treatment has no effect on basal tone and twitches in REM sleep. I found that basal tone of REM sleep was not affected in the masseter muscle nor in the neck muscle when treated with CNO (saline vs. CNO; n=4; masseter muscle: paired t test, p=0.286; neck muscle: paired t test, p=0.434) (Figure 12). The twitch frequency in REM sleep was also not affected in the masseter muscle nor in the neck muscle when treated with CNO (saline vs. CNO; n=4; masseter muscle: paired t test, p=0.899; neck muscle: paired t test, p=0.405) (Figure 13a&b). In regards to the amplitude of phasic twitches in REM sleep, it was also not affected in the masseter muscle nor the neck muscle when treated with CNO (saline vs. CNO; n=4; masseter muscle: paired t test, p=0.220; neck muscle: paired t test, p=0.843) (Figure 13a&b).
Finally, the temporal distribution of phasic twitches in a REM sleep episode was investigated. It is known that phasic twitches become more frequent as a REM sleep episode progresses in time (Brooks, 2011). Therefore, I wanted to ensure that the temporal pattern of twitches across a REM sleep episode is not altered with CNO. I went about observing this temporal distribution of phasic twitches by splitting grouped REM sleep episodes into quartile windows and quantifying the amount of phasic twitches per quartile window. I found that there was no change in the temporal pattern nor any significant difference in the amount of phasic twitches between saline and CNO for the masseter muscle (two-way RM-ANOVA; n=4, p=0.908) (Figure 14a) nor in the neck muscle (two-way RM-ANOVA; n=4, p=0.562) (Figure 14b). Together these results indicate that CNO treatment and AAV driven expression of a foreign protein in the GABA/Gly neurons of the VMM has no effects on sleep/wake amounts and motor activity in sleep.

Figure 12: CNO administration to mice expressing mCherry in GABA/Gly neurons of the VMM does not significantly alter basal tone during REM sleep. Group data showing a comparison of basal tone for the a) masseteric muscle (n=4) and the b) neck muscle (n=4) during REM sleep when treated with CNO. A.U: arbitrary units. All values are ± SEM.
Figure 13: Administration of CNO to mice expressing mCherry in GABA/Gly neurons of the VMM does not lead to a significant overall change in frequency or amplitude of phasic twitches. Group data showing a comparison of twitch frequency and amplitude for the a) masseter muscle (n=4) and b) neck muscle (n=4) during REM sleep when treated with CNO. A.U: arbitrary units. All values are ± SEM.

Figure 14: Administration of CNO to mice expressing mCherry in GABA/Gly neurons of the VMM does not change the temporal pattern of phasic twitches nor does it significantly change the twitch frequency across REM sleep episodes. Group data showing how phasic activity changes across a REM sleep episode by dividing REM sleep episodes into quartiles for the a) masseter muscle (n=4) and the b) neck muscle (n=4) when treated with CNO. All values are ± SEM.
SECTION 3.2: Results for inhibition of GABA/Gly neurons of the VMM

The major goal of this thesis is to test the hypothesis that GABA/Gly neurons of the VMM are responsible for inducing motor paralysis during REM sleep. To test this hypothesis, I expressed hM4Di in GABA/Gly neurons of the VMM and inactivated them by injecting mice with CNO (5 mg/kg). As stated before, immunohistochemistry to label GABA/Gly neurons was unsuccessful; therefore I could not confirm localization of hM4Di exclusively to GABA/Gly neurons. Hence, when I state that I inhibited GABA/Gly neurons of the VMM, it should be noted that it is presumed GABA/Gly neurons of the VMM.

Section 3.2.1- hM4Di was appropriately targeted to the VMM

To confirm that hM4Di was expressed in the VMM, I sacrificed mice after each behavioural experiment and used fluorescent microscopy to image the fluorophore mCherry, which provides indirect visualization of hM4Di. Figure 15 demonstrates that mCherry positive neurons were expressed in the VMM, hence demonstrating that the VMM was appropriately transfected with hM4Di (n=7).
Figure 15: Histological conformation that hM4Di was targeted to GABA/Gly neurons in the region of the VMM. a) Histological maps outlining the distribution hM4Di in and around the VMM (n=7). b) Representative example of hM4Di expression in the VMM. Bar= 500 μm.
Section 3.2.2- Waking and sleeping behaviour were unaffected by inhibition of GABA/Gly neurons of the VMM

Before determining if inactivation of GABA/Gly neurons of the VMM prevents REM sleep paralysis, I first wanted to demonstrate that this intervention did not affect general behaviour. I found that CNO injection into mice expressing hM4Di in the GABA/Gly neurons of the VMM did not affect overt behaviours in either sleep or wakefulness. Using videography with EEG/EMG, I watched mice for 2.5 hours after CNO injection and found that mice continued to engage in normal waking behaviours such as grooming, wheel running and feeding. Because VMM inactivation has been previously shown to cause RBD-like behaviours in cats during REM sleep (Lai et al., 2010), I also monitored mouse movements during sleep, and I found no obvious changes in overt movements during both NREM sleep and REM sleep.

Section 3.2.3- Inhibition of GABA/Gly neurons of the VMM impacts REM sleep, but not waking or NREM sleep

My next goal was to determine if VMM inhibition impacts sleep-wake amounts and I found that CNO injection had negligible effects on sleep/wake activity. Specifically, I showed that VMM inhibition did not significantly alter sleep-wake amounts (2-way RM ANOVA; n=7; p=0.78) (Figure 16).
Figure 16: Inhibition of GABA/Gly neurons of the VMM does not significantly alter sleep/wake amounts. Group data for mice (n=7) with hM4Di in the GABA/Gly neurons of the VMM does not show a significant alteration in overall sleep/wake amounts when treated with CNO. W=wake; NREM=non-rapid eye movement sleep; REM=rapid eye movement sleep. All values are mean ± SEM.

Even though VMM inhibition did not affect overall amounts of REM sleep, I nonetheless wanted to examine if the intervention impacted the micro-architecture of REM sleep. Therefore, I grouped REM sleep episodes by their duration, by grouping episodes into one minute time bins for both saline and CNO treatment. The reason why I looked at REM sleep with this fine of a temporal resolution is because a study by Weber et al., 2014 suggest that the GABA/Gly neurons of the VMM actually control REM sleep amounts. What I found was that administration of CNO displayed a global trend for a decrease in the frequency of REM sleep episodes across all time bins compared to saline (2-way RM ANOVA; n=7; p=0.004) (Figure 17). This evidence suggests that GABA/Gly neurons of the VMM are unlikely involved in controlling wakefulness and NREM sleep since its inhibition had no influence on these states. However, inhibition of GABA/Gly neurons of the VMM had an impact on REM sleep expression, which suggests that this brain area underlies REM sleep control.
The main goal of this thesis was to test the hypothesis that GABA/Gly neurons of the VMM are responsible for the generation of REM sleep atonia. I did this by inactivating VMM neurons using hM4Di receptors expressed on GABA/Gly neurons while monitoring both neck and masseter muscle activity. I found that CNO-induced inactivation of GABA/Gly neurons of the VMM increased muscle activity in the masseter but not in the neck muscle during REM sleep.

**Section 3.2.4-Inhibition of GABA/Gly neurons of the VMM leads to a general increase in muscle activity of the masseter muscle during REM sleep**

I found that inhibition of GABA/Gly neurons of the VMM significantly increased general muscle activity during REM sleep in the masseter muscle. General muscle activity during REM sleep between saline and CNO treatment, was $1.04 \pm 0.02$ a.u vs. $1.09 \pm 0.03$ a.u, respectively (saline vs. CNO; n=7; masseter muscle: paired t test, p=0.039) (Figure 18a, and table 1).

I also wanted to determine if VMM inhibition selectively affects muscle activity during REM sleep, so I examined how CNO administration impacts muscle activity during both waking
and NREM sleep. Under waking conditions I found that there was no significant change in general muscle activity in the masseter muscle with CNO treatment (saline vs. CNO; n=7; Wilcoxon matched pairs test, p=1.000). I also found that inactivation of the VMM neurons had no effect on muscle activity in the masseter muscle during NREM sleep (saline vs. CNO; n=7; paired t test, p=0.328) (Figure 18b). This suggests that GABA/Gly neurons of the VMM influence general muscle activity in REM sleep only.
a) REM

Figure 18: Inhibition of GABA/Gly neurons of the VMM leads to a significant increase in general muscle activity during REM sleep. Group data for the masseter muscle (n=7) shows a significant increase in general muscle activity during a) REM sleep when treated with CNO whilst there is no change in general muscle activity during b) NREM sleep when treated with CNO. *p < 0.05. All values are mean ± SEM.

<table>
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</tr>
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<tbody>
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<tr>
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<td>1.12 a.u</td>
</tr>
<tr>
<td>VGAT 17</td>
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<td>1.02 a.u</td>
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<td>1.18 a.u</td>
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<td>0.98 a.u</td>
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<tr>
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<td>1.15 a.u</td>
</tr>
<tr>
<td>VGAT 27</td>
<td>1.08 a.u</td>
<td>1.14 a.u</td>
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</table>

Table 1: Data representing general muscle activity during REM sleep in the masseter muscle for each mouse under saline and CNO treatment. a.u: arbitrary units
Section 3.2.5—Inhibition of GABA/Gly neurons of the VMM increases the proportion of REM sleep episodes to have a higher basal muscle tone

Since VMM inhibition increased general muscle activity in REM sleep, I needed to identify if this increase resulted from an increase in basal tone or increased phasic motor activity (i.e. twitches). Therefore, I quantified basal levels of massteric tone during REM sleep and compared it to saline treatment (i.e. baseline). I found that CNO-induced inactivation of GABA/Gly neurons of the VMM did not affect overall basal tone levels of the masster during REM sleep (saline vs. CNO; n=7; paired t test, p=0.220) (Figure 19, and table 2).

![Figure 19: Inhibition of GABA/Gly neurons of the VMM does not change overall REM sleep basal tone. Group data showing overall basal tone for the massteric muscle (n=7) during REM sleep when treated with CNO. A.U: arbitrary units. All values are ± SEM.](image)

<table>
<thead>
<tr>
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</tr>
</thead>
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<tr>
<td>VGAT 14</td>
<td>1.00</td>
<td>1.13</td>
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<td>VGAT 17</td>
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<td>VGAT 20</td>
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<td>VGAT 22</td>
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<td>VGAT 26</td>
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<td>1.02</td>
</tr>
<tr>
<td>VGAT 27</td>
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<td>0.98</td>
</tr>
</tbody>
</table>

Table 2: Data representing overall basal muscle tone during REM sleep in the masster muscle for each mouse under saline and CNO treatment. a.u: arbitrary units

Since I hypothesized that inactivation of the VMM would increase REM sleep basal tone and because levels of motor activity vary between individual REM sleep episodes, I decided to examine how CNO treatment affected basal muscle tone during individual REM sleep episodes. This approach is particularly important because patients with RBD do not experience REM
sleep without atonia during every REM sleep episode (Boeve, 2010); instead, they experience REM sleep without atonia during some of their REM sleep periods, suggesting that VMM inhibition may prevent REM sleep paralysis during a restricted number of REM sleep bouts. Therefore, I examined how levels of REM basal tone vary across time in both saline and CNO treated mice. Mice injected with saline experienced 74 REM sleep episodes during the 2h test period, whereas mice dosed with CNO experienced 46 REM sleep episodes. In order to determine if CNO treatment influenced REM sleep basal tone, I quantified the percentage of REM sleep episodes in which basal tone during individual REM sleep episodes was higher than the average basal tone of all saline REM sleep episodes in both saline and CNO treatment. I found that there was a significant 17% increase of individual REM sleep episodes being higher than the group mean with CNO injection compared to saline (saline vs. CNO, two proportion z-test, p=0.034) (Figure 20). This observation suggests that inhibition of GABA/Gly neurons of the VMM increases basal levels of muscle tone, but only during some episodes. This observation nicely fits the clinical observation in RBD patients showing that REM sleep atonia is not absent in all REM sleep episodes (American Academy of Sleep Medicine, 2005).
Figure 20: Inhibition of GABA/Gly neurons of the VMM increases the proportion of REM sleep episodes that have a higher basal muscle tone. a) Basal tone for each REM sleep episode was plotted against the time of its occurrence for each treatment (saline, CNO) for the masseter muscle (n=7). The dashed red line represents the average basal tone of all saline REM sleep episodes. b) Graphical representation of REM sleep episodes for both treatments being above the average basal tone of all saline REM sleep episodes. There was a 17% increase of REM sleep episodes being higher with CNO-induced inactivation of GABA/Gly neurons of the VMM compared to saline. c) Raw traces depicting an EMG representing 30 seconds at the start of a REM sleep episode in the masseter muscle under saline and CNO treatment. This figure illustrates how CNO-induced inactivation of GABA/Gly neurons of the VMM can cause an increase in masseter basal muscle tone during some REM sleep episodes. The dashed white line serves as a visual aid to see the increase in basal tone with CNO treatment. * p<0.05 A.U.: arbitrary units.
Section 3.2.6-Inhibition of GABA/Gly neurons of the VMM does not alter phasic twitches during REM sleep in the masseter muscle

During REM sleep, muscle atonia is punctuated by phasic twitches. Therefore, I had to determine whether inhibition of GABA/Gly neurons of the VMM led to an increase in phasic twitches during REM sleep. I found that CNO-induced inactivation of GABA/Gly neurons of the VMM did not increase overall phasic twitches during REM sleep in the masseter muscle (saline vs. CNO; n=7; Wilcoxon matched pairs test, p=0.578) (Figure 21, and table 3).

Figure 21: Inhibition of GABA/Gly neurons of the VMM does not significantly change overall phasic twitches during REM sleep. Group data showing overall phasic twitches for the masseteric muscle (n=7) during REM sleep when treated with CNO. A.U: arbitrary units. All values are ± SEM.

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<td>0.79 twitches/s</td>
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<td>VGAT 14</td>
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<td>2.13 twitches/s</td>
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<td>VGAT 17</td>
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<td>1.87 twitches/s</td>
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<td>VGAT 20</td>
<td>1.14 twitches/s</td>
<td>1.20 twitches/s</td>
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<td>VGAT 22</td>
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<td>0.63 twitches/s</td>
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<td>VGAT 26</td>
<td>1.13 twitches/s</td>
<td>3.24 twitches/s</td>
</tr>
<tr>
<td>VGAT 27</td>
<td>10.77 twitches/s</td>
<td>16.89 twitches/s</td>
</tr>
</tbody>
</table>

Table 3: Data representing overall twitch frequency (twitches/s) during REM sleep in the masseter muscle for each mouse under saline and CNO treatment.

Since I hypothesized that inactivation of the VMM could increase phasic twitches and because levels of motor activity vary between individual REM sleep episodes, I decided to examine how CNO treatment affected phasic twitches during individual REM sleep episodes.
Again this approach is particularly important because patients with RBD do not experience REM sleep with increased levels of movement during every REM sleep episode (Boeve, 2010). Instead, they experience it during some of their REM sleep periods only, suggesting that the VMM inhibition may cause increased movement during a restricted number of REM sleep bouts. Therefore, I examined how levels of phasic activity vary across time in both saline and CNO treated mice. Mice injected with saline experienced 74 REM sleep episodes during the 2h test period, whereas mice dosed with CNO experienced 46 REM sleep episodes. In order to determine if CNO treatment influenced the amount of phasic activity, I quantified the percentage of REM sleep episodes in which phasic twitches was higher than the average phasic twitches of all saline REM sleep episodes for both saline and CNO treatment. I found that there was an insignificant 4% increase of individual REM sleep episodes being higher than the group mean with CNO injection compared to saline (saline vs. CNO, two proportion z-test, p=0.28) (Figure 22). This observation suggests that inhibition of GABA/Gly neurons of the VMM does not increase levels of phasic activity in only a subset of REM sleep episodes, hence not mirroring any RBD-like phenotype.

![Figure 22: Inhibition of GABA/Gly neurons of the VMM does not increase the proportion of REM sleep episodes to have a greater twitch frequency.](image)

Twitch frequency (twitches/s) for each REM sleep episode was plotted against the time of its occurrence for each treatment (saline, CNO) for the masseter muscle (n=7). The dashed line (threshold value) represents the average twitch frequency of all saline REM sleep episodes. There was an insignificant increase in the number of REM sleep episodes being higher than the threshold value with CNO-induced inactivation of GABA/Gly neurons of the VMM compared to saline. A.U: arbitrary units.
In addition to measuring the frequency of phasic twitches during REM sleep, I also quantified how CNO administration influenced amplitude of phasic twitches during REM sleep. I found that CNO-induced inactivation of GABA/Gly neurons of the VMM did not alter overall amplitude of phasic twitches in the masseter muscle (saline vs. CNO treatment; n=7; paired t test, p=0.168) (Figure 23, and table 4).

**Figure 23:** Inhibition of GABA/Gly neurons of the VMM does not significantly change overall amplitude of phasic twitches during REM sleep. Group data showing overall amplitude of phasic twitches for the masseteric muscle (n=7) during REM sleep when treated with CNO. A.U: arbitrary units. All values are ± SEM.

<table>
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<th>Mouse #</th>
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<th>CNO (A.U)</th>
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<tbody>
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<td>4.57 a.u</td>
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<td>VGAT 22</td>
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<td>4.07 a.u</td>
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<td>VGAT 26</td>
<td>5.13 a.u</td>
<td>6.89 a.u</td>
</tr>
<tr>
<td>VGAT 27</td>
<td>4.24 a.u</td>
<td>3.74 a.u</td>
</tr>
</tbody>
</table>

**Table 4:** Data representing overall amplitude of phasic twitches during REM sleep in the masseter muscle for each mouse under saline and CNO treatment. A.U: arbitrary units.

I also decided to observe the amplitude of phasic twitches over many individual REM sleep episodes. This is because amplitude of phasic twitches can vary between episodes, and I decided to examine how CNO treatment affected the amplitude of phasic twitches during individual REM sleep episodes. This approach is useful because patients with RBD experience exaggerated movements in REM sleep, but not during every REM sleep episode (Boeve, 2010). Instead, they experience exaggerated levels of movement during some of their REM sleep periods, suggesting that VMM inhibition may cause exaggerated movement (i.e. greater
amplitude of phasic twitches) during a restricted number of REM sleep bouts. Therefore, I examined how levels of amplitude of phasic twitches vary across time in both saline and CNO treated mice. Mice injected with saline experienced 74 REM sleep episodes during the 2h test period, whereas mice dosed with CNO experienced 46 REM sleep episodes. In order to determine if CNO treatment influenced amplitude of phasic twitches, I quantified the percentage of REM sleep episodes in which the amplitude of phasic twitches was higher than the average amplitude of phasic twitches of all saline REM sleep episodes for both saline and CNO treatment. I found that there was an insignificant 5% increase of individual REM sleep episodes being higher than the group mean with CNO injection compared to saline (saline vs. CNO, two proportion z-test, p=0.28 (Figure 24). In conclusion, inhibition of GABA/Gly neurons of the VMM does not increase the amplitude of phasic twitches in only a subset of REM sleep episodes, hence not mirroring any RBD-like phenotype.

![Graph](image)

**Figure 24:** Inhibition of GABA/Gly neurons of the VMM does not increase the proportion of REM sleep episodes to have phasic twitches with greater amplitude. Average amplitude of phasic twitches for each REM sleep episode was plotted against the time of its occurrence for each treatment (saline, CNO) for the masseter muscle (n=7). The dashed line (threshold value) represents the average twitch frequency of all saline REM sleep episodes. There was an insignificant increase in the number of REM sleep episodes being higher than the threshold value with CNO-induced inactivation of GABA/Gly neurons of the VMM compared to saline. A.U: arbitrary units.

The temporal distribution of phasic twitches during a REM sleep episode is that the twitch frequency increases as the REM sleep episode progresses in time (Brooks, 2012). Hence, I determined if inhibition of the GABA/Gly neurons of the VMM could elicit a change in the
temporal pattern of phasic twitches during a REM sleep episode. Looking at the temporal
distribution of phasic twitches in quartile windows of REM sleep episodes, there was no change
in the temporal pattern nor any significant difference in the amount of phasic twitches in the
REM sleep quartile windows between saline and CNO treatments for the masseter muscle (two-
way RM-ANOVA; n=7; p=0.767) (Figure 25).

Figure 25: Inhibition of GABA/Gly neurons of the VMM does not change the temporal pattern of phasic
twitches nor does it significantly change the twitch frequency across REM sleep episodes. Group data showing
how phasic activity changes across a REM sleep episode by dividing REM sleep episodes into quartiles for the
masseter muscle (n=7). There was neither a change in the temporal pattern nor a significant change in twitch
frequency across REM sleep episodes when treated with CNO. All values are ± SEM.

Section 3.2.7-Inhibition of GABA/Gly neurons of the VMM did not increase general muscle
activity in the neck during REM sleep

In regards to inhibition of the VMM, I investigated two muscles, the masseter and neck muscle.
The reason being is that one muscle is a cranial muscle (i.e. the masseter muscle) and the other
being a postural muscle (i.e. the neck muscle); hence they could have different
mechanism/sources of inhibition during REM sleep. Unlike the masseter muscle, I found there
was no significant difference in general muscle activity during REM sleep in the neck muscle
(saline vs. CNO; n=6; neck muscle: paired t test, p=0.492) (Figure 26). This suggests that the
GABA/Gly neurons of the VMM are unlikely involved in controlling muscle activity in the neck
muscle during REM sleep. Data representing general muscle activity during REM sleep for each
individual mouse is listed in table 5 below.
Basal tone and phasic twitches is what contributes to general muscle activity in REM sleep, and since no change was observed in general muscle activity in REM sleep of the neck muscle with CNO treatment, I wanted to show that basal tone and phasic activity also remain unaffected. I found that overall basal tone of REM sleep was not affected in the neck muscle when treated with CNO (saline vs. CNO; n=6; paired t test, p=0.490) (Figure 27). The twitch frequency in REM sleep also remain unaffected in the neck muscle when treated with CNO (saline vs. CNO; n=6; paired t test, p=0.598 (Figure 28a). In regards to the amplitude of the phasic twitches, I also found that there was no significant change with CNO treatment (saline vs. CNO; n=6; Wilcoxon matched pairs test, p= 0.22) (Figure 28b). Data representing overall phasic twitches and amplitude of phasic twitches during REM sleep for each individual mouse is listed in table 7. The temporal distribution of phasic twitches in the neck muscle was not changed nor any significant difference in the amount of phasic twitches in the REM sleep quartile windows between saline and CNO treatments (two-way RM-ANOVA; n=6; p=0.986) (Figure 28c). This leads me to conclude that inhibition of the GABA/Gly neurons of the VMM does not affect twitch frequency, amplitude of phasic twitches or the temporal distribution of phasic twitches during REM sleep in the neck muscle.
Figure 26: Inhibition of GABA/Gly neurons of the VMM does not significantly change general muscle activity during REM sleep. Group data for the neck muscle (n=6) shows there is no change in general muscle activity during REM sleep. All values are mean ± SEM.

<table>
<thead>
<tr>
<th>Mouse #</th>
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<tbody>
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Table 5: Data representing general muscle activity during REM sleep in the neck muscle for each mouse under saline and CNO treatment. a.u: arbitrary units

Figure 27: Inhibition of GABA/Gly neurons of the VMM does not significantly change overall REM sleep basal tone. Group data showing overall basal tone for the neck muscle (n=6) during REM sleep when treated with CNO. A.U: arbitrary units. All values are ± SEM.
Table 6: Data representing overall basal tone during REM sleep in the neck muscle for each mouse under saline and CNO treatment. a.u: arbitrary unit.

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Table 7: Data representing overall phasic twitches and overall amplitude of phasic twitches during REM sleep in the neck muscle for each mouse under saline and CNO treatment. a.u: arbitrary units

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<td>VGAT 27</td>
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Figure 28: Inhibition of GABA/Gly neurons of the VMM does not significantly change phasic twitches during REM sleep. Group data for the neck muscle (n=6) showing a) overall twitch frequency b) overall amplitude of phasic twitches and c) temporal pattern of phasic twitches during REM sleep when treated with CNO. A.U: arbitrary units. All values are ± SEM.
SECTION 4: DISCUSSION

Section 4.1 - Summary of findings

This thesis project investigates GABA/Gly neurons in the VMM and its role in the generation of REM sleep atonia. There were two major findings resulting from this experimental paradigm. The first was an increase in general muscle activity during REM sleep in the masseter muscle with inhibition of GABA/Gly neurons of the VMM while no change was observed in the neck muscle. Second, which was an unexpected finding, was that inhibition of GABA/Gly neurons of the VMM resulted in a decrease in the frequency of REM sleep episodes.

Section 4.2 - GABA/Gly neurons of the VMM contributes to the generation of REM sleep atonia in the masseter muscle

The inhibition of the GABA/Gly neurons of the VMM led to a general increase in muscle activity during REM sleep in the masseter muscle. Hence, suggesting that the neurons of the VMM are playing a role in REM sleep atonia. But it was determined that neither an overall increase in basal muscle tone, nor in twitch frequency or the amplitude of the phasic twitches significantly contributed to the increase in muscle activity seen during REM sleep. A potential reason why this source of increase in general muscle activity was not seen at least in one of the three variables (basal tone, twitch frequency or amplitude of phasic twitches) was because all three variables could have contributed to overall muscle activity but on an individual basis its contribution was not statistically significant.

Another aspect that was looked at was if an RBD phenotype was present with CNO-induced inhibition of GABA/Gly neurons of the VMM. It was found that these mice did not exhibit a stereotypical RBD phenotype, such as gross body and limb movements, i.e. running, jerking, and chewing during REM sleep (Brooks and Peever, 2011). But what was found was
that these mice exhibited a greater proportion of REM sleep episodes with a higher basal tone with CNO treatment compared to saline in the masseter muscle. This phenotype was not evident with twitch frequency or amplitude of the phasic twitches. This suggests that dysfunction of the GABA/Gly neurons of the VMM can start to resemble a RBD reminiscent-like phenotype. Since only some REM sleep episodes can have an intermittent or complete loss of muscle atonia in RBD (American Academy of Sleep Medicine, 2005). This phenotype was imitated in CNO-induced inactivation of GABA/Gly neurons of the VMM because it increases the basal tone in only some REM sleep episodes, which resulted in an increased proportion of REM sleep episodes with a higher basal tone compared to saline.

It has been shown in the masseter muscle that REM sleep atonia is generated via the activation of GABA<sub>A</sub>/GABA<sub>B</sub>/Gly receptors at the level of the masseter motor pool in the rodent (Brooks and Peever; 2012). Therefore, it was expected that inhibition of the GABA/Gly neurons of the VMM would elicit a substantial change in general muscle activity in REM sleep, but only a minor increase (~5%) was observed in the masseter muscle. One possibility was that not an adequate amount of GABA/Gly neurons of the VMM were inhibited. Another possibility is that GABA/Gly neurons that are more caudal in the ventral medullary brainstem rather than the defined VMM in this thesis play a larger role in inhibition of the masseter muscle during REM sleep.

**Section 4.3-GABA/Gly neurons of the VMM does not contribute to REM sleep atonia in the neck muscle**

Surprisingly the inhibition of GABA/Gly neurons of the VMM did not increase general muscle activity in the neck muscle. This suggests that the GABA/Gly neurons of the VMM (defined in this thesis) are not involved in the generation of REM sleep atonia in the neck. One possibility is that GABA/Gly neurons that are more caudal in the ventral medullary brainstem are involved in
the generation of REM sleep atonia in the neck. But evidence from Vetrivelan et al., 2009 showed that GABA/Gly neurons in the VMM that were more caudal (i.e. AP: -6.64 mm to AP: -6.96 mm) than the VMM area defined in this thesis were also not responsible for REM sleep atonia in the neck. This area defined by Vetrivelan et al, 2009 only encompassing the region of the ventral gigantocellular (GiV) nucleus was termed by the authors as the “supraolivary medulla” (SOM) area. It was shown that another neural mechanism in the SOM area was at work in the generation of muscle suppression in the neck.

It was found that when they conditionally knocked out glutamatergic neurons from the SOM, the neck muscle presented with exaggerated phasic twitches during REM sleep. They also found that Sub-C neurons project to the spinal ventral horn and therefore could also be potentially involved in the generation of REM sleep atonia in the neck (Lu et al., 2006, Fort et al., 2009). Hence, they proposed a model for the generation of REM sleep atonia in the neck via Sub-C glutamatergic neurons directly projecting to the interneurons of the spinal cord and these in turn inhibiting the motoneurons of the neck. In addition, they also proposed that glutamatergic neurons of the Sub-C project also project to glutamatergic neurons of the SOM. These glutamatergic neurons of the SOM then project to interneurons of the spinal cold (Drew and Rossignol, 1990; Takukusaki et al., 2001) which in turn inhibit motoneurons of the neck. The evidence to support that interneurons at the level of spinal ventral horn can generate REM sleep atonia is a study done by Yamuy et al., 1991 that showed that interneurons of the spinal cord that potentially mediate motor inhibition also have increased c-Fos expression when REM sleep is induced via carbachol treatment. All this evidence suggests that these interneurons are potentially activated via a glutamatergic mechanism originating from the Sub-C and the VMM to generate REM sleep atonia in the neck.
Section 4.4—GABA/Gly neurons of the VMM modulate the frequency of REM sleep episodes

An unexpected observation made in this study was that inhibition of GABA/Gly neurons of the VMM led to a decrease in the frequency of REM sleep episodes. There are no published papers known that implicates the VMM in REM sleep control. There is only a recent 2014 abstract presented at the SFN conference by Weber et al., 2014 which has implicated the GABA/Gly neurons of the VMM in REM sleep control. The study was a gain of function approach using optogenetics, in which GABA/Gly neurons of the VMM were excited during NREM sleep. It was found that when these GABA/Gly neurons were excited during NREM sleep, there was a reliable entrance into REM sleep and it increased the duration of REM sleep episodes. In this thesis project, a similar phenotype was seen, in terms of frequency, in which inhibition of GABA/Gly neurons of the VMM showed a decreasing trend in the frequency of REM sleep episodes.

A hypothetical mechanism in which GABA/Gly neurons of the VMM can decrease the frequency of REM sleep episodes is via potential projections to the GABAergic neurons of the vLPAG and dDpMe nuclei. The GABAergic neurons of these two nuclei are known to project onto the Sub-C (Sapin et al., 2009; Boissard et al, 2003). If the excited GABA/Gly neurons of the VMM inhibit the GABAergic neurons of the vLPAG and dDpMe nuclei, then the GABAergic input onto the Sub-C would decrease. This would cause an increase propensity for the Sub-C to become activated, hence generating REM sleep. A continued activation of the GABA/Gly neurons of the VMM would increase the duration of REM sleep episodes as well. The inverse of this is that inactivation of GABA/Gly neurons of the VMM would not inhibit the GABA/Gly neurons of the vLPAG and dDpMe; hence the Sub-C would not be disinhibited. This would result in a decreased propensity for the Sub-C to become activated, hence reducing the frequency of REM sleep episodes. A caveat was that inhibition of the GABA/Gly neurons of...
the VMM in this experimental paradigm did not decrease the duration of REM sleep episodes; hence is not exactly congruent with the data from Weber et al., 2014. These differences in the data could possibly be explained by the fact that different techniques (DREADDs vs. optogenetics) were used. Despite these differences, these pieces of evidence are illuminating a new role for the VMM other than just a brain area facilitating muscle atonia during REM sleep.

Section 4.5—Technical considerations/future directions

A concern that could be raised is that hM4Di might not have potently inhibited the GABA/Gly neurons of the VMM. Hence, explaining why there is no substantial increase in general muscle activity during REM sleep in the masseter muscle with CNO treatment. However, previous extracellular studies from Dr. Peever’s lab at the University of Toronto (unpublished results) demonstrated that CNO-induced inactivation (via hM4Di) of all neurons of the VMM performed in-vivo in an anesthetised mouse was able to silence the neurons being recorded in the VMM. Additionally, it is known that GABA/Gly neurons of the VMM were indeed silenced since there was a decrease in the frequency of REM sleep episodes.

With the use of AAVs to drive expression of hM4Di into GABA/Gly neurons of the VMM, there was a great degree of undesirable spread within the medullary brainstem. This issue was also seen in the work by Vetrivelan et al., 2011 who also targeted the VMM. To combat this issue in future work it would be beneficial to use optogenetics to inhibit the GABA/Gly neurons of the VMM. Optogenetics uses light sensitive ion channels that either hyperpolarize or depolarize neurons when activated with a specific wavelength of light (Rogan and Roth, 2011). This technique conventionally uses an AAV to drive expression of light sensitive channels in neurons, and if the VMM was targeted there still can be a great degree of spread. But the solution to this issue is that optogenetics utilizes an optic fibre to deliver the specific wavelength of light within the brain and the location of the optic fibre determines which
specific brain region will either become activated or inhibited. Therefore, this technique will allow for greater spatial control in the medullary region, regardless of the spread of light sensitive ion channels amongst the neurons in the medulla. In addition, optogenetics can be used to exclusively target GABA/Gly neurons of the VMM, just like in the DREADDs technology. Another advantage in using optogenetics is that it would allow for a finer temporal resolution of control, which is in the order of milliseconds compared to DREADDs, which is in the order of hours. This temporal resolution afforded to one by the use of optogenetics would help in understanding the distinct role of the GABA/Gly neurons of the VMM in REM sleep control and its role in REM sleep motor control.

In this experimental paradigm, a VGAT-Cre\textsuperscript{+} mouse line was used in conjunction with Cre-dependent hM4Di. This mouse line expresses the enzyme Cre-recombinase in all GABA/Gly neurons; hence the Cre-dependent hM4Di should localize only to GABA/Gly neurons. Immunohistochemistry was performed to label all GABA/Gly neurons on tissue slices expressing mCherry in the VMM, which is an indirect maker of hM4Di expression in the VMM. Unfortunately, the immunohistochemistry was unsuccessful at labelling GABA/Gly neurons, therefore, one cannot claim that with absolute certainty that hM4Di localized to GABA/Gly neurons. However, it has been shown that an AAV Cre-dependent DREADD does localize to GABA/Gly neurons when a VGAT-Cre\textsuperscript{+} transgenic mouse line is used (Anaclet et al., 2014). Regardless, a future aim of this project is to label GABA/Gly neurons of the VMM by staining for VGAT via fluorescent in-situ hybridization (FISH) and show that hM4Di colocalizes to these GABA/Gly neurons.

As a future direction to this project, it might be worthwhile to investigate the role of the interneurons in the generation of REM sleep atonia, as these neurons have been suggested to play a role (Vetrivelan et al, 2009). An approach that can be used is optogenetics, where one can
exclusively target interneurons (via Cre-dependent method) of motor pools such as the easily accessible masseter motor pool and determine if inhibition of these interneurons could reverse REM sleep atonia. If inhibition of interneurons does reverse REM sleep atonia, it further adds to the wealth of literature that demonstrates that REM sleep atonia is facilitated via a GABA/Gly mechanism and demonstrates another source of GABA/Gly that is responsible for REM sleep atonia.

**Section 4.6-Cholinergic mechanism that can generate REM sleep atonia**

Despite the wealth of evidence supporting a GABA/Gly neurotransmitter mechanism in the generation of REM sleep atonia, it is important to note that a cholinergic mechanism has been implicated as well. Work done by *Grace et al., 2013* in freely behaving rats showed that REM sleep atonia in the hypoglossal muscle was reversed by antagonizing M2 and M4 cholinergic receptors (via reverse microdialysis) at the level of the hypoglossal motor pool. It was shown that activation of these M2 and M4 cholinergic receptors leads to REM sleep atonia via the subsequent activation of G protein inwardly rectifying potassium (GIRK) channels. GIRK channels function via increasing the efflux of potassium out of the neuron, which hyperpolarizes the neuron (Vazquez et al., 2012), hence generating REM sleep atonia. Therefore, this evidence suggests that REM sleep atonia is not mediated via one universal mechanism and that different muscles could have different neurotransmitters and circuitry at work to generate REM sleep atonia.
Section 5: Concluding remarks

Over the many years, much research has gone into understanding the brain region responsible for the generation of REM sleep atonia. It was initially isolated to the medial medulla (Maghoun and Rhines, 1946, Shenkel and Siegel 1989) and further isolated to the area of the VMM (Siegel and Lai, 2010; Lu et al., 2009). Further evidence suggested that a GABA/Gly mechanism mediates REM sleep atonia (Brooks and Peever, 2012). Therefore, this thesis aimed to test if the source of GABA/Gly generating REM sleep atonia was from the VMM and it was found at least in the terms of the masseter muscle that it is a source of inhibition. It must be noted however that there are lines of evidence that suggests other neural mechanisms are at work (Vetrivelan et al., 2009; Grace et al., 2013) possibly in concert with GABA/Gly neurotransmitter system to generate REM sleep atonia in other skeletal muscles. All this evidence suggests that the generation of REM sleep atonia is a complex process and there are potentially multiple neural mechanisms that govern REM sleep atonia across the whole neural axis. This thesis adds a novel contribution to understanding REM sleep paralysis by providing functional role for the VMM in the generation of REM sleep paralysis in the masseter muscle and unexpectedly illuminates a new role for the VMM, which is controlling REM sleep itself.
References


