Transduction of human alpha synuclein in the subcoeruleus region causes symptoms of REM sleep behaviour disorder in mice

by

Dillon McKenna

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

© Copyright by Dillon McKenna 2017
Transduction of human alpha synuclein in the subcoeruleus region causes symptoms of REM sleep behaviour disorder in mice

Dillon McKenna

Master of Science

Department of Cell and Systems Biology
University of Toronto

2017

Abstract

During rapid-eye-movement (REM) sleep, dreams and wake-like cortical activity accompany muscle paralysis (atonia) interspersed with phasic twitches, motor characteristics controlled by the pontine subcoeruleus (SubC) region. REM sleep behaviour disorder (RBD) is a disruption of motor control during REM sleep, manifesting as loss of atonia, increased twitches, and overt movements. Over 90% of RBD patients develop synucleinopathy, a neurodegenerative disorder associated with aggregates of the protein alpha-synuclein (αSyn). While brains of RBD patients show αsyn aggregates within the SubC region, there is currently no direct evidence of the effects SubC αSyn-related pathology will have on REM sleep. Using targeted overexpression of human αSyn in the SubC, I demonstrated αSyn-aggregation, excessive muscle twitches during REM sleep, and slowing of cortical activity in wild type mice. This project links RBD symptoms and αSyn in the SubC region, and has produced an RBD model that is clinically-relevant to the pathology of human synucleinopathy.
Acknowledgments

First I would like to thank my supervisor Dr. John Peever. His guidance and overwhelming support for me (and all his students) made for an environment where curiosity and creativity was encouraged, and where as a student my ideas felt valued and were allowed to develop. He is an amazing scientist and supervisor, and being a part of his lab was really an incredible opportunity.

I thank my committee members, Dr. Ali Salahpour and Dr. Joanne Nash for their guidance and for their valuable input on my project. I would like to give a special thank you to Dr. Richard Horner, for acting on my committee and for his continued support throughout the completion of my degree.

I would like to thank the CIHR Sleep and Biological Rhythms Toronto research and training program for a fantastic funding opportunity that supported this project financially.

Grad school could be rough. I could not have gotten through it without help from all the members of the Peever lab. I thank Dr. Jimmy Fraigne and Dr. Jennifer Lapierre for dedicating an incredible amount of time to making the lab run, and for training me and guiding me in my research. I’d like to give a special thank you to Zoltan Torontali who spent so much of his own time helping me and my project (he wrote the Spike script used in this project for twitch analysis). He created an environment where the lab members felt more like friends than coworkers, is a natural teacher and a great friend. Thank you to Matthew Snow for teaching me a lot of the techniques when I first started, to Simon Lui for his help with all the histology, and for all the members of the Peever lab during my time there: Sharshi Bulnar, Daniel Li, Sara Pintwala, Gabrielle Thibault-Messier, Linda Yang and Han-Hee Lee, for their help, friendship, and for making the lab a great place to work.

Also thank you to Dr. Patti Brooks for all her help while I was an undergrad, and for believing in me enough to recommend me as a student to her Ph.D. supervisor. Thank you to all the staff in the CSB department, with special thanks to Ian Buglass for his help throughout my time here.

I am very lucky to have an amazing family that supported me throughout this degree. Thank you to my mom Kelly, dad Bruce, sister Jocelyn, grandparents Bill, Carrie, Ivan, and Patricia. Your constant support and encouragement throughout my life has allowed me to pursue my interests with confidence. Any good qualities I have today are because of you, and I love you all.

Thank you to all my friends, who helped me forget how stressful school could be, and a special thank you to Katherine Lawrence who had to spend more time with me than anybody, often while I was stressed out, feeling hopeless, and going crazy. You stood by me through it all, helped me more than you can imagine, and I will be forever grateful, I love you.
# Table of Contents

Acknowledgments......................................................................................................................... iii  
List of Tables ................................................................................................................................... vii  
List of Figures .............................................................................................................................. viii  
List of Abbreviations .................................................................................................................. ix  
Chapter 1 – Introduction ............................................................................................................ 1  
1.1 Overview .................................................................................................................................. 1  
1.2 Control of muscle activity during REM sleep ............................................................................. 3  
   1.2.1 Muscle atonia in REM sleep ................................................................................................. 3  
   1.2.2 Phasic muscle twitches in REM sleep .................................................................................. 4  
1.3 REM sleep behaviour disorder ................................................................................................ 7  
1.4 Etiology of RBD ....................................................................................................................... 10  
   1.4.1 Synucleinopathies ............................................................................................................. 10  
1.5 Animal models of RBD .............................................................................................................. 14  
1.6 Animal models of synucleinopathy ........................................................................................... 15  
1.7 Hypothesis ............................................................................................................................... 16  
Chapter 2 – Materials and Methods ........................................................................................... 17  
2.1 Animals ..................................................................................................................................... 17  
2.2 Stereotaxic Injection Surgeries ................................................................................................. 17  
2.3 EEG and EMG Instrumentation surgery .................................................................................... 18  
2.4 Recording Protocol .................................................................................................................. 19  
2.5 Data Acquisition ....................................................................................................................... 19  
2.6 Data Analysis ............................................................................................................................ 20  
   2.6.1 Scoring sleep/wake states ..................................................................................................... 20
2.6.2 Analysis of sleep/wake architecture..........................................................20
2.6.3 EMG analysis...........................................................................................20
2.6.4 EEG analysis...........................................................................................22
2.7 Histology.......................................................................................................22
  2.7.1 Immunohistochemistry .........................................................................22
  2.7.2 Silver Staining .......................................................................................23
2.8 Statistical Analysis .......................................................................................23

Chapter 3 - Results...........................................................................................24
  3.1 Transduced αSyn expresses in the SubC region and aggregates within neurons. ..........24
  3.2 Transduction of asyn in the SubC region increases phasic muscle twitches during REM sleep...........................................................................................................27
    3.2.1 Masseter muscle: REM sleep twitches are exaggerated and happen more often in αSyn mice compared to controls..........................................................29
    3.2.2 Neck muscle: REM sleep twitches have a higher amplitude in αSyn mice compared to controls .................................................................31
  3.3 The effect of virally driven αSyn in the SubC on muscle activity is specific to phasic twitches during REM sleep.................................................................34
  3.4 Mice transduced with αSyn show a slowing of cortical activity across states........38
  3.5 Mice transduced with αSyn show longer REM sleep episodes compared to controls.....40

Chapter 4 - Discussion.......................................................................................42
  4.1 Mice transduced with human αSyn in the SubC show an RBD phenotype ..........45
    4.1.1 Transduced human αSyn forms intra-neuronal aggregates resembling an early form of those seen in human synucleinopathy ..............................................45
    4.1.2 Following αSyn transduction, mice display physiological signs of RBD ........45
  4.2 Limitations ..................................................................................................47
  4.3 Future directions..........................................................................................49
  4.4 Conclusions ................................................................................................50

References...........................................................................................................52
List of Tables

Table 1. Mice transduced with αSyn display symptoms resembling human RBD ...........................................43
List of Figures

Figure 1. Healthy REM sleep shows characteristic electrophysiological properties.................................2

Figure 2. Brainstem circuitry controls muscle activity during REM sleep....................................................6

Figure 3. RBD shows characteristic electrophysiological properties...........................................................9

Figure 4. α-Synuclein aggregation and brainstem pathology in RBD............................................................12

Figure 5. Virally driven human αSyn forms aggregates within SubC neurons.............................................24

Figure 6. Virally driven GFP expresses diffusely within SubC neurons......................................................25

Figure 7. Raw electrophysiological recordings display heightened phasic muscle activity (twitches) in αSyn mice during REM sleep.................................................................................................27

Figure 8. αSyn mice show an increased amount masseter muscle twitches during REM sleep....................29

Figure 9. αSyn mice show larger masseter muscle twitches during REM sleep.............................................30

Figure 10. The amount of neck muscle twitches during REM sleep is unchanged in αSyn mice....................32

Figure 11. αSyn mice show larger neck muscle twitches during REM sleep...............................................33

Figure 12. The effect of virally driven αSyn in the SubC on muscle activity is specific to phasic REM sleep.....36

Figure 13. The effect of virally driven αSyn in the SubC on muscle activity is specific to REM sleep...............37

Figure 14. Mice transduced with αSyn display slowing of cortical activity across sleep/wake states............39

Figure 15. αSyn mice show longer REM sleep episodes compared to controls............................................41

Figure 16. Silver staining results were inconclusive in determining amounts of αSyn mediated degeneration....48
List of Abbreviations

- µm: micrometer
- a.u.: arbitrary units
- AAV: adeno-associated virus
- ANOVA: analysis of variance
- AW: active wake
- CBA: chicken beta actin
- DAB: 3,3’-diaminobenzidine
- DLB: dementia with Lewy bodies
- EEG: electroencephalogram
- EMG: electromyogram
- EOG: electrooculogram
- G: gauge
- GABA: gamma-aminobutyric acid
- GFP: green fluorescent protein
- Glut: glutamate
- Gly: glycine
- Hz: hertz
- Kg: kilogram
- LC: locus coeruleus
- LDT: laterodorsal tegmental nucleus
- mg: milligram
- mL: millilitre
- mm: millimetre
- MN: motor-neuron
- MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydro-pyridine
- MSA: multiple systems atrophy
- nL: nanoliter
- NREM: non-rapid eye movement
- PBS: phosphate buffered saline
- PD: Parkinson’s disease
- PFA: paraformaldehyde
- PMnR: paramedian reticular area
- QW: quiet wake
- RBD: rapid eye movement sleep behaviour disorder
- REM: rapid eye movement
- RN: red nucleus
- rPCRt: rostral parvocellular reticular formation
- s: second
- SLD: sublaterodorsal nucleus
- SubC: subcoeruleus
- VGLUT2: vesicular glutamate transporter 2
- VMM: ventromedial medulla
- W: wake
- αSyn: alpha-synuclein
Chapter 1 – Introduction

1.1 Overview

In rapid eye movement (REM) sleep, vivid dreaming, wake-like cortical activation, and darting eye movements accompany skeletal muscle activity characterized by paralysis (atonia) interspersed with phasic muscle twitches$^{1-3}$ (Figure 1). Circuits in the brain stem function to control muscle activity during REM sleep, and the focal point of this circuit lies within the subcoeruleus (SubC) region of the pons$^{4-6}$. Failure of this control results in REM sleep behaviour disorder, a parasomnia where patients display heightened phasic muscle activity, a loss of muscle atonia and occasional bouts of complex, often violent dream enacting behaviours during REM sleep that risk injuring the patient and bed partner$^{7,8}$. Importantly, RBD predicts debilitating neurodegenerative diseases which often emerge years after RBD onset$^{9,10}$. These RBD-related neurodegenerative diseases are characterized by aggregates of the protein alpha-synuclein (αSyn) in midbrain and cortical regions that control waking motor function and cognition.

RBD patients display αSyn aggregates in the SubC region, however the role αSyn pathology has in the development of RBD symptoms remains untested. Furthermore, while a number of RBD-like phenotypes have been replicated in animals following manipulation of the SubC region, there are currently no αSyn-based animal models of RBD. Using targeted over expression of αSyn in the SubC of wild-type mice, this thesis examines the role of pathological αSyn in the breakdown of motor control during REM sleep.
Figure 1. Healthy REM sleep shows characteristic electrophysiological properties. Polysomnographic recordings display left and right eye movements (Electrooculogram, EOG; red traces) and small amplitude, high frequency cortical activity (Electroencephalogram, EEG; blue traces) during rapid eye movement (REM) sleep. Furthermore, during REM sleep, facial and limb muscles (Electromyogram, EMG; black traces) normally show paralysis (atonia) interspersed with phasic twitches. (Adapted from Peever et al., 2014).
1.2 Control of muscle activity during REM sleep

1.2.1 Muscle atonia in REM sleep

During REM sleep, a network of brainstem regions acts to directly inhibit the motor neurons innervating skeletal muscles. Early lesion studies identified the crux of this network as residing in the dorsal pontine tegmentum. Bilateral destruction of the region encompassing the locus coeruleus (LC), along with the area immediately ventral and medial to the LC resulted in a loss of muscle atonia combined with complex movements during REM sleep in cats\(^5,6\). A homologous region (ventral to both the LC and laterodorsal tegmental nucleus (LDT)) has since been identified in rodents\(^4\), called the sublaterodorsal nucleus (SLD) or subcoeruleus (SubC). In this thesis, I will use the term SubC when referring to this region. The behavioural effects of lesioning this region will be described in greater detail in section 1.5.

In addition to being necessary for the normal suppression of muscle activity during REM sleep, the SubC region contains a population of neurons that are highly active during REM sleep (REM-on neurons)\(^4\). Colocalization of the vesicular glutamate transporter (VGLUT2; marker for glutamatergic neurons) with c-fos (marker for neuronal activity) has indicated SubC REM-on neurons are glutamatergic\(^11\), which is supported by recent calcium imaging of VGLUT2+ neuronal activity during REM sleep\(^12\). Along with the SubC being endogenously active during REM sleep, stimulation of the SubC region induces muscle paralysis in awake animals\(^13\), suggesting the SubC region is both necessary and sufficient in producing the muscle atonia during REM sleep. Finally, SubC neurons project to REM-on inhibitory neurons outside of the pons that are involved in suppressing muscle activity: the ventromedial medulla (VMM), and inhibitory spinal interneurons\(^4\).

Downstream of the SubC, the ventromedial medulla (VMM) had been implicated as a crucial part of the circuit suppressing muscle activity during REM sleep. This area is targeted by excitatory glutamatergic signals\(^14,15\) from the pontine SubC region\(^4\). Intracellular recordings and the expression of c-fos show that VMM neurons are REM-on\(^16,17\). Furthermore, neurons in this region project to motor neurons\(^18\), and release the inhibitory transmitters glycine and gamma-aminobutyric acid (GABA) when electrically stimulated\(^14\). Finally, stimulation of this region suppresses muscle activity in freely moving animals\(^14,19\), while lesions produce muscle activity in REM sleep\(^20\). In addition to the VMM, glutamatergic signals from the SubC project to inhibitory
spinal interneurons\textsuperscript{4,15}, which have been demonstrated to be necessary for normal amounts of REM sleep atonia\textsuperscript{15}. This has led to the hypothesis that there are two pathways that stem from the SubC involved in generating atonia during REM sleep: SubC to VMM to motorneuron, and SubC to interneuron to motorneuron\textsuperscript{4,15}.

Upon entering REM sleep, motor neurons receive strong, hyperpolarizing signals\textsuperscript{21}. Motor neuron inhibition during REM sleep is the result of glycine and GABA release, which act on glycine, GABA\textsubscript{A} and GABA\textsubscript{B} receptors in tandem to strongly inhibit muscle activity\textsuperscript{22,23}. Only simultaneous pharmacologic blockage of these receptors creates a loss of muscle atonia along with increased phasic activity during REM sleep\textsuperscript{23}.

This circuit makes up the proposed core of active muscle inhibition during REM sleep: REM-on glutamatergic neurons of the SubC region send excitatory signals to the VMM and spinal interneurons, which then send inhibitory GABA and glycine signals to hyperpolarize motor neurons by simultaneously agonizing GABA\textsubscript{A}, GABA\textsubscript{B}, and glycine receptors, thereby strongly inhibiting muscle activity during REM sleep (Figure 2. A)

1.2.2 Phasic muscle twitches in REM sleep

Active suppression of tonic muscle activity during REM sleep is interrupted by bursts of phasic twitches. Muscle twitches are a defining characteristic of normal REM sleep, and while their physiological purpose is currently undefined, the relatively high abundance of twitches very early in life, the refinement and coordination of twitches in infancy, and decline in intensity with age suggests a role in motor development\textsuperscript{24,25}.

For these bursts of twitch activity to break through the active suppression of muscle tone, motor-neurons receive excitatory glutamatergic signals, the antagonism of which leads to a significant loss of twitch activity\textsuperscript{26}. Several areas have been implicated as sources for these excitatory, twitch-activating signals. Lesions of the rostral parvocellular reticular formation (rPCRt) and paramedian reticular area (PMnR), which send glutamatergic signals to trigeminal motor-neurons innervating the masseter muscle, significantly reduced masseter twitches in REM sleep while neck twitches were unaffected\textsuperscript{27}. Furthermore, specific inhibition of glutamate activity from rPCRt neurons greatly reduced masseter twitching\textsuperscript{27}. Another glutamatergic structure implicated in muscle twitches is the midbrain red nucleus. Here, targeted excitation of
glutamatergic neurons was recently found to increase the amount of twitches in both the masseter and neck muscles\textsuperscript{28}. Therefore, mounting evidence suggests multiple regions are involved in actively generating twitches in multiple muscles.

These excitatory signals have also been found to be actively opposed in normal REM sleep. When the temporal distribution of muscle twitches was analyzed, it was found that muscle twitches are mostly absent soon after entering REM sleep, and as REM episodes progress, twitches increase in size, number, and frequency\textsuperscript{29,30}. This suggests the organization of muscle twitches are controlled in part by a strong inhibitory signal early in REM sleep that gradually diminishes in strength\textsuperscript{29,30}. The signals responsible for this inhibition are likely glycine and GABA, as disrupting these signals at the level of the motor-neuron causes larger muscle twitches, as well as a loss in the normal distribution of twitches, with a greater numbers appearing earlier in REM sleep\textsuperscript{22,30,31}.

Taken together, both tonic and phasic muscle activity during REM sleep are tightly controlled phenomena organized by excitatory and inhibitory signaling between brainstem nuclei (Figure 2. A). The SubC region is critical for the circuit responsible for the suppression of muscle activity during REM sleep, and failure of this circuit could lead to abnormal, unrestrained motor activity during REM sleep.
Figure 2. Brainstem circuitry controls muscle activity during REM sleep. A. In Healthy REM sleep, the pontine subcoeruleus (SubC) region sends excitatory glutamatergic signals to the ventromedial medulla (VMM) and spinal interneurons (IN), which in turn send inhibitory GABA/glycine signals that inhibit motor neurons (MN), silencing muscle activity. Also during REM sleep, multiple regions (including the red nucleus (RN), the rostral parvocellular reticular formation (rPCRt), and paramedian reticular area (PMnR)) send excitatory glutamatergic signals to motor-neurons causing phasic muscle twitches. B. Hypothesized cause of RBD, where the SubC is compromised, causing disinhibition of motor-neurons and leading to heightened tonic and phasic activity, while allowing overt movements during REM sleep.
1.3 REM sleep behaviour disorder

First described by Schenck et al. (1986), RBD is a parasomnia characterized by abnormally high levels of muscle activity during REM sleep, culminating in overt and complex movements\(^7\). RBD patients display a variety of seemingly purpose-driven actions that include punching, kicking, gesturing, walking, jumping, laughing and talking all while remaining in REM sleep\(^7,8,32,33\). These behaviours are frequently reported to reflect dream content\(^32\). Patients also display excessive simple limb jerking movements in REM and NREM sleep\(^32\).

Polysomnographic recordings have revealed the majority of RBD cases show a decrease or absence of muscle atonia, coupled with large bursts of phasic activity during overt movements\(^32,33\) (Figure 3). Furthermore, the muscle twitches characteristic of normal REM sleep are exaggerated in size (duration and amplitude) and amount in RBD patients\(^34–36\) even in the absence of overt movements\(^37\). This excessive muscle activity (both tonic and phasic) during REM sleep has led to the prevailing thought that RBD patients suffer from failure of the brainstem circuitry that normally acts to suppress muscle activity in REM sleep, culminating in occasional bouts of dream enacting behaviours\(^32,38\) (Figure 2. B).

RBD is most prevalent in males over 60 years old, and the dramatic, often violent movements can be a risk of serious injury for both the patient and bed partner\(^7,32,39\). The excessive motor activity of RBD can be successfully treated in the majority of cases with clonazepam and melatonin\(^7,32,39,40\), however, long-term studies have also revealed a crucial link between RBD and neurodegenerative disease later in life\(^40\).

Monitoring groups of RBD patients over time has led to the striking discovery that RBD is highly predictive for developing a neurodegenerative disease in the future. First reported after a 5 year follow up with RBD patients, where 38% developed a synucleinopathic (described in section 1.4.1) neurodegenerative disease (predominantly Parkinson’s disease)\(^40\), ongoing monitoring of multiple groups has seen this number rise above 90%\(^9,41,42\). The motor symptoms of RBD worsen with time\(^43\) and RBD patients display slowing of cortical activity, an early sign of cognitive impairment\(^44\), suggesting RBD is an early and progressive component of neurodegenerative disease itself. The onset of RBD symptoms can precede synucleinopathy diagnosis by years or even decades\(^10\), and for this reason understanding the relationship of RBD to neurodegenerative disease, and how RBD develops within the nervous system can provide
important insight into how these neurodegenerative diseases progress, and into how this progression could possibly be stopped.
Figure 3. RBD shows characteristic electrophysiological properties. Polysomnographic recordings continue to display eye movements (Electrooculogram, EOG; red traces) and small amplitude, high frequency cortical activity (Electroencephalogram, EEG; blue traces) during rapid eye movement (REM) sleep in patients with REM sleep behaviour disorder. However, also REM sleep, facial and limb muscles (Electromyogram, EMG; black traces) show loss of atonia along with bursts of exaggerated phasic activity coinciding with limb movements. (Adapted from Peever et al., 2014).
1.4 Etiology of RBD

RBD has been documented in patients following a wide variety of acute pontine injury encompassing the SubC region. RBD has been described in a number of patients who were discovered to have tumors within the pontine tegmentum\textsuperscript{45,46}, and RBD symptoms have also been documented as developing following surgery for removal of tumors in this region\textsuperscript{47}. RBD has also been associated with lesions of the pontine/subcoeruleus region due to stroke\textsuperscript{48} and to demyelination injury in patients with multiple sclerosis\textsuperscript{49}.

1.4.1 Synucleinopathies

The neurodegenerative diseases associated with RBD belong to a category called synucleinopathy. Synucleinopathies include Parkinson’s disease (PD), dementia with Lewy bodies (DLB) and multiple systems atrophy (MSA), a group of diseases that can cause devastating motor and cognitive disabilities\textsuperscript{33}. The brains of patients with these diseases show degeneration associated with prominent dense intracellular inclusions called Lewy bodies along with the less dense pale bodies (precursors to Lewy bodies) (Figure 4. A). Motor symptoms primarily arise following degeneration of the midbrain substantia nigra (an important source of dopamine), while later degeneration of the cortex is associated with cognitive impairment and dementia\textsuperscript{50}.

While Lewy body-related diseases have been known for centuries\textsuperscript{51}, they were first linked to the protein alpha-synuclein (αSyn) in the mid 1990’s when an A53T mutation in the αSyn gene was identified in a family that were highly susceptible to developing PD\textsuperscript{52}. Soon after, αSyn was identified as a major component in Lewy body inclusions\textsuperscript{53}, highlighting the importance of this protein in this group of neurodegenerative diseases now known as synucleinopathies.

αSyn is an endogenous protein located throughout the healthy brain. While the exact function of αSyn has not been identified, it has been localized primarily to synaptic terminals (however it is also present in cell bodies) of mature neurons\textsuperscript{54,55}, and downregulation causes a reduction in available synaptic vesicles\textsuperscript{55}, suggesting a role in synaptic transmitter release. The structure of healthy αSyn contains several α-helices at the N-terminus\textsuperscript{56}, some of which are altered in PD-related mutations to form β-sheets that are susceptible to aggregation\textsuperscript{52,57}. 
Along with mutations of the protein, an abundance of un-mutated αSyn is also closely linked with aggregation and cell degeneration, as gene duplications and triplications are implicated in synucleinopathy development, with triplication more closely associated with early onset of disease. Additionally, mutant αSyn has been shown to be vulnerable to accumulation within cells, as the normal pathway involved with degradation of αSyn is less effective on the disease-related mutant form.

The role that aggregated αSyn plays in cell degeneration has not been pinpointed, however there has been a link established between αSyn and oxidative stress within cells. In vitro, neurons over-expressing αSyn have shown mitochondrial dysfunction, with abnormally large mitochondria and signs of oxidative stress including increased levels of free-radicals. Also, mice with αSyn-null mutations are resistant to the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of neurodegeneration, a neurotoxic model of PD that induces cell death by oxidative stress through mitochondrial dysfunction. Together, mounting evidence has shown that while it serves an endogenous function in the healthy brain, αSyn can also be highly detrimental, especially in an aggregated form.

Post mortem examination of PD patients has revealed that αSyn pathology advances in a distinct pattern throughout the brain. The earliest sign of αSyn aggregates appear in caudal medullary nuclei, and seem to advance rostrally through distinct regions of the brainstem and into the frontal cortex, coinciding with the progression of disease symptoms (Figure 4. B). Pontine inclusions and RBD symptoms appear early in PD, before the midbrain, forebrain and cortical pathology associated with the waking motor deficits and dementia of late-term PD (Figure 4. C). As these inclusions are present within the brainstem regions that are vital for regulating muscle activity during REM sleep, this supports the hypothesis that RBD itself may be an early manifestation of synucleinopathic neurodegenerative disease.

Synucleinopathic degeneration of the SubC region being the root of RBD is supported by recent imaging studies in RBD patients. Neuromelanin-sensitive magnetic resonance imaging has indicated a loss of structural integrity of the SubC region that correlates with severity of RBD symptoms in populations of patients having RBD with PD, as well as in RBD without concomitant diseases (Figure 4. D and E). Taken together, injury (acute and degenerative) to
the pontine SubC region appears to underlie RBD in humans, a finding supported by the manipulation of homologous regions in animal models.
Figure 4. *α*-Synuclein aggregation and brainstem pathology in RBD. *A*. Synucleinopathic neurodegenerative disease is characterized by the presence of large intra-neuronal inclusions that stain positive for αSyn (blue) known as Lewy bodies (black arrowhead) or pale bodies (precursors to Lewy bodies; black arrow). These inclusions are prominent in the midbrain dopaminergic substantia nigra, where degeneration causes motor symptoms of Parkinson’s disease (adapted from Braak et al., 2003). *B*. The earliest sign of αSyn aggregates appear in the caudal brainstem, and seem to advance rostrally with time through distinct regions of the brainstem and into the frontal cortex, coinciding with the progression of disease symptoms. The darker red indicates earlier appearance of αSyn aggregates in the corresponding brain region, with white arrows highlighting the direction of disease progression (adapted from Braak et al., 2003). *C*. Patients diagnosed with a synucleinopathy and concomitant RBD display aggregates of αSyn (dark brown) within the SubC region (adapted from Iranzo et al., 2013). *D*. Neuromelanin-sensitive imaging allows visualization of the SubC region in healthy subjects (white arrow pointing to white signal), which is depleted in *E*. patients diagnosed with RBD (adapted from Ehringer et al., 2016). Together, this indicates pathological changes within the SubC region due to αSyn aggregation underlies RBD symptoms that predict synucleinopathy diagnosis. μm: micrometers.
1.5 Animal models of RBD

The dorsal pontine tegmentum was first implicated as the hub for generating REM sleep atonia when cats displayed dream enacting behaviour during REM sleep following mechanical lesion encompassing the peri-locus coeruleus (a region homologous to the SubC)\(^6\). These animals experienced REM sleep without atonia, as well as overt “hallucinatory-like” behaviour during REM sleep that included limb movements and attempts to walk, side to side “searching” head movements, and leaping\(^6\). Similar REM sleep behaviours were reported following lesions of the sublaterodorsal nucleus (SLD; SubC homolog) of the rat\(^4\) with animals displaying increased phasic twitching, as well as complex behaviours (walking) during REM sleep\(^4\) analogous to humans with RBD. Finally, the ability to manipulate mice genetically has allowed for highly accurate cell-type specific lesioning. Transgenic mice with the VGLUT2 gene flanked by loxP (allowing gene silencing in the presence of Cre recombinase) had the SubC targeted with a virus to drive the expression of Cre-recombinase and inhibit SubC glutamatergic transmission\(^15\). These mice displayed both increased twitching behaviour and complex walking behaviours in REM sleep, supporting the hypothesis that glutamatergic neurons of the SubC regulate REM sleep atonia\(^15\).

Animal models have also displayed aspects of RBD when areas outside of the SubC region were targeted. Lesions encompassing the VMM in both cats and rats induces simple, jerking movements, however complex behaviours were not observed\(^20\). Similar behaviours were observed in mice that had GABA/glycine spinal interneurons silenced\(^15\), supporting the hypothesis that both SubC to VMM and SubC to spine pathways contribute to REM sleep muscle suppression. At the level of motor neurons, transgenic mice with altered GABA/glycine transmission display a phenotype similar to RBD, with heightened twitching and complex behaviours\(^31\), while total blockade of GABA and glycine signaling eliminated muscle atonia\(^23\).

Outside the REM atonia circuit, it was found that targeted dopaminergic lesions of the substantia nigra (mimicking PD) in marmosets elicited REM sleep bouts with heightened muscle tone, but neither increased phasic activity nor complex movements were observed\(^66\). Substantia nigra lesions in the rat, however, did not affect muscle activity during REM sleep\(^67\). Therefore, while midbrain and forebrain degeneration seen later in synucleinopathy may contribute to the
increased muscle activity seen in RBD, the SubC appears to be an evolutionarily conserved center for the control of REM sleep atonia, which when lesioned results in RBD symptoms.

While numerous animal models recreate the symptoms of RBD, they rely on lesioning that is not based on the αSyn-related degeneration seen in the majority of human RBD cases. Therefore, a valuable tool for understanding RBD development would be an animal model which reflects the natural development of the disease.

1.6 Animal models of synucleinopathy

Acute neurotoxin-induced lesions have been used to recreate the dopaminergic cell loss seen in synucleinopathy. Neurotoxins such as 6-hydroxydopamine and 1-methyl-4-phenyl-1,2,3,6-tetrahydro-pyridine (MPTP), which induce oxidative stress and rapid cell death of substantia nigra dopaminergic neurons, have been used to replicate the motor dysfunction of PD in multiple animal models. While these models can be useful in assessing treatments for the motor dysfunction seen in synucleinopathies, they lack the gradual onset and progression of disease symptoms, as well as the underlying pathology related to αSyn aggregation.

In addition, mutations and duplications of the human αSyn gene found in familial cases of synucleinopathy have been used in transgenic mouse models. Contrary to the neurotoxic models, these transgenic animals show aggregated αSyn pathology, however, cell loss is not usually seen, and regions are affected by aggregation that are normally spared in the natural disease progression.

The cell loss seen in the neurotoxic models, and the αSyn aggregation seen in transgenic models are both seen in animal models that use virally-driven targeted overexpression of αSyn. Adeno-associated viruses (AAVs) are able to package desired genes, which are then expressed episomally in transduced cells, and in this way have been used to drive expression of genes of interest in specific regions of the brain. This method has been used to successfully target midbrain dopaminergic and cortical regions to induce αSyn aggregation, cell loss and motor deficits in primate, rat and mouse models of PD and DLB. However, this method has not yet been used to target the brainstem nuclei that control muscle activity during REM sleep, which are compromised by αSyn aggregation in human RBD and later synucleinopathies. This
would allow unprecedented insight into the possible development and progression of RBD symptoms in a manner relevant to human disease progression.

1.7 Hypothesis

The SubC region of the pons is central to a circuit of brainstem nuclei that works to inhibit muscle activity during REM sleep. This inhibition is lost in RBD, where patients display heightened phasic twitches, loss of muscle atonia, and overt movements while in REM sleep. RBD patients are usually diagnosed with a synucleinopathy later in life, with RBD now considered an accurate biomarker for a developing neurodegenerative disease. Although RBD patients display αSyn aggregation within the SubC, the contribution of these aggregates to the development of RBD has not been tested. Furthermore, multiple animal models of RBD have been produced following manipulations of the SubC region, however a synucleinopathic model of RBD has not been described. The objective of this thesis is to investigate the role that pathological αSyn in the SubC region plays in the development of RBD symptoms.

I hypothesize that targeted over-expression of αSyn in the SubC region will elicit an RBD-like phenotype in wild type mice.

I used an AAV containing the unmuted human αSyn gene for targeted transduction of the SubC region in mice. I then used immunohistochemistry to evaluate αSyn expression and aggregation, followed by electrophysiological, and behavioural techniques to assess possible RBD-like symptoms.
Chapter 2 – Materials and Methods

2.1 Animals

The following experiments were performed using adult, male C57Bl/6 mice (n=13). All procedures were approved by the University of Toronto animal care committee. Animals were housed in transparent plastic, High-Efficiency Particulate Arrestance (HEPA)-filtered cages containing cob bedding, plastic shelter, and nesting/enrichment material (cotton). Cages and supplies were replaced with clean materials weekly. Both food (rodent diet 5001, LabDiet) and water were available at all times. Mice were kept on a 12:12 Light:dark cycle with the light period starting at 7:00a.m.

2.2 Stereotaxic Injection Surgeries

All surgeries were performed using sterile techniques while wearing appropriate PPE (i.e., gown, mask, bouffant, shoe covers, gloves). Mice were anesthetized with isoflurane (3%), had their weight recorded, and were prepared for surgery at a designated prep station. The scalp was shaved using electric trimmers and was wiped with gauze. The claws were trimmed with scissors to avoid future injury. Following preparation, the mice were transferred to the surgical table where they were head-fixed into a stereotaxic frame (model 902; David Kopf Instruments) and kept on an electric heating pad (TC-1000; CWE, INC, Andromore, PA). Mice were maintained on 1-2% isoflurane while breathing and rectal temperature was monitored closely throughout surgery. The eyes were covered in lubricating gel (Tears Naturale PM; Alcon) and the scalp was wiped with ethanol (70%) and betadine solution (10% povidone-iodine; Purdue Pharma) twice. When mice were deeply anesthetized (assessed via loss of toe pinch reflex), an incision was made down the middle of the scalp, the skin was parted, and the periosteum removed with a cotton swab dipped in hydrogen peroxide (3%). The head was leveled and holes were drilled into the skull above the injection site using a hand drill. A 28G cannula connected to a microinjection pump (Pump 11 Elite; Harvard Apparatus), was lowered into the SubC region (5mm posterior to bregma, ± 0.9mm lateral, and 4.27mm ventral).
To drive expression of αsyn, an AAV with serotype 2 containing the wild-type human αsyn gene under the chicken beta actin (CBA) promoter (AAV2-CBA-αsyn; 1.5x10^{13} vg/mg) was injected in the SubC region of experimental animals (n=7). Identical injections of a virus containing the green fluorescent protein (GFP) reporter gene in place of αsyn (AAV2-CBA-GFP; 8.1x10^{12} vg/mg) were given to a separate group of mice (n=6) as controls.

In total, 200nl of virus was injected either bilaterally into each SubC region (n=13) at a rate of 50nL per minute. Following injections, the cannula was allowed to remain in place for 4 minutes, and then slowly withdrawn. The animals were then given injections of ketoprofen (5mg/kg s.c.; Anafen; MERIAL Canada Inc.) in lactated ringers.

The scalp was sutured (#6-0, 3/8” tapered needle; Sofsilk) and cleaned with alcohol and betadine as previously mentioned. Topical anesthetic Emla cream (2.5% lidocaine and 2.5% prilocaine; AstraZeneca) and antibacterial polyderm (polymyxin B and bacitracin zinc) were then applied to the incision.

After surgery mice were transferred to an individual recovery cage and given both hydrating (hydrogel; Clear H2O) and high calorie (boost; Clear H2O) dietary gel, were monitored for pain and discomfort, and were administered subcutaneous 5mg/kg ketoprofen every 24 hours and over the next 48 hours.

2.3 EEG and EMG Instrumentation surgery

After a 5 week recovery period, mice were instrumented with EEG and EMG electrodes in order to identify and analyze of sleep/wake states. The masseter and neck extensor muscles were used to assess muscles activity in experimental and control animals across states. These muscles were chosen because their profile of activity has been well documented across sleep/wake states in rodents, with both masseter and neck muscles displaying the characteristic suppression of activity punctuated by twitches during REM sleep. Furthermore, in human RBD patients, heightened activity has been documented in a wide variety of muscles, including facial, head, trunk, and limb muscles, suggesting the heightened muscle activity associated with RBD symptoms are widespread throughout the skeletal muscles of the body. Therefore masseter and neck muscle activity can be used to give an accurate indication of RBD-like
changes in experimental animals, and has been used in past studies to assess RBD-like phenotypes following manipulation of the REM sleep circuit\textsuperscript{4,15,22,23,31}.

Mice were prepared for surgery, fixed into the stereotaxic frame and the skull exposed as described above. Electrodes consisted of stainless steel wire (AS 632; Cooner Wire) looped at one end, with the other end soldered to a micro-strip connector (CLP-105-02-L-D; Electrosonic). For EEG recordings, the loops of four electrodes were wrapped around stainless steel screws (P0090CE125; J.I. Morris) which were fastened into the center of the left and right frontal and the left and right parietal bones. A micro-strip connector was then affixed to the skull with dental cement (Ketac-cem and C&B Metabond Cement System;K-dental). For EMG recordings, the loops of two electrodes were sutured into the neck extensor muscles and two into the right masseter muscle. Incisions were closed and cleaned as described previously, and the mice given subcutaneous injections of 5mg/kg ketoprofen in lactated ringers.

After surgery mice were monitored and administered ketoprofen as previously described, and were given at least 2 weeks to recover before experiments began.

### 2.4 Recording Protocol

After the 2 week recovery period, mice were transferred to a plexiglass recording chamber with cob bedding, nesting material and unlimited access to food and water. Mice were allowed to habituate to the recording cage for at least 3 days prior to experimentation. In order to record EEG and EMG signals, the skull-fixed microstrip connector was tethered with light-weight cable, after which mice were allowed to habituate for a further 3 days.

Starting 8 weeks post-viral injection, EEG, EMG and video was recorded for 3 hours in the latter half of the light phase (14:00-17:00), where REM sleep tends to be most prevalent in mice\textsuperscript{83}. This time was chosen to increase the likelihood of observing possible RBD-like behaviours.

### 2.5 Data Acquisition

EEG and EMG signals were passed from electrodes and micro-strip connector through the cable tether to a Super-Z Head-Stage and a BMA-400 Bioamplifier (CWE Inc.), with signals
amplified by a factor of 500. All signals were digitized at 1000 Hz (Spike 2 Software, 1401 Interface, Cambridge Electronic Design Ltd.), had a DC offset applied with a time constant of 0.4 s. Both EEG and EMG signals were digitally filtered (EEG, 1-100 Hz; EMG, 30-1000 Hz), and EMG signals were rectified. Videos of the mice were captured simultaneously and were synchronized to EEG/EMG recordings, to identify any overt behaviours across states during recording.

2.6 Data Analysis

2.6.1 Scoring sleep/wake states

Combined EEG, EMG and video data were used to score behavioural states in 5s epochs using the Sleepscore v1.01 script with Spike 2 software (CED Inc.). Epochs were scored as 1 of 4 possible states. Active wake (AW) which was characterized by high frequency, low amplitude EEG signals along with high levels of EMG activity and overt movement (for example: eating, walking, grooming). Quiet wake (QW) which was characterized by EEG signals similar to AW, but in the absence of overt motor activity. NREM sleep which was defined by high amplitude, low frequency EEG signals combined with minimal EMG tone. REM sleep was distinguished by low amplitude, high frequency theta-like EEG activity with EMG showing atonia interspersed by periodic muscle twitches.

2.6.2 Analysis of sleep/wake architecture

Sleep/wake architecture was analyzed using the 5s epochs analysis script with Spike 2 software (CED Inc.) to output the scored epochs into a text document, combined with an Excel macro developed in the lab to compile the data output from the script to observe different aspects of sleep/wake architecture. By compiling the scored states, the script and macro calculated for each state the: number of episodes, percent time spent in each state, number of transitions between state, and average duration of each state.

2.6.3 EMG analysis

2.6.2.1 Analysis of overall muscle activity in REM sleep
Total average EMG activity for each state was quantified in 5s epochs. Raw EMG signals were full-wave rectified, integrated and quantified in arbitrary units (a.u.) and were compared between groups of animals for each state.

2.6.2.2 Analysis of muscle activity in REM sleep

EMG activity during REM sleep consists of both a suppression of tonic activity and bursts of phasic twitch activity, with both types of muscle activity which are increased in RBD\textsuperscript{32,38}. To determine the effect that virally driven αSyn in the SubC region had on both categories of REM sleep muscle activity, tonic and phasic EMG activity during REM sleep were isolated and analyzed separately. Using a spike 2 script developed in the lab, EMG activity during REM sleep was quantified in 10ms bins. Output from this script was entered into an Excel macro developed in the lab. This macro separated tonic and phasic EMG activity as previously described\textsuperscript{31}. Briefly, since phasic twitches are largely absent during the first 5s of REM sleep, this 5s period was used to calculate the 99th per centile of EMG activity (ie: the level of EMG activity below which 99% of 10ms epochs fall). This percentile was used as the threshold for tonic EMG activity, with above this level was designated as phasic activity. Phasic EMG activity was further analyzed for: amplitude, duration, frequency and number of twitches, which are excessive in RBD\textsuperscript{32,34}. Each animal had each episode of REM sleep analyzed individually for the average EMG properties of tone and twitches for that episode, which was then compiled together with all REM episodes from either the experimental or control group to analyze possible RBD-like changes in muscle activity following αSyn transduction. This method was chosen, rather than including only a single value for each individual animal (giving n=7 experimental and n=6 control animal vales for each EMG property analyzed) due to the infrequency of RBD episodes seen in many human cases. There is night-to-night variability in the presence of RBD symptoms, and while some RBD patients may show loss of atonia with overt movements several times per night, many patients only exhibit increased muscle activity during REM sleep on a very infrequent basis, ranging from nightly to monthly\textsuperscript{7,84}. Due to the possible infrequent nature of changes in muscle activity, effects could easily be masked if each animal was only considered as an average for all REM sleep episodes. Therefore we chose to include a value for every REM sleep episode within the experimental and control groups to increase the amount of observations and decrease the likelihood of overlooking infrequent changes in muscle activity. When analyzing the number or frequency of twitches, all REM episodes where considered (αSyn
n=136, control n=109), and when focusing on the neck muscle, one mouse was excluded due to signal noise in tat channel (for a total n=100). When analyzing the duration and amplitude of twitches, only periods of REM sleep with twitches present in the respective muscle were included (masseter, αSyn n=94, control n=60; neck, αSyn n=50, control n=43).

### 2.6.4 EEG analysis

Non-motor symptoms of RBD include slowing of cortical activity\(^{44}\). To assess the effect that SubC viral-αSyn had on cortical activity in the mice, EEG signals were analyzed using Spike 2 software (CED Inc.). Fast-Fourier transform was performed on EEG signals to generate a power spectra profile in 1.0Hz bins for each behavioural state. The EEG power for each frequency was then calculated as a percentage of total EEG power for each state.

### 2.7 Histology

Once behavioural experiments were complete, mice were anesthetized with injection of Avertin (0.025mL/g i.p.; 2,2,2-tribromoethanol and 2-methyl-2-butanol; Sigma-Aldrich), and level of anesthesia confirmed with the loss of foot withdrawal reflex. Mice were then immobilized, had the heart exposed, and were transcardially perfused with 0.1M phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA). Following perfusion, the brain was removed and stored in 4% PFA overnight. Brains were then transferred to 30% sucrose for 48 hours to protect tissue during freezing. Brains were immersed in Tissue-Tek OCT Compound (Electron Microscope Sciences) and snap-frozen on dry ice. Frozen brains were then sliced into coronal sections 40μm thick using a cryostat (CM3050 S; Leica).

#### 2.7.1 Immunohistochemistry

To assess the presence of virally transduced αSyn in the SubC region, slices were incubated with antibodies specific to human αSyn (1:1000, LB509 ab27766; Lot # GR281729-1; abcam) followed by biotinylated secondary antibodies (1:1000, BA-9200; Lot # Y0907; Vector Laboratories) and visualized by 3,3’-Diaminobenzidine (DAB) staining (SK-4100; Vector Laboratories). Identical staining was performed in control slices using a primary antibody against GFP (1:1000, MAB3580; Lot # 2615707; Millipore). To investigate whether viral αSyn is expressed in neurons, following DAB staining both experimental and control sections were incubated in primary antibody against the neuron-specific NeuN protein (1:1000, ab104225;
abcam). Sections were then incubated in biotinylated secondary antibodies and stained with Vector NovaRED peroxidase (SK-4800; Vector Laboratories). Stained sections were then mounted on glass slides, cover slipped with Permount (SP15; Fischer Scientific) and imaged using brightfield microscopy (AxioImager Z1; Zeiss). Expression was plotted on standardized coronal brain maps (Paxinos & Franklin, 2001).

2.7.2 Silver Staining

To investigate whether virally transduced αSyn caused neuronal damage in the SubC region, sections were stained with silver (FD Neurosilver Kit II; FD NeuroTechnologies Inc.), because neurons become argyrophilic as they degenerate\textsuperscript{85}. Coronal slices were stored in 4% PFA for at least 7 days then stained using the solutions provided by the kit (Solutions A-G). Stained sections were clarified with xylenes then mounted and imaged as described above, and staining quantified using ImageJ to calculate optical density.

2.8 Statistical Analysis

All statistical tests were performed using Prism 5.0 (GraphPad). A Kolmogorov-Smirnov test was used to determine whether data were normally distributed. For normally distributed data, an unpaired \( t \)-test was used to compare data between groups. For data not normally distributed, a non-parametric Mann-Whitney \( U \) test was performed. EEG power spectra was compared between groups using a non-repeated measures two-way ANOVA with Bonferroni post-test. All tests had a critical two-tailed alpha value of \( P<0.05 \) applied.
Chapter 3 - Results

3.1 Transduced αSyn expresses in the SubC region and aggregates within neurons.

The expression of AAV-driven human αSyn was confined to the SubC region. Using an antibody specific to human αSyn, AAV-mediated expression of human αSyn was confirmed in the SubC region (n=7) (Figure 5. A, B). To control for possible confounding behavioural effects related to SubC injection or to the expression of AAV-driven proteins in general, a second group of mice was injected with an AAV that drove GFP expression, which was also confined within the SubC region (n=6) (Figure 6. A, B).

A defining feature of synucleinopathies is the presence of aggregated αSyn within neurons of brain regions related to motor and cognitive function. Furthermore, RBD patients show similar αSyn aggregation in the SubC region. AAV driven human αSyn expresses within neurons (identified by the expression of the neuronal marker NeuN) of the SubC region as aggregated inclusion bodies (Figure 5. C). However, transduced GFP was found to express diffusely throughout SubC neurons (Figure 6. C).
Figure 5. **Virally driven human αSyn forms aggregates within SubC neurons.** A. Coronal maps (adapted from Paxinos & Franklin, 2001) of the mouse brain at the level of the SubC. Purple shading shows region with cells positive for expression of human αSyn, with colour corresponding to expression within individual animals. B. Histology showing SubC region with positive staining for human αSyn (black) and the neuronal marker NeuN (red). C. Individual neurons showing aggregates of αSyn (yellow arrows). wt: wild type. μm: micrometers
Figure 6. **Virally driven GFP expresses diffusely within SubC neurons.** A. Coronal maps (adapted from Paxinos & Franklin, 2001) of the mouse brain at the level of the SubC. Green shading shows region with cells positive for expression of GFP, with colour corresponding to expression in individual animals. B. Histology showing SubC region with positive staining for GFP (black) and the neuronal marker NeuN (red). C. Individual neurons show diffuse expression of GFP within SubC neurons. wt: wild type. µm: micrometers
3.2 Transduction of asyn in the SubC region increases phasic muscle twitches during REM sleep

Once human αSyn expression and synulceinopathy-like aggregation were confirmed within the SubC region, behavioural and electrophysiological recordings of mice were analyzed across the sleep-wake cycle to assess for possible RBD-like behaviours that might have arisen due to αSyn pathology.

While overt, complex movements were not seen in video recordings of any mice during periods of REM sleep, EMG recordings revealed heightened muscle activity in αSyn mice (Figure 7. A) compared to the normal twitch activity in control mice (Figure 7. B). Since RBD patients show both elevated tonic and phasic activity during REM sleep\textsuperscript{32}, phasic twitches (both amount and size) and basal tone were analyzed separately to determine how αSyn expression in the SubC affected REM sleep muscle activity.
Figure 7. Raw electrophysiological recordings display heightened phasic muscle activity (twitches) in αSyn mice during REM sleep. A. Example recording of REM sleep in mice transduced with αSyn displaying heightened muscle activity in the masseter (EMGₘ) and neck (EMGₙ) compared to B. recordings of mice transduced with GFP. Boxed regions correspond to close up traces displaying EEG with theta activity characteristic of REM sleep.
3.2.1 Masseter muscle: REM sleep twitches are exaggerated and happen more often in αSyn mice compared to controls

Mice transduced with αSyn showed a significantly higher amount of twitches in the masseter muscle during REM sleep compared to controls (αSyn median: 5.50 twitches per episode, n=136 episodes; control median: 1.00 twitches per episode, n=109 episodes; Mann-Whitney U test, U=5524, ***P<0.001) (Figure 8. A). The difference between αSyn and control populations was also seen when the frequency distribution of REM sleep episodes was plotted with respect to the number of twitches per episode with αSyn-expressing mice showing a shift toward more REM sleep episodes with a higher amounts of twitches (Figure 8. B). Twitches in the masseter muscle also occurred at a higher frequency compared to controls (αSyn median: 0.12 twitches per second, n=136 episodes; control median: 0.042 twitches per second, n=109 episodes; Mann-Whitney U test, U=5622, ***P<0.001) (Figure 8. C), which was also seen as a shift toward a higher percentage of REM sleep episodes having higher twitch frequency (Figure 8. D).

Along with αSyn mice having more masseter muscle twitches during REM sleep, these twitches were also found to be exaggerated compared to the twitches present in the masseters of control mice. These twitches in αSyn mice were found to have a 27% increase in duration (αSyn median: 0.070 seconds, n=94 episodes; control median: 0.055 seconds, n=60 episodes; Mann-Whitney U test, U=2116, **P<0.01) (Figure 9. A), with a clear difference between groups with the distribution of REM sleep episodes shifted toward longer twitch duration in αSyn mice (Figure 9. B). There was no significant difference in twitch amplitude between groups (αSyn median: 6.88 a.u., n=94 episodes; control median: 7.35 a.u., n=60 episodes; Mann-Whitney U test, U=2365, P=0.1530) (Figure 9. C), while αSyn mice show a wider distribution of REM sleep episodes with respect to twitch amplitude (Figure 9. D).
Figure 8. αSyn mice show an increased amount masseter muscle twitches during REM sleep. A. group data showing αSyn mice have a higher total number of twitches in the masseter per REM sleep episode (n=136) compared to controls (n=109). B. Frequency distribution of REM sleep episodes displaying a shift toward a higher percentage of REM sleep episodes with an increased number of twitches. C. αSyn mice also have a higher number of twitches per second in the masseter per REM sleep episode compared to controls. D. Frequency distribution of REM sleep displaying a shift toward episodes with higher twitches per second. Mann-Whitney U test. Boxplot whiskers show 1.5 IQR, black dots show outliers. s: seconds.
Figure 9. αSyn mice show larger masseter muscle twitches during REM sleep. A. Group data showing αSyn mice have longer individual twitches in the masseter per REM sleep episode (n=94) compared to controls (n=60). B. Frequency distribution of REM sleep episodes highlights the difference between the two populations, with αSyn mice showing a shift toward REM episodes with higher duration twitches. C. αSyn mice do not show a significant difference in the amplitude of twitches in the masseter compared to controls. D. Frequency distribution showing episodes of REM sleep in αSyn mice appear more varied in terms of twitch amplitude. Mann-Whitney U test. Boxplot whiskers show 1.5 IQR, black dots show outliers. s: seconds. a.u.: arbitrary units.

3.2.2 Neck muscle: REM sleep twitches have a higher amplitude in αSyn mice compared to controls

Despite the above findings in the masseter muscle, mice transduced with αSyn did not show a significant difference in the amount of neck muscle twitches during REM sleep compared
to controls ($\alpha$Syn median: 0.50 twitches per episode, $n=100$ episodes; control median: 0.00 twitches per episode, $n=109$ episodes; Mann-Whitney U test, $U=4739$, $P=0.073$) (Figure 10, A), or in the frequency of twitches ($\alpha$Syn median: 0.0040 twitches per second, $n=100$ episodes; control median: 0.00 twitches per second, $n=109$ episodes; Mann-Whitney U test, $U=4916$, $P=0.179$) (Figure 10, C). Also, the frequency distribution of REM sleep episodes with respect to number of twitches (Figure 10, B) and frequency of twitches (Figure 10, D), was very similar between the two groups of mice.

Unlike twitches in the masseter, $\alpha$Syn mice did not show a difference in the duration of twitches compared to controls ($\alpha$Syn median: 0.042 seconds, $n=50$ episodes; control median: 0.039 seconds, $n=43$ episodes; Mann-Whitney U test, $U=828$, $P=0.057$), although there was a trend toward longer duration twitches (Figure 11, A, B). There was however significantly larger amplitude neck muscle twitches in $\alpha$Syn mice compared to controls ($\alpha$Syn median: 3.63 a.u., $n=50$ episodes; control median: 3.23 a.u., $n=43$ episodes; Mann-Whitney U test, $U=820$, $P=0.049$) (Figure 11, C) and a clear difference between groups with a shift in the distribution of REM sleep episodes toward having larger amplitude twitches in $\alpha$Syn mice (Figure 11, D).
Figure 10. The amount of neck muscle twitches during REM sleep is unchanged in αSyn mice. A. Group data showing the amount of twitches in the neck per REM sleep episode (n=100) in αSyn mice is not significantly different from controls (n=109). B. Frequency distribution of REM sleep episodes with respect to number of twitches displaying similarity between αSyn and control mice. C. The number of twitches per second does not differ significantly between αSyn mice and controls, also seen by D. REM sleep episodes showing a similar distribution in terms of twitches per second. Mann-Whitney U test. Boxplot whiskers show 1.5 IQR, black dots show outliers. s: seconds.
3.3 The effect of virally driven αSyn in the SubC on muscle activity is specific to phasic twitches during REM sleep

Along with increased phasic muscle activity, a defining symptom of RBD is an increase in basal muscle tone during REM sleep\textsuperscript{32,33}. Furthermore, lesions in the SubC area results in a
loss of atonia in experimental animals. To investigate whether transduction with αSyn can result in a loss of atonia, tonic activity was analyzed in both the masseter and neck muscles for αSyn and control mice. I found that there was not a significant difference in the tonic activity of the masseter (αSyn median: 0.96 a.u., n=100 episodes; control median: 0.97 a.u., n=109 episodes; Mann-Whitney U test, U=7071, P=0.60) (Figure 12. A), or in the neck muscle (αSyn median: 0.88 a.u., n=100 episodes; control median: 0.83 a.u., n=109 episodes; Mann-Whitney U test, U=5206, P=0.66) (Figure 12. C) between the two groups.

In addition to increases in tonic and phasic muscle activity separately, RBD patients also show an increase in combined tonic and phasic activity (“all” muscle activity) during REM sleep. Total muscle activity was analyzed, but no significant difference was found in either masseter (αSyn median: 1.05 a.u., n=136 episodes; control median: 1.01 a.u., n=109 episodes; Mann-Whitney U test, U=6654, P=0.16) (Figure 12. B) or neck muscle activity (αSyn median: 0.91 a.u., n=100 episodes; control median: 0.89 a.u., n=109 episodes; Mann-Whitney U test, U=5271, P=0.69) (Figure 12. D).

While the defining characteristics of RBD occur during REM sleep, RBD patients also show occasional bursts of muscle activity in NREM sleep. To investigate possible effects on muscle activity outside of REM sleep, muscle activity in the masseter and neck was analyzed during NREM sleep and wake in αSyn and control mice. There was no significant difference between groups in the masseter muscle activity during wake (αSyn median: 2.01 a.u., n=136 episodes; control median: 1.87 a.u., n=109 episodes; Mann-Whitney U test, U=5710, P=0.88) (Figure 13. A) or during NREM sleep (αSyn median: 1.04 a.u., n=136 episodes; control median: 1.01 a.u., n=109 episodes; Mann-Whitney U test, U=5178, P=0.19) (Figure 13. B). Similar to the masseter muscle, there was no significant difference in neck muscle activity during either wake (αSyn median: 2.32 a.u., n=100 episodes; control median: 2.07 a.u., n=109 episodes; Mann-Whitney U test, U=5451, P=0.55) (Figure 13. C) or during NREM sleep (αSyn median: 0.96 a.u., n=136 episodes; control median: 0.97 a.u., n=109 episodes; Mann-Whitney U test, U=7071, P=0.60) (Figure 13. D).
Masseter

A

REM sleep basal tone (a.u.)

control \(\alpha\) Syn

P = 0.6018

B

All REM sleep EMG activity (a.u.)

control \(\alpha\) Syn

P = 0.1626

Neck

C

REM sleep basal tone (a.u.)

control \(\alpha\) Syn

P = 0.6629

D

All REM sleep EMG activity (a.u.)

control \(\alpha\) Syn

P = 0.6910
Figure 12. The effect of virally driven αSyn in the SubC on muscle activity is specific to phasic REM sleep. 
A. Group data showing basal tone of the masseter is unaffected during REM sleep episodes (n=136) in αSyn mice compared to controls (n=109). B. Also, “all” EMG activity (the total average EMG activity including both tonic and phasic activity) during REM sleep is not different between αSyn and control mice. C. Tonic activity in the neck during REM sleep episodes (n=100) of αSyn mice does not differ from tonic activity during REM sleep episodes (n=109) of control animals. Finally, D. “all” EMG activity in the neck during REM sleep is not different between αSyn and control mice. Mann-Whitney U test. Boxplot whiskers show 1.5 IQR, black dots show outliers. a.u.: arbitrary units.
3.4 Mice transduced with αSyn show a slowing of cortical activity across states

In addition to motor symptoms, most RBD patients also exhibit a slowing of cortical activity\textsuperscript{44}, and animal models of RBD have also displayed EEG slowing\textsuperscript{31}. To analyze the effect of virally driven αSyn in the SubC on cortical activity, the EEG signals of both αSyn and control mice were calculated in each state, as a percent of total power according to frequency (0-16Hz). Mice transduced with αSyn exhibited slowing of cortical activity across all states. In wake, αSyn mice showed a significant increase in total power in the low delta range (2Hz; αSyn average: 8.63\%, SEM: 0.44, n=7 mice; control average: 7.27\%, SEM: 0.26, n=6 mice; two-way ANOVA with Bonferroni post-test, **P<0.01) (Figure 14. A). Similarly, αSyn mice also showed a significant increase in power in the delta range during NREM sleep (2Hz; αSyn average: 9.06\%, SEM: 0.76, n=7 mice; control average: 7.59\%, SEM: 0.44, n=6 mice; two-way ANOVA with Bonferroni post-test, **P<0.01) (Figure 14. B). Finally, the biggest difference in EEG activity between groups was seen during REM sleep. Here, αSyn mice showed an increase in power in the delta/low theta range (2Hz; αSyn average: 5.87\%, SEM: 0.69, n=7 mice; control average: 3.71\%, SEM: 0.27, n=6 mice; two-way ANOVA with Bonferroni post-test, *P<0.05; 3Hz; αSyn average: 6.84\%, SEM: 0.76, n=7 mice; control average: 4.84\%, SEM: 0.47, n=6 mice; two-way ANOVA with Bonferroni post-test, *P<0.05; 5Hz; αSyn average: 7.88\%, SEM: 0.72, n=7 mice; control average: 5.63\%, SEM: 0.42, n=6 mice; two-way ANOVA with Bonferroni post-test, **P<0.01) as well as a decrease in the high theta/low alpha range (7Hz; αSyn average: 13.50\%, SEM: 1.11, n=7 mice; control average: 16.90\%, SEM: 0.83, n=6 mice; two-way ANOVA with Bonferroni post-test, ***P<0.001; 8Hz; αSyn average: 10.10\%, SEM: 0.86, n=7 mice; control average: 12.55\%, SEM: 0.48, n=6 mice; two-way ANOVA with Bonferroni post-test, **P<0.01).
Despite these changes, the overall EEG profile in each state retained the characteristic properties previously described, which allowed each state to be identified during scoring.
3.5 Mice transduced with αSyn show longer REM sleep episodes compared to controls

Despite the overt, often violent movements during REM sleep in RBD, the overall sleep/wake architecture of RBD patients is generally unaffected\textsuperscript{8,32,44,65}. To assess sleep architecture in αSyn and control mice, the duration, number, percent of time spent in, and the number of transitions between sleep wake states was analyzed. Surprisingly, unlike human RBD patients, αSyn mice showed a 58\% increase in the average duration of REM sleep episodes (αSyn average: 83.28 seconds, SEM: 5.60, n=7 mice; control average: 55.87 seconds, SEM: 7.05, n=6 mice; unpaired t-test, **P=0.0088), but without a significant difference in wake (αSyn average: 58.44 seconds, SEM: 5.87, n=7 mice; control average: 45.08 seconds, SEM: 4.01, n=6 mice; unpaired t-test, P=0.092) or NREM sleep (αSyn average: 47.92 seconds, SEM: 4.40, n=7 mice; control average: 39.68 seconds, SEM: 2.56, n=6 mice; unpaired t-test, P=0.14) episode length (Figure 15. A). However sleep/wake architecture was not different between groups in terms of the overall number of episodes of any state (wake: αSyn average: 100.88 episodes, SEM: 6.13, n=7 mice; control average: 113.57 episodes, SEM: 7.96, n=6 mice; unpaired t-test, P=0.22; NREM: αSyn average: 100.75 episodes, SEM: 6.20, n=7 mice; control average: 114.14 episodes, SEM: 7.96, n=6 mice; unpaired t-test, P=0.20; REM: αSyn average: 20.63 episodes, SEM: 2.81, n=7 mice; control average: 20.57, SEM: 3.56, n=6 mice; unpaired t-test, P=0.99) (Figure 15. B), the percent time spent in any state (wake: αSyn average: 47.20\%, SEM: 3.59, n=7 mice; control average: 47.41\%, SEM: 1.31, n=6 mice; unpaired t-test, P=0.96; NREM: αSyn average: 38.72\%, SEM: 2.92, n=7 mice; control average: 41.94\%, SEM: 1.31, n=6 mice; unpaired t-test, P=0.36; REM: αSyn average: 14.07\%, SEM: 1.76, n=7 mice; control average: 10.64\%, SEM: 0.71, n=6 mice; unpaired t-test, P=0.11) (Figure 15. C), or the transitions between states (wake to NREM: αSyn average: 100.36 transitions, SEM: 6.18, n=7 mice; control average: 113.42 transitions, SEM: 7.99, n=6 mice; unpaired t-test, P=0.21; NREM to wake:...
αSyn average: 79.75 transitions, SEM: 4.93, n=7 mice; control average: 92.71 transitions, SEM: 5.55, n=6 mice; unpaired t-test, P=0.10; NREM to REM: αSyn average: 20.50 transitions, SEM: 2.81, n=7 mice; control average: 20.57 transitions, SEM: 3.55, n=6 mice; unpaired t-test, P=0.99; REM to wake: αSyn average: 20.62 transitions, SEM: 2.80, n=7 mice; control average: 20.57 transitions, SEM: 3.55, n=6 mice; unpaired t-test, P=0.99 (Figure 15 D).
Chapter 4 - Discussion

Multiple lines of experimental evidence have pinpointed the pontine SubC region as being central to a brainstem circuit that suppresses muscle activity during REM sleep. Lesions of this region induce heightened muscle activity during REM sleep in multiple species (rats, cats and mice), resembling the symptoms of RBD in humans\(^4,5,15\). Additionally, RBD has been shown to be a sign of developing synucleinopathic neurodegenerative disease, with the majority of RBD cases preceding the diagnosis of synucleinopathy by years or decades\(^9,10,38,41\). There is a predictable caudal to rostral spreading of αSyn pathology in these diseases, with intra-neuronal αSyn aggregates present in the SubC region of patients diagnosed with synucleinopathy and RBD\(^9,50,63\). However, until now there has been no direct link made between αSyn aggregation in the SubC region and RBD symptoms, and there have been no αSyn-based models of RBD. The results presented in this thesis show - for the first time - that symptoms of RBD arise in wild type

Figure 15. αSyn mice show longer REM sleep episodes compared to controls. A. Mice transduced with αSyn (n=8) show significantly longer REM sleep episodes compared to control animals (n=7). There is no significant difference between groups in the duration of wake or NREM episodes. There is no difference between groups in B. the number of episodes of each state, C. the percent of time spent in each state or D. the number of transitions between states. Unpaired t-test. ns: not significant. W: wake. NR: non-REM sleep. R: REM sleep.
mice following transduction of human αSyn in the SubC region. The similarities between human RBD and mice transduced with αSyn are summarized in Table 1. This model provides important insight into the development of RBD symptoms, and could be clinically important for investigating methods of preventing the progression of αSyn-related disease.
Table 1. Mice transduced with αSyn display symptoms resembling human RBD

<table>
<thead>
<tr>
<th>Symptom</th>
<th>Human RBD</th>
<th>αSyn mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-neuronal aggregates of α-synuclein in the SubC region</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Overt behaviours during REM sleep</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Loss of muscle atonia</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Excessive amounts of phasic muscle twitches during REM sleep</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Larger phasic muscle twitches during REM sleep</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Slowing of cortical activity</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Increased duration of REM sleep episodes</td>
<td></td>
<td>✓</td>
</tr>
</tbody>
</table>

Table 1. Mice transduced with αSyn display symptoms resembling human RBD
4.1 Mice transduced with human αSyn in the SubC show an RBD phenotype

4.1.1 Transduced human αSyn forms intra-neuronal aggregates resembling an early form of those seen in human synucleinopathy

Formation of the dense intra-neuronal Lewy body aggregates that are the hallmark of synucleinopathies is a gradual process. Pathological αSyn aggregation begins as small granules within the cell body, which accumulate into large, loosely compact “pale bodies”, which then become densely compact, spherical, and highly ubiquitinated Lewy bodies classically associated with synucleinopathy. In our study, at 8 weeks post injection of AAV2-CBA-αSyn, SubC neurons show inclusions of human αSyn that resemble the early stage of Lewy body formation. The appearance of small intracellular aggregates was specific to virally driven αSyn, as transduced GFP appeared diffusely throughout cells. These aggregates resemble those previously describes at similar time points following transduction of dopaminergic neurons in rodent models of PD. Importantly, the small αSyn positive aggregates seen in our study also resemble those described by Braak et al. (2001) in the SubC and VMM of PD patients. Along with larger Lewy bodies, the lower brainstem nuclei of these patients displayed small granular aggregates of αSyn, which were specific to neuromelanin-containing neurons. Recent evidence has shown that compromised integrity of the neuromelenin positive SubC region is a marker for severity of RBD symptoms in humans. Together, this suggests neurons in the SubC region that are vital to normal muscle suppression during REM sleep appear to be especially conducive to fostering aggregates of αSyn, supporting the hypothesis that RBD symptoms are the result of a developing synucleinopathy. Furthermore, this validates our model as an important tool for gaining insight into RBD development, as the transduced αSyn mimics the early-stage aggregation seen in humans, allowing for the early progression of aggregate formation to be observed alongside the early progression of any RBD-related symptoms.

4.1.2 Following αSyn transduction, mice display physiological signs of RBD

Mice transduced with αSyn did not display a loss of atonia in masseter or neck muscles, nor were any overt movements observed in any of the mice during REM sleep. However, mice with virally driven αSyn in the SubC displayed consistent elevation of phasic twitch activity in both of the muscles measured during REM sleep. While a loss of muscle atonia during REM
sleep is observed in many cases of human RBD, it is not present in all of them, and the total absence of muscle atonia is not required for excessive phasic twitching to occur\textsuperscript{32,37}. Elevated twitch activity is seen consistently in RBD, including cases where elevated tone and overt movement are absent\textsuperscript{32,90}, and exaggerated twitches are an important factor in correctly identifying RBD\textsuperscript{34}. Therefore, an increase in REM sleep muscle twitches is a defining characteristic of human RBD, a characteristic that is reproduced in our mouse model.

Following αSyn transduction, mice displayed a significant increase in the amount, frequency and duration of muscle twitches in the masseter, higher amplitude twitches in the neck, and a shift toward an increased percentage of REM episodes that displayed high levels of twitch activity. The presence of heightened twitch activity with preserved atonia, and the differing effect on twitch activity between masseter and neck muscles would be expected in the early stages of gradual loss of REM sleep muscle suppression. In healthy REM sleep, motor neurons naturally receive bursts of excitatory signals that overcome active, atonia-causing inhibition, and cause twitches to occur\textsuperscript{15,26}. When inhibitory signals are partially removed via pharmacological blockade or genetic manipulation, excessive twitching emerges, while atonia remains\textsuperscript{32,31}, suggesting that in a gradual decline of muscle suppression, excessive twitches would appear before basal tone was elevated. Furthermore, different muscles naturally show different levels of twitch activity, and there is not a region of the brain universally responsible for twitching in all muscles, but rather evidence suggests specific brain regions incite twitches in specific muscles\textsuperscript{27,29}. Thus, in a gradual decline of REM sleep muscle suppression, excessive twitching would likely precede the loss of atonia, and would likely occur non-uniformly across different muscle groups. Indeed, many cases of human RBD are reportedly preceded by excessive twitching, that occurs for years before complex, dream-like behaviours arise\textsuperscript{32}. In this way, our results support the hypothesis that αSyn pathology in the SubC underlies the development of RBD symptoms, and our model appears to be important for observing the early stages of RBD.

In addition to abnormal motor behaviours, RBD patients also display a slowing of cortical activity\textsuperscript{44}. Cortical slowing is an important feature of RBD, as it is also seen in PD and DLB patients, where higher amounts of activity in the delta and theta range, along with decreased activity in the alpha range is observed compared to controls\textsuperscript{91,92}. Cortical slowing is clinically significant, because the degree of slowing is associated with cognitive decline and
dementia. Furthermore, RBD patients with higher degrees cortical slowing were found to be more likely to transition to diagnosed synucleinopathy after a 3.5 year follow up.

Similar to human RBD, in our study mice displayed a slowing of cortical activity across states, with a higher percentage of total power in delta and theta ranges compared to controls. While the cause of cortical slowing in synucleinopathy is not understood, there is greater slowing in DLB than in Alzheimer’s disease, which suggests structures that are affected specifically in synucleiopathy may contribute to these EEG abnormalities.

The SubC region has been shown to be vital for the generation of normal brain activity during REM sleep, and our results suggest that pathological αSyn in the SubC region could contribute to the EEG slowing seen in human RBD along with later synucleinopathy and related dementia. Therefore, mice transduced with human αSyn in the SubC region show early forms of synuclein aggregation, excessive muscle activity and cortical dysfunction that appear in humans developing a synucleinopathic disease. Furthermore, our model supports the hypothesis that early motor and cognitive symptoms of RBD arise from developing synucleinopathy in the brainstem.

4.2 Limitations

RBD patients show αSyn aggregates and degeneration in the SubC. While we observed αSyn inclusions within SubC neurons, it was not determined how the viability or activity of these neurons were affected following aggregation, and therefore the underlying cause of RBD symptoms following SubC αSyn transduction could not be inferred. As neurons degenerate, they more readily take up silver, and because of this quality, silver stains have been used extensively to identify neurodegeneration. In our study, we used silver staining in an attempt to identify whether changes in motor and cortical function could be explained by degeneration of SubC neurons. However, due to high levels of non-specific background staining in the majority of both control and αSyn tissue (Figure 16. A and B), along with similar amounts of silver-positive staining in control and αSyn tissue without universal background staining (Figure 16. C and D), our silver staining results were inconclusive, and degeneration of the SubC region could not be supported or ruled out as the source of RBD-like symptoms in the mice. Since silver staining can only identify degenerating neurons for a small window of time before cellular debris is removed, future experiments investigating the relationship between αSyn overexpression and
Figure 16. Silver staining results were inconclusive in determining amounts of αSyn mediated degeneration. High background silver-staining (black; marker for neurodegeneration) was present universally in the majority of A. control tissue and B. αSyn tissue tested. Furthermore, similar staining in the few C. control and D. αSyn tissue samples without high background meant the RBD-like symptoms that arose following transduction of αSyn in the SubC region being due to degeneration could not be supported or refuted in our study.
degeneration should observe tissue at a wider array of time points, and the silver staining procedure would need to be fine-tuned to decrease non-specific staining in the brainstem.

Another limitation of our study is that the virus was targeted directly to the SubC, and therefore the caudal medullary regions that show αSyn pathology preceding that seen in the SubC, were not affected. This could potentially explain several discrepancies between our model and human RBD. First, although the SubC has been pinpointed as a region necessary for the normal suppression of muscle activity\(^4,5\) the VMM is also involved\(^14\). Furthermore, the VMM also shows αSyn aggregation in RBD and PD\(^50,63\), and therefore pathology in this region could contribute to increased muscle activity in human RBD. The VMM being spared in our model could plausibly explain why some characteristic symptoms of RBD (ie: loss of atonia, presence of overt movements) are absent from our model. Second, while human RBD cases generally do not show changes in sleep architecture\(^32,38,64\), the mice transduced with αSyn in our study exhibited episodes of REM sleep that lasted significantly longer than controls, while other aspects of sleep architecture (duration of wake and NREM sleep episodes; the number, percent time spent in, and transition between all states) were unaffected. It has been demonstrated that one population of descending neurons within the SubC controls the suppression of muscle activity during REM sleep, while another ascending population promotes REM sleep itself\(^4,94\). The preservation of REM sleep architecture in human RBD suggests the REM sleep promoting region of the SubC is spared, while the atonia promoting population is compromised. In this way, the natural caudal to rostral spreading of αSyn pathology from medulla to pons to midbrain to cortex may progress through specific descending neurons of the SubC associated with muscle suppression. Interestingly, abnormal αSyn has been linked to increased neuronal firing and neurotransmitter release\(^95,96\), which could explain why the REM promoting activity of the SubC appears to be enhanced following transduction of αSyn. Therefore driving αSyn overexpression in indiscrete SubC neurons could account for the symptoms seen in our mice that are not present in humans.

### 4.3 Future directions

Despite the limitations described above, transduction of αSyn in the SubC of wild type mice resulted in the emergence of several early signs of RBD. Therefore this is a clinically-relevant model for studying the development of RBD that is based on underlying pathology seen
in the human disease and could be a valuable tool in future RBD research. To more accurately recreate the disease progression seen in humans, future studies utilizing this model could target other brain regions effected in synucleinopathy that control muscle activity in REM sleep, namely the VMM. By targeting a wider array of brain regions, this could allow for the development of a wider array of RBD symptoms to be studied. The ability to drive overexpression of αSyn in desired regions is a powerful tool that until now has not been described in RBD research, and could provide valuable insight into specific symptoms that arise following the appearance of αSyn aggregates in specific regions. On this note, future studies could also gain important information on the progression of RBD symptoms by examining this model at earlier and later time points than we observed in this study. Since the pathology and RBD-like symptoms we observed in our study appear to be similar to those described in early synucleinopathy and RBD (small αSyn aggregates, excessive twitching, and cortical slowing) and since αSyn aggregation and RBD symptoms worsen with time, observation of this model at later time points could uncover the development of RBD symptoms not observed at 8 weeks post-transduction (ie: loss of atonia, complex movements).

4.4 Conclusions

The results of this study support the original hypothesis that targeted over-expression of αSyn in the SubC region will elicit an RBD-like phenotype in wild type mice. Transduction of the mouse SubC with human αSyn caused aggregation of αSyn resembling an early stage in the formation of Lewy bodies. Following transduction, these mice showed heightened twitch activity in the masseter and neck muscles during REM sleep, mimicking the excessive twitch activity seen in human RBD. Furthermore, these mice displayed slowing of cortical activity, an early sign of declining cognition and progressing degenerative disease in human RBD and later synucleinopathy. In conclusion this thesis project has produced a number of findings that are important to RBD research:

1) This project has demonstrated for the first time a direct link between the emergence of RBD symptoms and pathological αSyn in the SubC region.

2) This project has produced an animal model of RBD that is clinically relevant to the underlying pathology seen in the human disease.
This model of RBD provides important insight into the development of RBD, displaying symptoms in mice that are described early on in humans as part of a disease that gets progressively worse with time. Therefore, this model could be extremely valuable for investigating preventative methods of halting the progression of αSyn-related pathology, and in turn halting the progression of early RBD symptoms into more devastating motor and cognitive dysfunction.
References


10. Postuma RB. Prodromal Parkinson’s disease--using REM sleep behavior disorder as a


