On the Generation of Rapid Eye Movement Sleep and the Reversal of Sleep-Related Hypotonia in Muscles of the Upper Airway

by

Kevin P. Grace

A thesis submitted in conformity with the requirements for the degree of Doctorate of Philosophy

Institute of Medical Science
University of Toronto

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Abstract

Rapid eye movement (REM) sleep - characterized by vivid dreaming, motor paralysis, and heightened neural activity - is one of the fundamental states of the mammalian central nervous system. One of the major consequences of REM sleep is an augmentation of the muscle hypotonia that occurs throughout sleep. Hypotonia in muscles of the upper airway is a precipitating cause of obstructive sleep apnea (OSA): a prevalent syndrome characterized by repeated episodes of upper airway narrowing and closure that occur only during sleep. The studies described in this thesis aimed to: (i) identify the role of cholinergic input to the pontine subcoeruleus (SubC) region in the generation of REM sleep, (ii) determine the involvement of the ventrolateral periaqueductal gray (vlPAG) and deep mesencephalic reticular nucleus (DpMe) in the bistability of sleep, and (iii) identify pharmacological targets for the reactivation of the upper airway musculature throughout sleep. The data reported here will show that contrary to the prevailing hypothesis of cholinergic involvement in REM sleep, cholinergic input to the SubC does not induce transitioning into REM sleep but rather reinforces transitions after their initiation, making them quicker and less likely to fail. In a separate study, evidence is presented
indicating that vlPAG/DpMe neurons, namely the REM sleep-inactive subpopulation, are not critical regulators of non-REM (NREM)-to-REM sleep transitioning. In contrast, the results of vlPAG/DpMe inactivation instead support a major involvement of REM sleep-active neurons in preventing breakdowns in sleep bistability specifically during REM sleep. Lastly, proof-of-principle is established that targeted blockade of certain potassium channels at the hypoglossal motor pool is an effective strategy for reversing upper airway hypotonia and causing sustained reactivation of upper airway muscles throughout NREM and REM sleep. These findings identify an important new direction for translational approaches to the pharmacological treatment of OSA.
I’d like to thank the longstanding and ongoing support from my supervisor, Dr. Richard Horner. Richard has been my mentor, my teacher, and my friend for nearly 10 years. Working with Richard I have come to believe that he genuinely prioritizes the success of his trainees above even his own professional advancement. The lengths that he will go to in order to promote his trainees go far beyond the traditional supervisor-student relationship. I’ll be forever grateful for and indebted to Richard for his role in creating a foundation for my scientific career.

I’d also like to thank the members of my doctoral advisory committee, Drs. John Peever and Richard Stephenson. Dr. Peever has been an influential mentor of mine, for many years, dating back to my Master’s work. Dr. Peever has always been one of my strongest advocates, promoting me and my work within the broader scientific community. I have always been able to depend on Dr. Stephenson for a careful and honest appraisal of my work and ideas. I respect and admire the level of intellectual rigor that he commits himself to and I’ve done my best to live up to that standard.
Contributions

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Chapter 1: Introduction

This chapter contains text segments and figures adapted from several published papers listed below. In the case of multi-authored papers, this chapter contains only those portions written solely by Kevin P. Grace.

Citations:


Grace KP, Hughes SW, Shahabi S, Horner RL. (2013) K+ Channel Modulation Causes Genioglossus Inhibition in REM Sleep and is a Strategy For Reactivation. Respiratory Physiology and Neurobiology. 188(3)

Citations (figures only):


Chapter 2: Aims & Hypotheses

This chapter was written solely by Kevin P. Grace for this dissertation.

Chapter 3: Endogenous Cholinergic Input to the Pontine REM Sleep Generator Reinforces, But Does Not Initiate, Transitioning into REM Sleep

This chapter presents research that was published by Kevin P. Grace, Lindsay E. Vanstone, and Richard L. Horner. Kevin P. Grace designed the study. Kevin P. Grace performed the research and analyzed the data with assistance from Lindsay E. Vanstone (under the supervision of Kevin P. Grace). Kevin P. Grace wrote the paper and Richard L. Horner participated in editing the manuscript.

Citation:


Chapter 4: REM Sleep-active vIvPAG/DpMe Neurons are Required to Maintain Sleep Bistability During REM sleep
This chapter presents research by Kevin P. Grace and Richard L. Horner that is being submitted to the Journal of Neuroscience. Kevin P. Grace designed the study, performed the research, analyzed the data, and wrote the paper. Richard L. Horner participated in editing the manuscript.

**Chapter 5: Identification of a Pharmacological Target for Genioglossus Reactivation Throughout Sleep**

This chapter presents research by Kevin P. Grace, Stuart W. Hughes and Richard L. Horner. Kevin P. Grace, Stuart W. Hughes and Richard L. Horner designed the study. Kevin P. Grace performed the research and analyzed the data. Kevin P. Grace and Richard L. Horner wrote the paper and Stuart W. Hughes participated in editing the manuscript.

*Citation:*


**Chapter 6: Additional Discussion and Future Directions**

Apart from section 6.2, this chapter was written solely by Kevin P. Grace for this dissertation. Section 6.2 contains text, written solely by Kevin P. Grace, which was published in collaboration with Andrew Wellman and Richard L. Horner.

*Citation:*

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Chapter 1
Introduction

1.1 Characterizing REM sleep as a unique state of the central nervous system

REM sleep, characterized by vivid dreaming, electroencephalographic activation and motor atonia, is a fundamental state of the mammalian central nervous system. Before REM sleep was discovered, sleep was assumed to be a homogenous brain state (Naquet, 2004). However, electrophysiological recording of activity in the sleeping brain revealed sleep’s dual nature (Aserinsky and Kleitman, 1953; Dement and Kleitman, 1957). Pioneering electroencephalography (EEG) studies showed that cortical activity in sleeping subjects is dominated by high amplitude, low frequency oscillations; however, beginning in the 1930’s, several groups reported that, during sleep, such slow-wave activity is periodically interrupted by periods of low amplitude fast-waves resembling waking EEG (Loomis et al., 1938; Rimbaud et al., 1955; Dement and Kleitman, 1957; Roth, 1964). The seminal characterization of this novel sleep stage and its physiological correlates were made by Aserinsky, Kleitman, and Dement in the 1950’s. They observed that periodic EEG activations during sleep occurred together with rapid, jerky, and binocularly symmetrical eye movements, which differed from the slow, rolling, and pendular eye movements that had been previously described in sleeping subjects. The authors also observed other phenomena typical of the state including surges in respiratory rate, heightened arousal threshold, and the occurrence of “fine movements” in the extremities (i.e., muscle twitching). Perhaps their most intriguing observation was the association between this collection of phenomena and dreaming. Subjects awoken from REM periods reported an increased incidence of highly visual and detailed dreams (Aserinsky and Kleitman, 1953).
The discovery of the REM stage of sleep presented a conceptual challenge to the field of sleep research. Whether this stage was a distinct sleep state, or whether all sleep stages stemmed from a common neural mechanism was still uncertain. The distinction between these two perspectives is nontrivial. REM sleep being a distinct state would mean that distinct neural circuitry is responsible for its generation, circuitry giving rise to unique physiology, pathophysiology and functionality. Aserinsky, Kleitman, and Dement described the REM and NREM sleep stages, not as distinct states but, as different “depths” of the same biological phenomenon. It was suggested that sleep cycling is a process by which the brain progresses through deeper and deeper stages of sleep followed by a “lightening” associated with the occurrence of EEG activation with rapid eye movements (Dement and Kleitman, 1957). The REM sleep stage was characterized as a “manifestation of a particular level of cortical activity which is encountered normally during sleep” (Aserinsky and Kleitman, 1953) — that level of cortical activity being one that is intermediate between wakefulness and NREM sleep. Moreover, they characterized the activated EEG associated with rapid eye movements as being equivalent to the electroencephalographic signature of Stage 1 sleep, which corresponded to the “drowsy” stage that had been previously described by Gibbs (1950). Speaking of the Stage 1 sleep that occurs at sleep onset, Dement and Kleitman commented that, “this Stage 1 EEG seemed to be identical with those occurring later in the night concomitant with actual eye movements” (Dement and Kleitman, 1957).

The suggestion that REM sleep represents one of the lightest stages of sleep conflicted with data showing that arousal threshold is highest during periods of rapid eye movement and EEG activation (Dement and Kleitman, 1957). Building on the descriptive work of Aserinsky, Kleitman, and Dement, a series of experiments and philosophical arguments (1958-1960) authored by Michel Jouvet is largely responsible for the current conception of REM sleep as a
brain state distinct from both NREM sleep and wakefulness (Jouvet, 1960; Jouvet et al., 1960). Jouvet reported that the occurrence of rapid cortical activity together with so-called “somato-vegetative phenomena” (i.e., the disappearance of all somatic muscle activity as well as variation in cardiac and respiratory rhythms) persisted following various supra-pontine transections in cats, but were eliminated following sectioning between the pons and trapezoid bodies (Jouvet and Michel, 1960b). Subsequent lesion experiments showed that destroying portions of the pontine tegmental field (PTF) (specifically the caudal pontine reticular formation and the posterior part of the oral pontine reticular formation) caused a selective loss of the REM sleep stage (Jouvet and Mounier, 1960). Moreover, the REM sleep stage could be selectively triggered by timed electrical stimulation of this same region. These findings were difficult to reconcile with the perspective of Kleitman and colleagues that REM sleep is an intermediary stage between wakefulness and NREM sleep produced by the combined action of wake and NREM sleep circuitry. These data indicated that the REM sleep stage was mechanistically unique because it depended upon the integrity of pontine control circuitry that was not involved in the generation of other sleep stages. Having identified a neuroanatomical locus for the REM sleep stage, Jouvet was able to argue that the phenomenological components of the REM sleep stage constituted a distinct state of the central nervous system (Jouvet, 1960; Jouvet and Michel, 1960a; Jouvet, 1962, 2004).

### 1.2 The distinct characteristics of REM sleep

#### 1.2.1 A physiological perspective

In contrast to NREM sleep, REM sleep is a state of homeostatic dysregulation (Phillipson and Bowes, 1986). For example, thermoregulation is strongly suppressed in REM sleep (Siegel,
Autonomic thermoregulatory responses to changes in ambient temperature are blunted (i.e., body temperature regulation becomes poikilothermic) stemming from reduced sensitivity in hypothalamic control circuitry (Parmeggiani, 2003). Cardiorespiratory control exhibits significant instability in REM sleep (Verrier and Harper, 2011). Concomitant with the phasic neural activations occurring in REM sleep, surges in sympathetic drive result in transient accelerations in heart rate and spikes in mean arterial blood pressure. Conversely, vagally-mediated decelerations in heart rate occur during tonic periods of REM sleep (Verrier and Harper, 2011). The phasic phenomena of REM sleep are also associated with increases in the rate and variability of breathing (Phillipson and Bowes, 1986). These changes in ventilation persist under hypoxic and hypercapnic conditions and are not the product of respiratory reflexes of the metabolic control system. In fact, the homeostatic metabolic control of breathing is often interrupted in REM sleep; during phasic REM sleep the ventilatory response to hypercapnia is strongly attenuated (Phillipson et al., 1977; Sullivan et al., 1979). Similar interruptions in metabolic ventilatory control occur in wakefulness in conjunction with behaviours such as phonation, which engage the behavioural respiratory control system. Therefore, metabolic homeostatic feedback to respiratory control centers may be overridden in REM sleep by activation of behavioural feed forward control mechanisms that prevent the achievement of respiratory steady states (Phillipson and Bowes, 1986). Indeed the inability to achieve steady-state conditions across multiple physiological systems is a general characteristic of REM sleep.

1.2.2 A systems neuroscience perspective

In contrast to NREM sleep, REM sleep and wakefulness are characterized by elevated levels of neural activity (Steriade et al., 2005), cerebral energy requirements (Maquet et al., 1990),
neuronal desynchronization and cortical effective connectivity (Massimini et al., 2010). Furthermore, both REM sleep and wakefulness are supportive of vivid conscious experience. Despite these similarities, the underlying functional neuroanatomy and connectivity that gives rise to the REM sleep state differs from wakefulness in many important ways. Functional neuroimaging of brain activity across sleep-wake states shows that resting wakefulness is associated with significant activation in the frontal and parietal cortices (Shulman et al., 1997), whereas REM sleep is associated with deactivation in those same regions (Maquet et al., 1996; Braun et al., 1997). During REM sleep, significant activation occurs in the mesopontine tegmentum, thalamic nuclei, limbic and paralimbic structures, as well as posterior cortical areas (Maquet et al., 1996; Braun et al., 1997; Maquet, 2000). REM sleep is associated with the loss of some functional relationships between brain regions operating in wakefulness and by the emergence of new functional connections. For instance, in a reversal of the functional relationship operating in wakefulness, activity in extrastriate cortex during REM sleep becomes negatively correlated with activity in regions at either end of the visual hierarchy mediating interactions with the environment (i.e., primary visual cortex and high level integrating centers in the frontal lobe) (Braun et al., 1998). Meanwhile, extrastriate and temporal cortical regions develop strong functional connectivity with the limbic system during REM sleep. From a systems neuroscience perspective, REM sleep can be viewed as an interoceptive (i.e., inwardly-oriented) processing state characterized by information flow in a closed loop between limbic centers and select posterior cortical areas that is ‘firewalled’ from sources of information about the external world (Braun et al., 1998).
1.3 A cholinergic mechanism of REM sleep generation

1.3.1 Seminal developments in our understanding of REM sleep generative circuitry

The recognition of REM sleep as an independent sleep state led to speculation regarding the possible mechanisms responsible for its generation. At the CIBA Foundation symposium on the nature of sleep in 1960, Michel Jouvet reported that the induction of REM sleep by electrical stimulation of the PTF was followed by a “refractory” period during which identical stimulations resulted in wakefulness accompanied by hypertonia and behavioural agitation. He remarked that the existence of this refractory period was suggestive of a “neurohumoral mechanism [of REM sleep generation] which would ‘discharge’ periodically during behavioural sleep but which could not be brought into play until a sufficient ‘stock’ of neurohormones was gathered”. He further speculated that this neurohumoral mechanism may importantly involve cholinergic neurotransmission (Jouvet, 1960). Stemming from Jouvet’s initial hypothesis, numerous studies have investigated the potential involvement of cholinergic neurotransmission in REM sleep generation; however, there is presently no consensus viewpoint. It is my position that a consensus viewpoint can be reached because the bulk of available evidence can be brought into agreement when appropriately interpreted and considered in light of findings presented in Chapter 3: “Endogenous Cholinergic input to the Pontine REM Sleep Generator Reinforces, But Does Not Initiate, Transitioning into REM Sleep”.

Speculation over cholinergic involvement in the mechanism of REM sleep generation stemmed from studies showing the REM sleep modulating effects of systemically administered cholinergic drugs (Jouvet and Michel, 1960a; Jouvet, 1962). In cats, systemic administration of the cholinergic receptor antagonist atropine strongly disrupts sleep and wakefulness.
Systemically administered atropine results in what was described as “dissociation between the behaviour of the animal and its electrical activity” (Jouvet, 1962). While the EEG of atropinized cats was marked by slow-waves and bursting activity similar to that observed during natural sleep and anesthesia, they were nevertheless awake. These animals had tonically elevated muscle activity, adopted standing and crouching postures, and remained responsive to auditory stimulation. However, auditory or noxious stimulation did not elicit cortical activation. Atropinized cats did not have REM sleep. Nevertheless, consistent with their being some capacity of cholinergic neurotransmission to promote REM sleep generation, increasing endogenous levels of acetylcholine, by systemic administration of an acetylcholinesterase inhibitor, increased REM sleep bout durations as well as the intensity of phasic REM phenomena (Jouvet and Michel, 1960; Jouvet, 1962).

1.3.2 Features of REM sleep induction by cholinergic agonism in the pontine tegmental field

1.3.2.1 Overview of seminal findings

The reported link between cholinergic neurotransmission and REM sleep generation gave rise to the hypothesis that cholinergic activation of the putative REM sleep generating circuitry in the PTF is the critical event leading to the initiation of the state. To-date the bulk of evidence cited in support of this hypothesis comes from studies showing that stimulating sites in the pons with cholinergic agonists is sufficient to induce REM sleep. The seminal cholinergic stimulation study by George et al. (1964) showed that microinjections of cholinomimetics — carbachol and oxotremorine — in cats, at PTF sites where lesioning eliminated REM sleep, produced extended REM sleep-like states. These states were characterized by atonia, electrocortical activation,
reflex inactivation, and rapid eye movements. Induced REM sleep persisted for 45-50 minutes and occurred at a short latency following microinjection (1-5 minutes). The latency to, and magnitude of, the induced REM sleep are important details because they suggest that the PTF REM sleep circuitry is structured such that cholinergic inputs are capable of overwhelming competing influences. From a systems dynamics perspective, cholinergic induction of REM sleep is an indicator of the controllability of sleep/wake circuitry in the cat. Controllability is a measure of the minimum number of nodes needed to drive a network from one dynamical state to another in a finite amount of time (Kumar et al., 2013). Therefore, when considering the brain as a dynamical system, cat studies suggest that cholinergic interactions with the PTF are capable of strongly driving network trajectory towards the state of REM sleep. This system controllability by cholinergic neurotransmission was the primary indicator that cholinergic neurotransmission might play a major role in REM sleep generation. Cholinergic PTF stimulation also induced dissociated states where motor atonia or a REM sleep-like EEG theta rhythm occurred apart from other REM sleep phenomena. The finding that dissociated states could be triggered by acetylcholine in addition to fully orchestrated REM sleep is significant for three reasons. Firstly, the induction of dissociated states suggests that the PTF is a source of multiple efferent pathways, originating from functionally distinct neuronal pools, which can independently control components of the REM sleep state. Secondly, the occurrence of fully orchestrated REM sleep (i.e., marked by the synchronized onset and offset of the full complement of REM sleep components) indicates that the PTF is also a likely source of controller/switching circuitry — responsible for coordinating the activation of REM sleep components, while preventing the intrusion of other behavioral states. Finally, these findings suggested that the phenomenological afferents and the executive controller are cholinceptive.
REM sleep induction by cholinergic PTF stimulation has been repeated by numerous other studies in both cats and rats (Cordeau et al., 1963; Kostowski, 1971; Amatruda et al., 1975; Van Dongen et al., 1978; Silberman et al., 1980; van Dongen, 1980; Hobson et al., 1983; Baghdoyan et al., 1984b; Baghdoyan et al., 1984a; Gnadt and Pegeram, 1986) (Shiromani and Fishbein, 1986; Shiromani and McGinty, 1986; Baghdoyan et al., 1987; Vanni-Mercier et al., 1989; Velazquez-Moctezuma et al., 1989; Yamamoto et al., 1990; Imeri et al., 1994; Mastrangelo et al., 1994; Reinoso-Suarez et al., 1994; Bourgin et al., 1995; Deurveilher et al., 1997; Garzon et al., 1998; Marks and Birabil, 1998). The experimental details of these studies are listed in Table 1. REM sleep can be elicited by microinjection of the mixed acetylcholine receptor agonist carbachol, selective muscarinic acetylcholine receptor agonists (e.g., oxotremorine and bethanecol), and acetylcholinesterase inhibitors (e.g., neostigmine). REM sleep effects exhibit dose dependency and can be blocked by co-application of cholinergic receptor antagonists (e.g., atropine). REM sleep-like states have also been induced in decerebrate and anesthetized animals (Kubin, 2001).

1.3.2.2 Neuroanatomy of REM sleep induction sites

Figure 1 shows the region of the PTF in which delivery of cholinomimetic drugs can induce REM sleep in cats (Fig. 1A) and rats (Fig. 1B). Table 1 provides more specific details about the locations of cholinomimetic drug delivery and the resulting effects on sleep and motor behaviour.

Initial studies generated disagreement regarding the location of the pontine site(s) in the cat where cholinergic stimulation generates REM sleep. Studies by Baghdoyan et al. (Baghdoyan et al., 1984b; Baghdoyan et al., 1984a) and Hobson et al. (1983) reported potent REM sleep
inducing effects — 40-50 minute episodes and 4-9 fold increases in REM sleep time — of cholinergic receptor agonism in the posterior portion the ventral PTF (roughly from the posterior boundary of the laterodorsal tegmental nucleus (LDT; P2 according to (Berman, 1968) to the bisection of the PTF by the facial nerve (P7) and ventral to the level of the trigeminal motor pool). However, lesioning studies have showed that unlike electrolytic ablation (that destroy neurons and axons of passage) of the ventral PTF, kainic acid (which destroy neurons only) lesions in these regions did not affect REM sleep or its component phenomena (Sastre et al., 1981; Drucker-Colin and Pedraza, 1983). These findings suggested that fibres passing through the ventral PTF are important for REM sleep generation rather than the neuronal perikarya. In contrast to posteroventral sites, REM sleep time is greatly reduced or eliminated by chemical lesioning of the anterodorsal pontine tegmentum (dorsal to the ventral extent of the trigeminal motor pool and anterior to P4) (Carli and Zanchetti, 1965; Webster and Jones, 1988; Lu et al., 2006b). While REM sleep is induced at both anterodorsal and posteroventral PTF sites, the former are associated with inducing REM sleep at the shortest latencies following drug microinjection (i.e., < 20 minutes) (Vanni-Mercier et al., 1989; Yamamoto et al., 1990). Due to drug diffusion following microinjection, longer latencies to REM sleep induction raise the possibility that the site of drug action is at a distance from the delivery site. Gnadt and Pegram (1986) showed that a relatively small volume of solution (0.1µl) containing titrated choline and
<table>
<thead>
<tr>
<th>Experiment type</th>
<th>Reference</th>
<th>Drug delivery</th>
<th>Drug</th>
<th>Species</th>
<th>PTF region</th>
<th>Sleep effects</th>
<th>Motor effects</th>
<th>Latency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Local gain-of-function</td>
<td>Cordeau et al.</td>
<td>Microinjection</td>
<td>Acetylcholine (20 μg)</td>
<td>Cat</td>
<td>mPnC</td>
<td>Injections in awake cats; induced NREM sleep and sleep attacks; injections in sleeping cats: no effect other than further EEG slowing; REM sleep (&quot;desynchronized&quot; EEG pattern, no movement, twitching) induced in 4 cats</td>
<td></td>
<td>1–5 min</td>
</tr>
<tr>
<td>GEORGES et al.</td>
<td>Microinjection</td>
<td>Carbachol (0.2–0.5 μg); oxotremorine (0.2–10 μg)</td>
<td>Cat</td>
<td>PrnO/C</td>
<td>Long bouts (45–50 min) of a REM sleep-like state (atonia, lost reflexes, &quot;low voltage fast&quot; EEG pattern, &quot;hyper-synchronous&quot; hippocampal activity); Mixed states: (i) atonia only, (ii) escalating to rhythm + sensory responsiveness</td>
<td>As the REM sleep state waned, a severe tremor emerged</td>
<td>1–5 min</td>
<td></td>
</tr>
<tr>
<td>Kostowski</td>
<td>Microinjection</td>
<td>ACh (5–15 μg); nicotine (5 μg); eserine (10 μg)</td>
<td>Cat</td>
<td>mPnO/C</td>
<td>Eserin and ACh: induced signs of sedation and sleep; nicotine: biphasic effect, excitation followed by sedation</td>
<td>Nicotine: stiffening of the tail + torsion of the head</td>
<td>3–6 min</td>
<td></td>
</tr>
<tr>
<td>Mitter and Dement</td>
<td>Microinjection</td>
<td>Carbachol (6 μg/μl)</td>
<td>Cat</td>
<td>Peri-LCo/dPNO</td>
<td>Induced wakefulness and arousal</td>
<td>Motor inhibition followed arousal (40%); flaccidity and areflexia lasting 20+ h</td>
<td>&lt;10 min</td>
<td></td>
</tr>
<tr>
<td>Aramdua et al.</td>
<td>Microinjection</td>
<td>Carbachol (3–9 μg)</td>
<td>Cat</td>
<td>PrnO/C</td>
<td>Persistent atonia with EEG desynchronization; REM sleep time (3.5–4.5 times (close dependent)</td>
<td></td>
<td>22.3 ± 25.9 min</td>
<td></td>
</tr>
<tr>
<td>van Dongen et al.</td>
<td>Microinjection</td>
<td>Carbachol (0.05–0.5 μg) + physostigmine (2–20 μg)</td>
<td>Cat</td>
<td>Peri-LCo/dPNO</td>
<td>Carbachol: episodes of motor atonia lasting 7–10 min following 60% of injections; effects blocked by atropine but not by mecamylamine; cats appeared awake at all times; Physostigmine: no effects</td>
<td>Asymmetric body fixon, ocirion, agression</td>
<td>&lt;1–32 min</td>
<td></td>
</tr>
<tr>
<td>van Dongen</td>
<td>Microinjection</td>
<td>Carbachol (0.05–0.5 μg)</td>
<td>Cat</td>
<td>Peri-LCo/dPNO</td>
<td>Motor atonia following 30% of injections; EEG not recorded</td>
<td>Asymmetric body fixon with/without ocirion (30%); agression (9%)</td>
<td>&lt;15 min</td>
<td></td>
</tr>
<tr>
<td>Silberman et al.</td>
<td>Microinjection</td>
<td>Carbachol (4 μg/0.25–1 μl)</td>
<td>Cat</td>
<td>PrnO/C</td>
<td>REM sleep time [2–12 x; length [2–21 x</td>
<td>Highly variable</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hobson et al.</td>
<td>Microinjection</td>
<td>Bethanechol (1.4–7 μg)</td>
<td>Cat</td>
<td>vPnC</td>
<td>Long bouts (40–50 min) of a REM sleep-like state (atonia, PGO waves, unresponsive, desynchronized EEG, reversible); REM sleep time [3–5 times (close dependent)); Mixed states: induced at lowest dose (1.4 μg), details not specified</td>
<td></td>
<td>&gt;25 min</td>
<td></td>
</tr>
<tr>
<td>Baghdoyan et al.</td>
<td>Microinjection</td>
<td>Nicotigmine (0.2–20 μg)</td>
<td>Cat</td>
<td>mPnO/C</td>
<td>REM sleep time [19 x (bout length and frequency 1); NREM sleep time [85%; effects were dose dependent and were blocked by atropine</td>
<td></td>
<td>18.5 ± 6.6 min</td>
<td></td>
</tr>
<tr>
<td>Baghdoyan et al.</td>
<td>Microinjection</td>
<td>Carbachol (4 μg/0.5 μl)</td>
<td>Cat</td>
<td>PrnO/C</td>
<td>REM sleep time [14 x (bout length and frequency 1); NREM sleep time [85%</td>
<td></td>
<td>~45 min</td>
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</tr>
<tr>
<td>Gnedt and Pálegam</td>
<td>Microinjection</td>
<td>Carbachol (0.1–5 μg)</td>
<td>Rat</td>
<td>PeriO/C</td>
<td>REM sleep 11.52 x only with caudal pontine injections of 0.5 and 1.0 μg doses; wake 11.5 x at the 0.5 μg dose while REM and NREM sleep were reduced</td>
<td>Asymmetric body fixon, tmsue tone, ocirion</td>
<td>39 min</td>
<td></td>
</tr>
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<td>Experiment type</td>
<td>Reference</td>
<td>Drug delivery</td>
<td>Drug</td>
<td>Species</td>
<td>PTF region</td>
<td>Sleep effects</td>
<td>Motor effects</td>
<td>Latency</td>
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<tr>
<td>Shiromani</td>
<td>Microinjection</td>
<td>Carbachol (6 µg/1 µl)</td>
<td>Cat</td>
<td>PrOC</td>
<td></td>
<td>Long bouts (11–47 min) of a REM sleep-like state (atonia, PGO waves, desynchronized EEG pattern); Mixed states: (i) atonia only (lasting – 50 min), (ii) escalating REM sleep with sensory responsiveness</td>
<td>6.1 ± 1.8 min</td>
<td></td>
</tr>
<tr>
<td>Baghdadyan et al.</td>
<td>Microinjection</td>
<td>ACh (5 µg); carbachol (4 µg, 0.25–0.5 µg)</td>
<td>Cat</td>
<td>PrOC</td>
<td></td>
<td>REM sleep time 13 x (bout frequency 1) (latencies), REM sleep time for rostral dorsal raphe relative to ventralcaudal injections</td>
<td>42 ± 33 min</td>
<td></td>
</tr>
<tr>
<td>Vanni-Mercier et al.</td>
<td>Microinjection</td>
<td>Carbachol (0.4 µg/0.2 µl)</td>
<td>Cat</td>
<td>PnL-Go, PrOC</td>
<td></td>
<td>Site-specific REM sleep effects (REM sleep enhancement or suppression, or no effect); para-LC injections were most effective; REM sleep time 13 x (bouts length 0.25–0.5 µg or bout frequency); NREM sleep time independent of site; Mixed states: (i) atonia, REMs, PGO waves, hippocampal REM sleep with sensory responsiveness; (ii) atonia and hippocampal REM sleep only with sensory responsiveness; (iii) hippocampal REM sleep persisting into NREM sleep</td>
<td>Peri-LCo: 5.5 ± 0.9 min</td>
<td></td>
</tr>
<tr>
<td>Velezquez-Močetzs et al.</td>
<td>Microinjection</td>
<td>Carbachol (3.8 µg/0.1 µl); McN-A-343 (1 µg/0.1 µl), oxotremorine (0.05 µg/0.1 µl)</td>
<td>Cat</td>
<td>unknown</td>
<td></td>
<td>Oxotremorine and carbachol: REM sleep time 13 and bout frequency, respectively (bouts length and frequency effects were not reported); McN-A-343: no response</td>
<td>Carb: 18.1 ± 6.4 min; Cx: NSD; Mth: NSD</td>
<td></td>
</tr>
<tr>
<td>Yamamoto et al.</td>
<td>Microinjection</td>
<td>Carbachol (4 µg/0.25 µl)</td>
<td>Cat</td>
<td>PnL-Go, PrOC</td>
<td></td>
<td>REM sleep time 10.5 x on average; effect latency and magnitude positively and negatively correlated, respectively, with the distance of injection sites from an oblique line running anterodorsally to posteroventrally</td>
<td>1–40 min</td>
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<tr>
<td>Reinoso-Suarez et al.</td>
<td>Microinjection</td>
<td>Carbachol (0.5–16 µg/0.02–0.03 µl)</td>
<td>Cat</td>
<td>PrOC</td>
<td></td>
<td>Dorsal sites: persistent wakefulness with periodic automatic atonia; REM sleep time (2.5–3 h x 4 h after injection); 5–40 min REM sleep episodes; Mixed states: atonia only; Ventral sites: REM sleep time (5 x); Mixed states: PGO activity with muscle tone and activated EEG</td>
<td>Dorsal: 2.2 ± 1 min; ventral: 4.7 ± 2.2 min</td>
<td></td>
</tr>
<tr>
<td>Lopez-Rodriguez et al.</td>
<td>Iontophoretic microinjection</td>
<td>ACh (2M); norepinephrine (2M) 200–500 nA current</td>
<td>Cat</td>
<td>PnL-Go, PrOC</td>
<td></td>
<td>Induced multiple states at identical sites depending on initial conditions; Injections during NREM = REM sleep (39% of cases), Wake (17%), N-Dis (12%), W-Dis (11%); no effect (22%); Injections during Wake = REM sleep (17% of cases), NREM (5%), N-Dis (17%), W-Dis (41%); no effect (20%); Mixed states: desynchronized EEG with atonia (W-Dis); synchronized EEG with PGO waves and muscle atonia (N-Dis)</td>
<td>4–6 min</td>
<td></td>
</tr>
<tr>
<td>Imeri et al.</td>
<td>Microinjection</td>
<td>Carbachol (0.5 µg/0.1 µl)</td>
<td>Rat</td>
<td>vPrC</td>
<td></td>
<td>REM sleep time 11.5 x; Wake and NREM sleep were reportedly unaffected local loss of function</td>
<td>NA</td>
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(Continued)
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<th>Reference</th>
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<th>Drug</th>
<th>Species</th>
<th>PTF region</th>
<th>Sleep effects</th>
<th>Motor effects</th>
<th>Latency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mastrangelo et al.</td>
<td>Microinjection</td>
<td>Carbachol (1 µg/0.5 µl)</td>
<td>Rat</td>
<td>unknown</td>
<td>No response in 25% of rats tested; Carbach induced 20-60 min of wakefulness following injection; REM sleep 1.4 x thereafter (bout frequency 1.3 x)</td>
<td>Circulating</td>
<td>60 min</td>
<td></td>
</tr>
<tr>
<td>Bourgin et al.</td>
<td>Microinjection</td>
<td>Carbachol (1–500 ng/50 nl)</td>
<td>Rat</td>
<td>PhO/C SubC</td>
<td>REM sleep time 1.2 x (bouf frequency 1.2 x); effects were dose dependent and were blocked by atropine; highest doses induced wakefulness</td>
<td>NSD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deveruegger et al.</td>
<td>Microinjection</td>
<td>Carbachol (0.005–3 µg/0.1 µl)</td>
<td>Rat</td>
<td>PhO/C SubC</td>
<td>No response (74%); carbachol wakefulness (2 x) (13%); often associated with motor disturbances; REM sleep 1.5–2 x (13%)</td>
<td>Asymmetric body flexion (8%), circling (3%), hyperactivity (1%)</td>
<td>NSD</td>
<td></td>
</tr>
<tr>
<td>Marks and Braili</td>
<td>Microinjection</td>
<td>Carbachol (1.1 mM/0.06 µl)</td>
<td>Rat</td>
<td>mPrO</td>
<td>REM sleep time 1.2 x and REM sleep bout frequency 1.2 x in 50% of injections; effects blocked by atropine</td>
<td>NSD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Garzon et al.</td>
<td>Microinjection</td>
<td>Carbachol (0.04–4 µg/0.02 µl)</td>
<td>Cat</td>
<td>vPrO</td>
<td>Induced alternating periods of wakefulness, REM sleep, and REM sleep-like states. REM sleep effects were not dose dependent. REM sleep 1.4–5 x (bout duration and frequency 1.5 x). NREM sleep was suppressed (40–100% reduction). Mixed states: desynchronized EEG, PGO waves, behavioral quiescence with muscle tone (at doses &gt;0.06 µg)</td>
<td>5–10 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Boissaud et al.</td>
<td>Iontophoretic microinjection</td>
<td>Carbachol (100 mM; 100–200 nl current)</td>
<td>Rat</td>
<td>SubCα</td>
<td>At sites where bicuculline/gabazine induced REM sleep carbachol induced a wake-like state with suppressed E, θ, and δ EEG power</td>
<td>[muscle tone]</td>
<td>&lt;0.5 min</td>
<td></td>
</tr>
<tr>
<td>Pollock and Meisberger</td>
<td>Microinjection</td>
<td>Neostigmine (8.8 µg/0.06 µl)</td>
<td>Mouse</td>
<td>PrO</td>
<td>Induced wakefulness and suppressed NREM and REM sleep for 3 h post injection; neostigmine induced state characterized by “very low-amplitude” EEG</td>
<td>Suppressed motor activity, occasional circling</td>
<td>Delayed by 3 h</td>
<td></td>
</tr>
<tr>
<td>Grace et al.</td>
<td>Microdialysis</td>
<td>Carbachol (tissue concentration – 1.8 µg/h)</td>
<td>Rat</td>
<td>PhO/C SubC</td>
<td>Persistent wakefulness/hyperarousal blocked by scopolamine (1 mM)</td>
<td>Asymmetric body flexion, circling, high muscle tone</td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>

Local loss-of-function

George et al. Microinjection Atropine (1 µg) – blocked the effects of carbachol | Cat | PrO/C | No “visible effects” reported (no data shown) | NA
| Kostowski Microinjection Atropine sulfate | Cat | mPrO/C | “Caused no constant behavioral effects” (no data shown) | NA
| Ghadiri and Pogani Microinjection Atropine (0.4 µg/0.1 µl) – blocked the effects of carbachol | Rat | PrO/C SubC | No effect on REM sleep time (baseline: 8.75%; total recording time vs. atropine: 10% total recording time) | NA
<p>| Imrani et al. Microinjection Pirenzepine (M1 antagonist; 1.6 µg/0.1 µl); methoctramine (M2 antagonist; 1–15 µg/0.1 µl; p-F-HHSD: M3 antagonist; 1.6 µg/0.2 µl) | Rat | vPrO | Methocuramine: [NREM sleep latency and wake time for three highest doses; REM sleep decreased as a % of total sleep time from ~16 to ~12% on average (across three highest doses); pirenzepine and p-F-HHSD: no effect | [2–6 x] Latency relative to first NREM episode | (Continued) |</p>
<table>
<thead>
<tr>
<th>Experiment type</th>
<th>Reference</th>
<th>Drug delivery</th>
<th>Drug</th>
<th>Species</th>
<th>PTF region</th>
<th>Sleep effects</th>
<th>Motor effects</th>
<th>Latency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bourgin et al.</td>
<td>Microinjection</td>
<td>Atropine (0.1–2 μg/0.1 μl) – blocked the effects of carbachol</td>
<td>Rat</td>
<td>PnO/C SubC</td>
<td>No effect on REM sleep time (no data shown)</td>
<td></td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Marks and</td>
<td>Microinjection</td>
<td>Atropine (4.3 mM/60 nl) – blocked the effects of carbachol</td>
<td>Rat</td>
<td>mPnO</td>
<td>No effect on REM sleep time (no data shown)</td>
<td></td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Gracce et al.</td>
<td>Microdialysis</td>
<td>Scopolamine (M1 antagonist); 1 mM (–) blocked the effects of (i) carbachol and (ii) selective activation of cholinergic PPT neurons by Urethran II</td>
<td>Rat</td>
<td>PnO/C SubC</td>
<td>No change in REM sleep time or bout frequency; NREM-to-REM sleep transitions: (i) duration increased by 25% and (ii) efficiency decreased by 30%; The increase in EEG α power in REM sleep relative to NREM sleep by 25%</td>
<td></td>
<td>NA</td>
</tr>
</tbody>
</table>
Table 1. The effects of modulating pontine cholinergic neurotransmission on REM sleep

A complete chronological listing of pharmacological studies testing the effects on REM sleep of focally delivered cholinomimetic drugs and/or cholinergic receptor antagonists into the pontine tegmental field. In the motor effects column, percentages listed with behaviors indicate the percentage of cases in which that behavior occurred. Anatomical designations correspond to labeled regions enclosed within the shaded areas in Figure 1. Anatomical abbreviations: Peri-LCa, peri-locus coeruleus alpha (cat); PnC, caudal part of the pontine reticular nucleus; PnO, oral part of the pontine reticular nucleus; PTF, pontine tegmental field; SubC, subcoeruleus nucleus including alpha, dorsal and ventral parts (rat). Prefixes: m, medial; d, dorsal; v, ventral.
Figure 1. PTF regions sensitive to cholinomimetic induction of REM sleep.

Coronal (left) and sagittal (right) maps of the cat (A) and rat (B) pons. The shaded regions are inclusive of all the effective REM sleep inducing injection sites from the studies listed in Table 1. Anatomical abbreviations: 5M, 5th motor nucleus; 7M, 7th motor nucleus; BC, brachium conjunctivum; DpMe, deep mesencephalic reticular nucleus; FTC, central tegmental field; FTG, gigantocellular tegmental field; FTM, magnocellular tegmental field; LC, locus coeruleus; LDT, laterodorsal tegmental nucleus; Me5, mesencephalic 5 nucleus; Peri-LCα, peri-locus coeruleus alpha; PnC, caudal part of the pontine reticular nucleus; PnO, oral part of the pontine reticular nucleus; PPT, pedunculopontine tegmental nucleus; PTF, pontine tegmental field; RM, raphe magnus; RPo, raphe pontis; scp, superior cerebellar peduncle; SubC, subcoeruleus nucleus including alpha (A), dorsal (D) and ventral parts (V); Tv, ventral tegmental nucleus of Gudden; Tz, nucleus of the trapezoid body. Anatomical maps were adapted from (Paxinos and Watson, 1998) and (Berman, 1968).
carbachol spread as much as 1-1.5mm over the course of an hour. Similarly, Myers and Hoch (1978) reported that a 0.5µl volume of radiolabeled dopamine spread 0.5-1.0mm after 15 minutes, while Macklis and Quattrochi (1991) found that 0.05µl of radiolabeled carbachol spread into an area with a 4-5mm diameter within 1 hour of the microinjection. Therefore, longer latencies to REM sleep induction following microinjection into posteroverentral PTF sites (injection volume range = 0.25-3 µl) may be the result of the time taken for drug to accumulate at in the anterodorsal PTF. However this interpretation has been challenged by Reinoso-Suárez et al., (1994). They found that carbachol microinjections within the dorsal PTF induced persistent waking states punctuated by periodic bouts of muscle atonia, consistent with the findings of Mitler (1974) and Van Dongen (1978). Only after several hours of such arousing effects were increases in REM sleep time observed. Moreover, they showed that carbachol microinjections in the ventral portion of the PTF strongly induced REM sleep, with latency to induction being less than 5 minutes on average. Garzon et al., (1998) also found that REM sleep could be induced at a short latency following ventral PTF microinjections of carbachol.

As in cats, REM enhancing sites in the rat extend along an anteroposterior axis from the posterior pole of the ventral tegmental nucleus of Gudden (Tv) to the root of the facial nerve (7n). There is no particular zone within this portion of the rat PTF where REM sleep can be reliably enhanced; responses to cholinergic stimulation range from no effect, to REM sleep enhancement, to arousal and motor impairment (for details on response variety, see the following sections and Table 1). However, despite the lack of reliability, REM sleep enhancing sites do tend to cluster in the subcoeruleus (SubC)/sublaterodorsal (SLD) portion of the rat PTF medial to the trigeminal motor pool (Deurveilher et al. 1997).
1.3.2.3 The differences between cat and rodent studies including wakefulness effects

There are notable differences in the features of cholinergic-induced REM sleep between cats and rodents. Unlike in cats, where REM sleep episodes can be triggered within minutes of drug microinjection, reported latencies to REM sleep induction in rats exceed 30 minutes (Table 1). As noted previously for cats, episodes of cholinergic-induced REM sleep can last as long as 50 minutes and REM sleep time increases to levels 3-9 times normal. By contrast, following PTF cholinergic stimulation in rats, REM sleep time increases to only 1.5-2 times normal levels and extended bouts of REM sleep do not occur (Table 1). Rather, increases in REM sleep time stem from increased bout frequency. Recall that the magnitude of REM sleep effects in cats — system controllability by cholinergic neurotransmission — was the primary indicator that cholinergic neurotransmission might play a major role in REM sleep generation. Consequently, the weaker effects of cholinergic stimulation observed in rats have led to alternative hypotheses suggesting that cholinergic neurotransmission in the PTF serves a modulatory, rather than a critical, role in REM sleep generation. Again, from a systems dynamics perspective, this implies that, in the rat, either cholinergic neurotransmission *per se* is less influential in terms of driving network state, or that the rat control circuitry for sleep is less controllable in general. Reduced controllability of the rat sleep control circuitry would imply that control network structure in the rat is more distributed as compared to the cat, such that a greater number of network nodes need to be simultaneously modulated in order to steer network trajectory towards REM sleep.

In rodents, the primary effect of cholinergic stimulation is often increased wakefulness rather than increased REM sleep. (Gnadt and Pegram, 1986; Bourgin et al., 1995; Deurveilher et al., 1997; Boissard et al., 2002). Pollock and Mistlberger (2005) reported that microinjection of
neostigmine into the PTF of mice induces extended periods of wakefulness characterized by elevated muscle tone, delaying REM sleep onset for up to 3 hours. Similarly, Boissard et al. (2002) showed that at dorsal PTF sites where gamma-Aminobutyric acid (GABA) receptor antagonism induced REM sleep, carbachol microinjection induced a waking state characterized by heightened muscle tone. Mastrangelo et al., (1994) showed that carbachol microinjections in the rat PTF induced extended periods of wakefulness lasting 20-80 minutes; thereafter, REM sleep time increased by 40%. In a large mapping study of the rat PTF, Deurveilher et al., (1997) showed that carbachol microinjections induced wakefulness as often as REM sleep (13% of cases); 74% of injections produced no effect. Persistent wakefulness following cholinergic stimulation of the PTF has also been reported in the cat. Baghdoyan et al., (1984a) found that carbachol microinjections at posterodorsal PTF sites induced wakefulness marked by ataxia, poor hind limb control, and aggressive behaviors. Mitler and Dement (1974) showed that carbachol microinjection into the peri-locus coeruleus region of the anterodorsal PTF induced a persistent waking state within 1-10 minutes of injection that could last upwards of 30 hours. In that study, motor atonia did appear but only in 6 of 15 injections after extended periods of wakefulness.

The arousing effects of cholinergic stimulation in the PTF are often accompanied by unusual motor activity. Reporting on the effects of carbachol microinjection into the PTF of the rat, Gnadt and Pegram (1986) noted that cholinergic stimulation sometimes produced “ipsilateral contraction of the axial musculature, particularly in the neck and thorax. In its mildest form, this appeared as an ipsiversive circling behaviour. In its most severe form, the contracture completely drew the rat into an incapacitating ipsilateral flexion of the body”. Similar results have been reported in cats by Van Dongen et al., (1980). Reports of increased wakefulness following
delivery of cholinomimetics into the PTF are often associated with such motor abnormalities. In the mapping study by Deurveilher et al. (1997), induced wakefulness was accompanied by asymmetric body flexion or hyperactive circling in 90% of cases. The association between motor effects and arousal raises the possibility that cholinomimetic-induced arousal is an epiphenomenon of motor disruption. Therefore, particularly in rats, the activation of motor circuitry and the resulting state of arousal may conceal what capacity exists for cholinergic stimulation to induce REM sleep in the PTF. It is important to acknowledge that cholinoceptive REM sleep-related neurons in the pons are intermingled amongst cholinoceptive locomotion-related reticulospinal neurons that provide input to spinal central pattern generators (Lai and Siegel, 1991). Experimental stimulation of these reticulospinal cells and the mesencephalic locomotor region that drives their activity produces similar patterns of muscle contraction and behaviour as those noted above (Depoortere et al., 1990).

1.3.2.4 State dissociation
In addition to the study by George et al., (1964) several additional studies in cats have reported state dissociation following cholinergic stimulation of the PTF. Cholinergic stimulation produced dissociated REM sleep states at sites scattered throughout the PTF; however, state dissociation was associated most frequently with microinjections in the peri-locus coeruleus region of the anterodorsal PTF (i.e., the same region associated with cholinomimetic-induced motor abnormalities and arousal). Vanni-Mercier et al. (1989) reported that carbachol microinjection at points near the peri-locus coeruleus alpha in cats produced several distinct REM sleep-like states. In some cases, cats entered a state very similar to normal REM sleep; however, hippocampal theta activity was more reminiscent of wakefulness, being described as medium amplitude and
lower frequency (i.e., 3-5 Hz). This state contrasted with periods where carbachol induced a REM sleep-like hippocampal theta rhythm that persisted into NREM sleep. In other cases, cats entered a state characterized by the occurrence of atonia, EEG desynchronization, pontogeniculo-occipital waves, and rapid eye movements typical of normal REM sleep; however, the animals were otherwise alert, able to track visual stimuli, and orient towards low intensity auditory stimuli. The most common form of state dissociation reported in cats following cholinergic PTF stimulation is persistent motor atonia — occurring in 80% of studies reporting state dissociation.

1.3.3 Interpreting gain-of function stimulation studies

The induction of REM sleep by cholinergic stimulation of the PTF is often regarded as indirect evidence supporting the involvement of endogenous PTF cholinergic neurotransmission in REM sleep generation. Many investigators interpret the results of gain-of-function cholinergic stimulation studies as supporting the same conclusions as loss-of-function experiments, just to a lesser degree. I will argue that this should be considered a misinterpretation of gain-of-function stimulation studies. I submit that, in principle, the results of cholinergic stimulation studies do not necessarily support or oppose any measure of endogenous cholinergic involvement in REM sleep generation.

Cholinergic stimulation of the PTF tests whether or not the endogenous cholinergic inputs to this region have a capacity, under the conditions of the experiment, to generate REM sleep or its component parts. In spite of the variable and species-dependent nature of cholinergic REM sleep enhancement, the capacity of endogenous cholinergic inputs to the PTF to generate REM sleep
has been established. However, we should not infer the function of cholinergic PTF afferents in REM sleep generation from their demonstrated capacity (Grace, 2015). The function, if any, of PTF cholinergic afferents in REM sleep generation is a product of the REM sleep control network’s response to their input. Gain-of-function stimulation studies make a *ceteris paribus* (i.e., all other things being equal) assumption with regards to the initial conditions present in the surrounding network. In other words, even though the initial state of the surrounding network is other than that normally associated with the regular activity of the node, this approach assumes that the natural function of the node can nevertheless be approximated by its stimulation. In principle, we should expect that the functional contribution of any group of neurons, to the dynamics of the network in which it is embedded, to be dependent on the timing and the magnitude of spiking within the group. However, in the specific case of cholinergic PTF stimulation studies, their results provide support for their own inappropriateness in determining the endogenous function of cholinergic PTF input. The variety of responses to cholinergic stimulation of the PTF illustrate the functional dependency of cholinergic input on initial conditions in the surrounding network (i.e., show the *ceteris paribus* assumption to be invalid). For instance, this dependency is illustrated by the refractory period following carbachol-induced REM sleep. During the refractory period an additional bolus of carbachol induces a waking state marked by high muscle tone rather than REM sleep (George et al., 1964). Also, optogenetic stimulation of PTF cholinergic afferents in the LDT and pedunculopontine tegmental nucleus (PPT) is only effective at enhancing REM sleep when stimulation occurs in NREM sleep — waking and REM sleep stimulations have no effect (Van Dort and Brown, 2015; Van Dort et al., 2015). Similar results were obtained by Lopez-Rodriguez et al., (1994) who showed that direct cholinergic stimulation in the PTF most often induced REM sleep when microinjections were
made during NREM sleep. Subsequent microinjections at the same locations during wakefulness preferentially induced a dissociated state characterized by motor atonia and sensory awareness. The importance of stimulus intensity is illustrated by the fact that, depending on the dose applied, cholinergic input to the PTF can have no effect, induce REM sleep, induce wakefulness, or produce dissociated states.

The effects of cholinomimetic stimulation of the PTF will approximate the function of endogenous cholinergic inputs only in the case that those inputs are a major source of feed-forward inductive REM sleep drive to the PTF. However, a priori, it is equally possible that cholinergic inputs are only minor sources of inductive drive relative to other parallel inductive inputs. It is also possible that cholinergic PTF input is non-inductive, meaning that the activation of REM sleep generating circuits in the PTF may precede the activation of their cholinergic afferents. In either of these alternative cases (i.e., where cholinergic PTF input is minor or non-inductive), stimulation of the PTF with cholinomimetic drugs could still have a major capacity to induce REM sleep despite endogenous cholinergic input to the PTF being potentially insignificant in the natural initiation of the state.

Determining the function of PTF cholinergic inputs in REM sleep generation requires loss-of-function experiments that remove or block this afferent input. Loss-of-function experiments support a qualitatively different conception of functional attribution in neural networks. The results of inactivating a network node can be taken to reflect the withdrawal, not of the node’s activity per se, but of the interaction between the node’s activity and the endogenous initial conditions in the surrounding network, which may or may not have given rise to that activity in the first place. In network analysis terms, inactivation studies reveal the embeddedness of a functional node, which is a measure of the impact of the node’s spiking activity on the activity in
the surrounding network (Kumar et al., 2013). Again, gain-of-function studies assume that we can effectively understand complex networks by de-emphasizing the importance of endogenous initial conditions and attributing functional significance to the nodes themselves rather than their dynamic interactions. This approach, relative to loss-of-function studies and the measures of nodal embeddedness that they produce, provide little basis for deriving higher order network logic, which can be considered the ultimate goal of interrogating circuit functionality. Addressing the problem of deriving higher order circuit logic from reductionist experimental data is further discussed in Chapter 5: “Additional Discussion and Future Directions”.

1.3.3.1 Epistemological case study: optogenetics and the involvement of MCH containing neurons in REM sleep generation

There is an emerging consensus that melanin concentrating hormone (MCH) containing neurons in the hypothalamus play a role in the generation and maintenance of REM sleep. Studies reporting REM sleep enhancement following optogenetic stimulation of MCH neurons are cited in support of this claim. Konadhode et al. (2013) have shown that optogenetic stimulation of MCH containing neurons shortens sleep latency and increases time spent in both NREM and REM sleep at the expense of wakefulness. A similar study by Jego et al. (2013) showed that optogenetic activation of MCH neurons during NREM sleep facilitated NREM-to-REM sleep transitions, while activation at the onset of REM sleep bouts increased their duration. Likewise, Tsunematsu et al. (2014) showed that optogenetic activation of MCH neurons induced transitions into REM sleep and increased REM sleep time at the expense of time spent in NREM sleep. Many in the field have interpreted these findings as implicating hypothalamic MCH neurons in
the mechanism of REM sleep generation (Fraigne and Peever, 2013; Jego et al., 2013; Jones and Hassani, 2013; Konadhode et al., 2013; Luppi et al., 2013; Tsunematsu et al., 2014).

I argue that while these data demonstrate the capacity of MCH containing neurons to generate and maintain the state of REM sleep, they do not necessarily implicate MCH neurons in this function. More importantly, the role of MCH containing neurons in REM sleep generation ought to be inferred from loss-of-function experiments rather than gain-of-function experiments. However, neither optogenetic silencing (Jego et al., 2013) nor targeted ablation (Tsunematsu et al., 2014) of MCH neurons has any effect on the generation or maintenance of REM sleep. These loss-of-function silencing experiments demonstrate that MCH neurons do not play a major role in the induction or maintenance of REM sleep, in spite of the REM sleep enhancing effects of MCH neuron stimulation.

It may be argued that, taken together, these data show that MCH-containing neurons are a sufficient, but not a necessary, cause of REM sleep. I would argue that this framing of function, into sufficient and necessary components, has limited usefulness towards modelling the logic of neural circuitry. Again, it implies that functional significance can be meaningfully attributed to MCH-containing neurons per se, irrespective of their interactions with the surrounding network. Gain-of function interventions that are used to test sufficiency necessarily vary the timing and/or the magnitude of a node’s activity, thereby inducing an abnormal set of network interactions relative to the endogenous network interactions that are the target of loss-of-function interventions, which test necessity. Therefore, generally speaking, as soon as we acknowledge that the functional contribution of a neuronal group to network dynamics cannot be divorced from its interactions with the surrounding network, we are forced to acknowledge that the terms necessity and sufficiency rarely apply to the same causal factors (e.g., the above conclusion, that
MCH-containing neurons are a sufficient but not a necessary cause of REM sleep, becomes: 

*MCH-containing neurons activity at time (1) and level (A) is sufficient for REM sleep generation while MCH-containing neurons active at time (2) and level (B) are not necessary for REM sleep generation.*

### 1.3.4 Blockade of cholinergic input to the putative REM sleep generator

Identifying the function of endogenous cholinergic inputs to the PTF in REM sleep generation requires the removal or blockade of cholinergic inputs. A small number of studies have examined the effects of antagonizing cholinergic receptors in the PTF. In the seminal study of cholinomimetic-induced REM sleep, George et al. (1964) anecdotally reported that, in two cats, atropine microinjections into the PTF had no “visible effects”. Similarly, Gnadt and Pegram (1986) microinjected atropine in five rats and reported that it had no effect on REM sleep time (no data provided). Bourgin et al. (1995) reported that in three rats, microinjection of atropine alone did not affect REM sleep time at the same sites where carbachol induced REM sleep (no data provided). In contrast, two studies have reported decreases in REM sleep following pharmacological blockade of cholinergic receptors in the pons. Firstly, Shiromani and Fishbein (1986) reported reductions of REM sleep in rats following pontine infusions of the muscarinic acetylcholine receptor antagonist scopolamine. However, infusions took place continuously over five days using a chronically implanted osmotic mini-pump. Over such an extended period of time, the infused scopolamine would have spread far beyond the target site located in the posteromedial PTF. Similar reductions in REM sleep occurred with medullary and ventricular infusions. In a second study, Imeri et al. (1994) reported increases in REM sleep latency in rats
following pontine microinjections of the muscarinic type-2 receptor antagonist methoctramine. Methoctramine microinjection resulted in a dose dependent increase in the latency to REM sleep. Total REM sleep time was also reduced following methoctramine microinjections. However, the reduced REM sleep time and the increased REM sleep latencies may have been a secondary effect of arousal, because NREM sleep time was also reduced while wake time increased, particularly at the highest doses. This study represents the strongest evidence available that cholinergic input to the PTF is involved in generating REM sleep. Nevertheless, taken together, these data neither confirm nor refute the involvement of pontine cholinergic neurotransmission in the generation of REM sleep.

1.3.5 Cholinergic afferents of the Pontine Tegmental Field

1.3.5.1 Non-selective modulation of the PPT and LDT

The claim that cholinergic neurotransmission in the PTF plays a major role in REM sleep generation entails that PTF cholinergic afferents located in the PPT and LDT are principal sources of the inductive drive that initiates REM sleep. PPT and LDT cell groups contain subpopulations of neurons that increase their activity in anticipation of and during REM sleep (i.e., REM sleep-active) (el Mansari et al., 1989; Thakkar et al., 1998; Datta and Siwek, 2002). The activation of cholinergic REM sleep-active PPT and LDT neurons is likely responsible for the increase in endogenous acetylcholine release in the PTF during REM sleep (Leonard and Lydic, 1997). Electrical or pharmacological stimulation of PPT and LDT neurons has been shown to increase REM sleep (Thakkar et al., 1996; Datta and Siwek, 1997; Datta, 2002; Datta and Siwek, 2002), induce acetylcholine release in the PTF (Lydic and Baghdoyan, 1993), and evoke scopolamine-sensitive excitatory postsynaptic potentials in PTF neurons (Imon et al.,
1996). These data further support the claim that cholinergic PTF afferents have a capacity to generate REM sleep.

Determining the function of cholinergic PPT and LDT neurons in REM sleep generation requires loss-of-function experiments. Reductions in REM sleep have been reported following electrolytic or chemical lesioning of the PPT and the LDT in cats, consistent with their causal involvement in REM sleep generation (Webster and Jones, 1988; Shouse and Siegel, 1992). However, in these studies lesions were not restricted to cholinergic cell areas and are therefore difficult to interpret. Lesions included other important components of the REM sleep generating circuitry including the deep mesencephalic reticular nucleus (DpMe), the ventrolateral periaqueductal gray (vlPAG), and the anterodorsal PTF including the peri-locus coeruleus region.

The effects of smaller, more selective chemical lesions of the PPT and LDT in rats (Lu et al., 2006) indicate that neither of these cell groups is needed to generate REM sleep. Lu and colleagues reported that LDT lesions did not affect REM sleep *per se*, but did increase the number of total state transitions per hour. Surprisingly, PPT lesions increased, rather than decreased, REM sleep time. This finding raises the possibility that some PPT subpopulations may function to suppress, rather than promote, REM sleep. An inhibitory influence on REM sleep by PPT neurons is further supported by the finding that pharmacological inactivation of the PPT, by GABA_A receptor agonism, increases REM sleep both as a percentage of total recording and total sleep time — i.e. independent of concurrent changes in wakefulness (Torterolo et al., 2002; Pal and Mallick, 2004, 2009). By contrast, Petrovic et al., (2014) reported no change in REM sleep amounts following bilateral PPT lesions; however, PPT lesions resulted in an increased number NREM-to-REM sleep and wake-to-REM sleep transitions. Moreover, REM
sleep split into two electroencephalographically distinct states: “sigma coherent” and “theta coherent” REM sleep.

The lack of consistency between PPT/LDT modulating studies notwithstanding, the REM sleep effects of manipulating the PPT or LDT en masse should not be taken to reflect the function of the REM sleep-active cholinergic neurons in these regions. Cholinergic REM sleep-active PPT/LDT neurons are vastly outnumbered by other cell types (Maloney et al., 1999; Verret et al., 2005; Boucetta et al., 2014). The composition of the LDT and PPT cell groups is highly heterogeneous. While the boundaries of the PPT and LDT are defined by their cholinergic subpopulations, 74-81% of PPT/LDT neurons are GABAergic or glutamatergic (Wang and Morales, 2009). Furthermore, immunostaining for c-Fos following REM sleep deprivation and recovery has revealed that only a small proportion (5-15%) of PPT/LDT neurons exhibiting heightened activity in REM sleep are cholinergic (Maloney et al., 1999; Verret et al., 2005). Much larger proportions (50-85%) are GABAergic (Maloney et al., 1999; Torterolo et al., 2001; Sapin et al., 2009). Moreover, Clement et al., (2011) have shown that more than 50% of LDT neurons found to be c-Fos positive after REM sleep recovery are glutamatergic.

REM sleep-active neurons in the PPT and LDT can be subdivided into two groups based on their firing rate profile across the sleep wake cycle (el Mansari et al., 1989; Thakkar et al., 1998; Datta and Siwek, 2002; Boucetta et al., 2014). Firstly, some REM sleep-active neurons exhibit maximal activity in REM sleep and minimal activity in wakefulness (i.e., REM sleep-max active). Within this group some neurons have firing rates that increase gradually from active wakefulness through NREM sleep to REM sleep, while other neurons only markedly increase their firing immediately prior to and during REM sleep (Boucetta et al., 2014). Secondly, some REM sleep-active neurons in the PPT and LDT discharge minimally during NREM sleep.
Neurons of this type exhibit increased firing during wakefulness in association with muscle activation, firing rate deceleration in transition to NREM sleep, and acceleration of firing rate in transition to REM sleep (i.e., REM sleep/wake-max active). Within this group some neurons discharge maximally in active wakefulness while others discharge maximally in REM sleep (Boucetta et al., 2014). In addition to REM sleep-active neurons, some PPT and LDT neurons discharge minimally in REM sleep and maximally during active wakefulness (Boucetta et al., 2014). Wake-active subpopulations of the PPT/LDT are hypothesized components of the ascending reticular activating system that maintains wakefulness (Steriade and McCarley, 2005c).

Using juxtacellular recording and labeling in the PPT and LDT, Boucetta et al., (2014) reported that these groups are neurochemically diverse, consisting of cholinergic, GABAergic and glutamatergic cell types. Previously, cholinergic neurons in the PPT and LDT were assumed to be both REM sleep-max active and REM sleep/wake-max active. However, Boucetta et al., (2014) found that recorded cholinergic neurons in the LDT and PPT were exclusively REM sleep/wake-max active. Since the sample size was small (6 neurons; LDT(5) and PPT(1)) further studies are needed to definitively determine whether or not any REM sleep-active cholinergic PPT/LDT neurons are REM sleep-max active. Boucetta et al., (2014) showed that the majority (4/6) of REM sleep/wake-max active cholinergic neurons in the LDT and PPT discharged maximally in REM sleep. On average, discharge rates of cholinergic REM sleep/wake-max active LDT and PPT neurons in REM sleep were 63% and 348% greater than those in active and quiet wake respectively. The existence of PPT and LDT cholinergic neurons that discharge maximally in REM sleep (irrespective of their activity in wakefulness) is also supported by positive immunostaining for c-Fos in cholinergic LDT/PPT neurons following recovery from
selective REM sleep deprivation. If REM sleep-active cholinergic neurons in the LDT and PPT were exclusively REM sleep/wake-max active and did not discharge at significantly higher rates in REM sleep as compared to wakefulness, it would be difficult to explain the observations of Maloney et al, (1999) and Verret et al, (2005) that c-Fos expression increases in rebound versus deprived conditions when the combined time spent in wakefulness and REM sleep remains constant. Taken together these studies demonstrate that there are distinct subpopulations of neurons defined by unique combinations of neurotransmitter expression and activity profile within the PPT and LDT. These subpopulations could be functionally distinct and therefore the inconsistencies between studies that manipulate these subpopulations *en masse* could be a reflection of this functional diversity.

### 1.3.5.2 Selective modulation of PPT and LDT subpopulations

Determining the functional importance of individual PPT and LDT subpopulations requires that they be targeted selectively. PPT and LDT cholinergic neurons selectively express receptors for Urotensin II, a vasomodulatory peptide (Clark et al., 2001). Urotensin II has been shown to selectively excite PPT and LDT cholinergic neurons in vitro (Clark et al., 2005; Huitron-Resendiz et al., 2005). Local infusion of Urotensin II in the PPT of rats increased the number of REM sleep episodes and the total time spent in REM sleep both as a percentage of total recording and total sleep time (Huitron-Resendiz et al., 2005). Consistent with the effects of selective pharmacological activation of PPT and LDT cholinergic neurons, selective optogenetic activation of PPT and LDT cholinergic neurons increased the frequency of REM sleep episodes as well as time spent in REM sleep, at the expense of time spent in NREM sleep (Van Dort et al., 2015). Optogenetic stimulation increased the probability of transitioning into REM sleep only
when stimulation occurred in NREM sleep. REM sleep was unaffected by stimulations occurring in wakefulness or REM sleep ((Van Dort et al., 2015) for commentary see: (Grace, 2015; Van Dort and Brown, 2015)). Kroeger et al. (2017) reported that targeted chemogenetic activation of cholinergic PPT neurons did not alter total amounts of wake, NREM, or REM sleep; however, EEG spectral power in NREM sleep was affected and the frequency of state transitions doubled. EEG power underwent a general suppression, with the exception of EEG power at around 7Hz, which was unchanged. The largest suppression of EEG power occurred in the δ-band. These EEG changes are typical of the NREM/REM sleep transitionary state (see data presented in section 4.3.4); therefore, it is a possible that the chemogenetic activation of cholinergic PPT neurons by Kroeger et al. (2017) induced frequent entries into a NREM-to-REM sleep transitionary state, although a more detailed analysis of EEG dynamics would be required to make such a determination. Taken together, these gain-of-function interventions confirm that, free of the potentially confounding effects of modulating intermingled non-cholinergic neurons, cholinergic PPT and LDT neurons have the capacity to induce REM sleep and enhance NREM-to-REM sleep transitioning. However, I submit that these studies do not imply that cholinergic LDT and PPT neurons are involved in the induction of REM sleep. It is not known what would be the effect, if any, of selectively inhibiting cholinergic PPT and LDT subpopulations — e.g., optogenetic/chemogenetic silencing or selective neurotoxic ablation with Urotensin II conjugated to diphtheria toxin (Clark et al., 2007).

Kroeger et al. (2017) also reported that targeted chemogenetic activation of GABAergic PPT neurons reduced REM sleep time, while chemogenetic activation of glutamatergic PPT neurons produced a 3-fold increase in time spent awake relative to control. Chemogenetic silencing of
glutamatergic PPT neurons produced a modest suppression of wakefulness along with a corresponding decrease in REM sleep as a proportion of total sleep time.

REM sleep-max active PPT and LDT neurons can be selectively inhibited by the 5-HT$_{1A}$ agonist 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT), leaving the activity of REM sleep/wake-max unaffected (Thakkar et al., 1998). Surprisingly, selective inhibition of the REM sleep-max active subpopulation of the PPT increased REM sleep, as a percentage of total recording and total sleep time (Grace et al., 2012). The increase in REM sleep time stemmed from an increase in the frequency of REM sleep episodes particularly during periods of low REM sleep drive/propensity, ((Grace et al., 2012) quantified electroencephalographically using a modified version of the algorithm developed by: (Benington and Heller, 1994; Benington et al., 1994)) .

1.4 The REM sleep switch
1.4.1 Pacemaker hypotheses
Mapping the neuroanatomical network responsible for REM sleep generation, although necessary, is insufficient for an understanding of the network dynamics that actually give rise to cycling into and out of REM sleep. In 1975, Hobson and McCarley proposed the reciprocal interaction model: a structural/mathematical hypothesis meant to provide an explanation for the cyclical generation of REM sleep. This model posited that a reciprocal interaction between REM sleep-inactive cell groups and REM sleep-active cell groups form a pacemaker circuit that drives oscillations between sleep stages (McCarley and Hobson, 1975) (Fig. 2A). Aminergic neurons in the locus coeruleus and dorsal raphe were hypothesized as the REM sleep-inactive cells. REM sleep-active neurons in the PTF innervating the LC were presumed to be cholinergic and
excitatory (Hobson et al., 1975). The model proposed that during wakefulness ‘cholinergic’ PTF neurons would be inhibited by activated aminergic neurons. At NREM sleep onset, waning aminergic neuron activity would disinhibit ‘cholinergic’ PTF neurons. At a critical point, the combination of this disinhibition and auto-excitation within the ‘cholinergic’ cell group would enable the exponential rise in PTF neuron activity that triggers the onset of REM sleep (Steriade and McCarley, 2005a). During REM sleep, ‘cholinergic’ PTF neurons would excite aminergic neurons resulting in their own inactivation and the termination of the REM sleep episode.

The reciprocal interaction model was proposed before cholinergic neurons could be immunohistochemically identified. The discovery that PTF neurons are predominately non-cholinergic forced a revision of the model (Armstrong et al., 1983) (Fig 2B). REM sleep-active cholinergic neurons in the PPT and LDT were included in the amended structural model as the main REM sleep promoting groups (Pace-Schott and Hobson, 2002; Steriade and McCarley, 2005a). In the revised model, PPT and LDT cholinergic neurons triggered REM sleep by exciting REM sleep-related non-cholinergic neurons in the PTF. In this version of the structural model, a mutually excitatory connection between the PTF and the PPT/LDT was considered responsible for the exponential rise in PTF neuron activity at REM sleep onset (Hobson et al., 1975; Steriade and McCarley, 2005b). This pacemaker circuit was modeled mathematically using equations of the Lotka-Volterra type derived from population models of predator-prey interactions (McCarley and Hobson, 1975). The simple Lotka-Volterra model formed the basis for the more robust Limit Cycle model, which incorporated circadian modulation (Massaquoi and McCarley, 1992). The time course of neuronal activity in the REM sleep-active and REM sleep in-active cell groups predicted by the mathematical models, coincide with the actual long-term recordings of REM-sleep active and inactive neurons. However, models of this type have
been criticized on the basis that they rely on the tuning of unconstrained parameters to achieve a particular ultradian period. Several lines of evidence argue against the reciprocal interaction model as being a workable explanation for REM sleep generation. Most notably, loss-of-function interventions targeting the structural components of the model — the PPT, LDT, LC, DRN — either have no effect, or have effects inconsistent with the predictions of the model (Torterolo et al., 2002; Pal and Mallick, 2004; Lu et al., 2006b; Pal and Mallick, 2009; Grace et al., 2012).

1.4.2 Flip-Flop hypotheses
More recently it has been suggested that mutual inhibition between REM sleep-active and REM sleep-inactive GABAergic cell groups creates a flip-flop switch that is critical for REM sleep generation (Lu et al., 2006b; Sapin et al., 2009). Unlike a pacemaker circuit, like that of the reciprocal interaction model which is intrinsically capable of generating an ultradian rhythm, a flip-flop switch is not necessarily intrinsically capable of switching state; switching may be triggered by an input signal external to the circuit. In other words, pacemaker network models assume that the periodicity of the sleep cycle is an intrinsic property of the circuit’s topology, whereas flip-flop mechanisms can account for cycling between sleep stages that occurs in response to external triggers. A flip-flop switch can convert a graded input signal, which changes slowly over time, into a two-state output that reduces time spent in intermediate states (i.e., bistability) (Brandman and Meyer, 2008). In comparison with a pacemaker model, a flip-flop mechanism may provide a better explanation for (i) the occurrence of a ‘rebound’ after REM sleep deprivation (Dement et al., 1966; Morden et al., 1967; Brunner et al., 1990; Endo et al., 1998) and (ii) the increasing frequency of brief REM sleep episodes over the interval between sustained bouts of REM sleep lasting greater than 30 seconds (Benington and Heller, 1994).
A. neural pool

+ excitation  - inhibition

A. Rem-ON PTF Cholinergic neurons

Rem-ON Locus Coeruleus & Dorsal Raphe Aminergic neurons

Auto-excitatory positive feedback

Pacemaker Switch

Generator

Generate REM sleep

Auto-inhibitory negative feedback

B. Rem-ON PPT/LDT Cholinergic neurons

Rem-ON Locus Coeruleus & Dorsal Raphe Aminergic neurons

positive feedback

Auto-inhibitory negative feedback

Pacemaker Switch

Generator

Generate REM sleep

C. Rem-ON Sublaterodorsal PTF neurons

GABAergic (vIPAG/DpMe)

Rem-ON GABAergic (vIPAG/DpMe)

External inductive Drive

Flip-flop Switch

Generator

Glutamatergic neurons

Generate REM sleep

D. GABAergic (vIPAG/DpMe)

Rem-ON

positive feedback

Rem-ON Glutamatergic PTF neurons

Rem-ON PPT/LDT Cholinergic neurons

Flip-flop Switch

Generator

Regulator: reinforcement

Generate REM sleep
Figure 2. Hypothesized REM sleep control circuits

A, The original reciprocal interaction hypothesis. B, modified reciprocal interaction hypothesis. C, Flip-flop circuit proposed by (Lu et al., 2006b). D, Version of flip-flop circuit proposed by (Sapin et al., 2009), modified by (Grace et al., 2014a). Anatomical abbreviations: DpMe, deep mesencephalic reticular nucleus; LDT, laterodorsal tegmental nucleus; PPT, pedunculopontine tegmental nucleus; PTF, pontine tegmental field; vlPAG, ventrolateral periaqueductal gray.
In 2006, Lu and colleagues proposed that REM sleep is generated by a mutually inhibitory interaction between REM sleep-active GABAergic neurons in the SLD region of the PTF and REM sleep-inactive GABAergic neurons in the vlPAG and the adjacent dorsal region of the DpMe (Fig 2C). In support of this arrangement, retrograde and anterograde tracing experiments showed that the SLD and vlPAG/DpMe neuronal pools mutually innervate one another (Lu et al., 2006). Moreover, Biossard et al. (2003) have shown that DpMe projections to the sublaterodorsal PTF are GABAergic. It ought to be noted, however, that the vast majority of DpMe projections to the SLD were found to be non-GABAergic and that no GABAergic projections from the PAG to the SLD were detected. Nevertheless, Lu et al., (2006) showed that half of the SLD cells retrogradely labeled from the DpMe and vlPAG contain glutamic acid decarboxylase (GAD)67 messenger ribonucleic acid (mRNA).

In contrast to sublaterodorsal PTF inactivation, which suppresses REM sleep, vlPAG and DpMe inactivation potently increases REM sleep time. Inactivation of the vlPAG/DpMe reliably increases REM sleep amounts in rat (Lu et al., 2006b; Sapin et al., 2009), mouse (Weber et al., 2015), cat (Sastre et al., 1996; Crochet et al., 2006), and guinea pig (Vanini et al., 2007) by means of lesioning, focal muscimol delivery, and optogenetic stimulation of ventral medullary GABAergic axons in the vlPAG/DpMe. Reported increases in REM sleep time ranged from 130-380% of baseline and, in most cases, resulted from increases in both the duration and frequency of episodes. vlPAG/DpMe inactivation also suppresses wakefulness and increases the incidence of wake to REM sleep transitioning (Sastre et al., 1996; Crochet et al., 2006; Lu et al., 2006; Sapin et al., 2009). While these effects are consistent with those that one would expect in the case that the sublaterodorsal PTF and vlPAG/DpMe neuronal pools form a mutually inhibitory flip-flop switch, it has been shown that REM sleep-active neurons in the sublaterodorsal PTF are
predominately glutamatergic rather than GABAergic (Clement et al., 2011). It is therefore unlikely that REM sleep-active sublaterodorsal PTF neurons form a mutually inhibitory flip-flop switch together with vlPAG/DpMe neurons. Sapin and colleagues (2009) alternatively proposed that a mutually inhibitory flip-flop switch might be formed between GABAergic REM sleep-inactive vlPAG/DpMe neurons and the large number of GABAergic REM sleep-active neurons also located in the vlPAG/DpMe (Fig 2D). In either case, activation of REM sleep-related PTF neurons and initiation of REM sleep would result from the withdrawal of GABAergic inhibition by REM sleep-inactive vlPAG/DpMe neurons. Consistent with such an inductive mechanism, GABA\textsubscript{A} receptor antagonism in the PTF terminates NREM sleep and induces persistent REM sleep (Xi et al., 1999; Boissard et al., 2002; Pollock and Mistlberger, 2003; Sanford et al., 2003).

Flip-flop hypotheses of REM sleep generation remain speculative; however, a recent modeling study has tested the theoretical validity of flip-flop switch involvement in the generation of the sleep cycle. Dunmyre et al. (2014) constructed a physiological model based on coupled flip-flop oscillators for the control of transitioning between wakefulness, NREM and REM sleep. With the inclusion of separate NREM and REM sleep homeostatic drives, the modeled circuit was able to faithfully reproduce experimentally measured rodent sleep architecture under normal and sleep deprived conditions. Importantly, unlike previous flip-flop models of behavioural state transitioning (Rempe et al., 2010; Kumar et al., 2012), this study does not incorporate intrinsic oscillatory capability into the neuronal groups. Therefore, this study provides theoretical validation that homeostatically regulated interlocking flip-flop oscillators are sufficient to explain behavioral state transitioning.
1.5 Causes and consequences of upper airway motor suppression in sleep

Sections 1.2.1 and 1.2.2, gave an overview of the phenomenological characteristics of REM sleep, in contrast to those of wakefulness and NREM sleep, from both physiological and systems neuroscience perspectives. One of the other major phenomenological changes associated with sleeping brain states, particularly REM sleep, is a suppression of motor output. The root cause of motor suppression in sleep is the suppression of cranial motor neuron activity by state-dependent changes in synaptic input (Horner, 2009; Dempsey et al., 2010). In NREM sleep, hypoglossal motor neurons are disfacilitated by withdrawal of excitatory inputs, with noradrenergic, serotonergic, and glutamatergic influences being a particular focus of previous studies in cranial motor pools (Fenik et al., 2005; Chan et al., 2006; Burgess et al., 2008; Steenland et al., 2008). In periods of REM sleep, motor output is further suppressed when REM sleep specific inhibitory mechanisms hyperpolarize motor neurons, producing a state of motor atonia. Motor atonia is critically important for the maintenance of REM sleep because it prevents the unwanted behavioural manifestation of the REM sleep-related activity in upstream central motor circuitry. The prevention of outward behavioural manifestations of centrally generated motor commands further supports the view of REM sleep, introduced earlier (section 1.2.2), as an inwardly-oriented state of the brain, characterized by information flow in closed loops.

It has been argued that the major, or even the singular, mechanism responsible for REM sleep motor paralysis is the post-synaptic glycineric/GABAergic inhibition of motor neurons. This view is supported by studies combining intracellular recording of lumbar motor neurons, to measure intrinsic membrane properties, and juxtacellular drug application during REM sleep (Chase et al., 1989; Soja et al., 1991). Intracellular recording has shown that during REM sleep,
hypoglossal motor neurons are hyperpolarized in association with barrages of large amplitude inhibitory post-synaptic potentials, which are reversed by intracellular injection of chloride ions (Fung and Chase, 2015). There are several lines of evidence, however, suggesting that fast-synaptic inhibition is neither the singular nor primary mechanism of motor paralysis. Firstly, glycine receptor antagonism does not block REM sleep-specific inhibitory post-synaptic potentials in a majority of sampled motor neurons, nor does it fully reverse motor neuron hyperpolarization during REM sleep (Chase, Soja et al. 1989; Soja, Lopez-Rodriguez et al. 1991). Also, population level blockade of fast-synaptic inhibition at the hypoglossal (Kubin et al., 1993; Morrison et al., 2003b; Morrison et al., 2003a) and trigeminal motor pools (Brooks and Peever, 2008) does not reverse REM sleep paralysis in the genioglossus and masseter muscles respectively. Together, these studies indicate that additional mechanism(s) importantly contribute to the genesis of REM sleep muscle paralysis; I have previously identified such a mechanism (Grace et al., 2013a), which is described in the following section.

1.5.1 Evidence for a muscarinic cholinergic mechanism mediating paralysis of the upper airway musculature in REM sleep in comparison with competing hypotheses

Cholinergic modulation of motor neuron activity is complex, involving excitatory and inhibitory effects on intrinsic membrane properties (Chevallier et al., 2006). Cholinergic inhibition could play a critical role in mediating REM sleep muscle paralysis in some, if not all, motor pools. At the hypoglossal motor pool, muscarinic receptor stimulation strongly inhibits tongue muscle activity (Liu et al., 2005). In spinal motor neurons, activation of post-synaptic muscarinic type-2 (M₂) receptors induces an inwardly rectifying potassium current (Chevallier et al., 2006; Miles et
al., 2007) that hyperpolarizes membrane potential and decreases input resistance (Chevallier et al., 2006; Miles et al., 2007). Interestingly, post-synaptic cholinergic M2 receptors are present on all α-motor neurons except those in the oculomotor nuclei (Vilaro et al., 1992; Hellstrom et al., 2003), i.e., motor neurons that are spared inactivation during REM sleep (Carskadon and Dement, 2011). In addition to post-synaptic inhibitory mechanisms, activation of pre-synaptic M2 receptors produces motor neuron disfacilitation at cranial and spinal motor pools via inhibition of excitatory neurotransmitter release (Bellingham and Berger, 1996; Quinlan and Buchanan, 2008). Local muscarinic receptor antagonism, at the hypoglossal motor pool, fully rescued rhythmic motor activity in the genioglossus muscle and prevented motor paralysis (Figure 3) (Grace et al., 2013a). Similar rescue of REM sleep paralysis does not occur with the blockade of fast-synaptic inhibition (Morrison et al., 2003b; Morrison et al., 2003a), as would be expected according to the prevailing hypothesis that the inhibition is caused by a recruitment of a REM sleep-specific glycinergic and/or GABA acid receptor-mediated inhibition. We should expect the activating effects of reversing a genuine mechanism of REM sleep motor inhibition to be greatest during REM sleep; otherwise, reversal of REM sleep paralysis could be explained by a state-independent increase in motor neuron gain rather than a prevention of REM sleep paralysis per se. For instance, GABAB receptor antagonism increases activity of the masseter muscle in REM sleep but only in the presence of co-applied GABA and glycine receptor antagonists at the trigeminal motor pool (Brooks and Peever, 2012). It was also identified that such combined glycine, GABA and GABAB receptor antagonism strongly increased motor activity in wakefulness and NREM sleep, such that the response occurring also in REM sleep is not evidence for an effect that relates to mechanism operating in REM sleep per se, particularly as the effects were of the smallest magnitude in that state.
Figure 3: Prevention of genioglossus motor suppression during REM sleep with micro-perfusion of a muscarinic receptor antagonist into the hypoglossal motor pool.

(A) Examples from one rat showing the EEG and the EMG of the genioglossus muscle in each sleep-wake state during both ACSF (control) and scopolamine microperfusion. Also shown are expanded examples of genioglossus activity during transitions from NREM sleep to REM sleep while micro-perfusing the hypoglossal motor pool with ACSF (B) and scopolamine (C). Note that normal suppression of the genioglossus muscle in REM sleep present under control conditions was reversed by muscarinic receptor antagonism. Group data for respiratory-related (D,E) and tonic (F,G) genioglossus activities show that the activating effect of scopolamine is most prominent in REM sleep. D and F show mean levels of tonic and respiratory genioglossus muscle activity in ACSF and scopolamine conditions across sleep-wake states. E and G plot the changes (Δ) in activity between ACSF and scopolamine across sleep-wake states, emphasizing the prominence of the effects in REM sleep.
The mechanism of REM sleep motor atonia may be most meaningfully understood as an interaction between a REM sleep-specific component and some set of motor pool initial conditions (i.e., resting potassium conductance or tonic GABA currents), the combination of which gives rise to muscle paralysis. We can expect to reverse motor paralysis by manipulating either the initial enabling conditions for motor atonia or the REM sleep-specific component(s) of the mechanism. For instance, Chapter 5 details experiments where blockade of voltage-gated and inwardly rectifying potassium channels at the hypoglossal motor pool (channel classes that are not rationally implicated in REM sleep motor atonia) increased genioglossus muscle activity across all sleep–wake states, and prevented genioglossus motor atonia in REM sleep (Grace et al., 2014b). To conclude that voltage-gated or inwardly rectifying potassium channels cause motor atonia at the hypoglossal motor pool, would be to conflate enabling conditions with REM sleep-specific factors. This is not to say, however, that enabling conditions are less critical, only that their distinction from REM sleep-specific causal factors is necessary for effective modelling of the mechanisms of REM sleep atonia. The objective in blocking cholinergic inputs to the hypoglossal motor pool was to determine whether or not they contributed to the REM sleep-specific component of the motor atonia mechanism. It is therefore significant that the activation of rhythmic motor activity by local muscarinic receptor antagonism occurred mainly during sleep (Figure 3).

Muscarinic inhibition is functionally linked to GIRK channel activation and is a classic signaling pathway (Egan and North, 1986; Nikolov and Ivanova-Nikolova, 2007). This coupling can be interrogated using the GIRK channel blocker tertiapin-Q (Jin et al., 1999; Jin and Lu, 1999). A functional coupling of muscarinic receptor-mediated inhibition with GIRK channel activation at the hypoglossal motor pool has been demonstrated (Grace et al., 2013a). This result was also
consistent with previous reports showing that muscarinic receptor-mediated inhibition of spinal motor neurons depends in part on the activation of inwardly rectifying potassium current (Chevallier et al., 2006; Miles et al., 2007). Importantly, blocking GIRK channels at the hypoglossal motor pool while leaving cholinergic neurotransmission intact mimicked the effects of muscarinic receptor antagonism; i.e., it restored respiratory and tonic genioglossus activities throughout REM sleep with minimal or no effects in other states (Figure 4). As with muscarinic receptor antagonism, it is also important to emphasize that the genioglossus activating effects were observed mainly in REM sleep. Again this distinguishes the GIRK channel mechanism as a causal mediator of genioglossus muscle atonia in REM sleep rather than a tonic and state-independent form of inhibition. Taken together, these findings indicate that muscarinic receptor-mediated activation of GIRK channels is an essential and major component of the mechanism producing atonia of the genioglossus muscle in REM sleep. It should be noted that, intracellular recordings of hypoglossal motor neurons showing that their inactivation in REM sleep is also caused by chloride-dependent postsynaptic inhibition were performed in nonrespiratory/tonically active hypoglossal motor neurons (Fung and Chase, 2015), whereas the aforementioned REM sleep-specific cholinergic inhibition primarily affected the respiratory component of genioglossus muscle activity — i.e., the component of genioglossus muscle activity which undergoes the greatest suppression in REM sleep in rats. This raises the possibility that mechanisms of REM sleep motor atonia may differ between respiratory and tonic motor units. In brain stem slices from neonatal mice, the response of hypoglossal motor neurons to muscarinic receptor stimulation is excitatory (Ireland et al., 2012). It remains to be identified whether the muscarinic receptor-mediated inhibition of hypoglossal motor activity is pre and/or post-synaptic in adult rodents across natural states of behavior. It is known that pre-synaptic M2
Figure 4: Prevention of genioglossus motor suppression during REM sleep with microperfusion of a GIRK channel blocker into the hypoglossal motor pool.

(A) Examples from one rat showing the EEG and the EMG of the genioglossus muscle in each sleep-wake state during both ACSF (control) and tertiapin-Q microperfusion. Also shown are expanded examples of genioglossus activity during transitions from non-REM sleep to REM sleep while micro-perfusing the hypoglossal motor pool with ACSF (B) and tertiapin-Q (C). Note that normal suppression of the genioglossus muscle in REM sleep present under control conditions was reversed by GIRK channel blockade. This result mimics the effects of muscarinic receptor antagonism (figure 3A-C). Group data for respiratory-related (D,E) and tonic (F,G) genioglossus activities show that the activating effect of GIRK channel blockade is most prominent in REM sleep. D and F show mean levels of respiratory-related and tonic genioglossus muscle activities in ACSF and tertiapin-Q conditions across sleep-wake states. E and G show the changes (Δ) in activity between ACSF and tertiapin-Q across sleep-wake states, emphasizing the prominence of the effects in REM sleep.
receptors inhibit excitatory glutamate inputs to hypoglossal motor neurons in-vitro (Bellingham and Berger, 1996), i.e., cholinergic mechanisms can produce an indirect disfacilitation via reducing excitatory glutamatergic transmission. This point is relevant because glutamate inputs mediate transmission of respiratory drive to the hypoglossal motor pool (Greer et al., 1991; Funk et al., 1993), and also contribute to tonic pharyngeal motor activity (Burgess et al., 2008; Steenland et al., 2008).

1.5.2 Pathogenesis of Obstructive Sleep Apnea and failed attempts at pharmacotherapy

Clinically, one of the most significant consequences of the normal hypotonia occurring in sleep, is that suppression of upper airway muscle activity can precipitate repeated episodes of upper airway narrowing and closure during sleep in predisposed individuals — i.e., obstructive sleep apnea (OSA). OSA is a serious clinical problem due to its high prevalence and association with adverse cardiovascular, metabolic, and cognitive outcomes (Brooks et al., 1997; Teran-Santos et al., 1999; Peppard et al., 2000; Malhotra and White, 2002). The causative factors for OSA are variable and complex within individuals (Eastwood et al., 2010). These causative factors include: (i) structural predispositions in airway anatomy, (ii) the insufficiency of compensatory neural mechanisms that are recruited to protect against or reverse airway compromise, (iii) respiratory control instability, (iv) reduced arousal threshold resulting in repeated cycles of respiratory instability, (v) reductions in airway caliber stemming from low functional residual capacity, particularly in obese patients (Patil et al., 2007; Dempsey et al., 2010; White and Younes, 2012; Wellman et al., 2013; Owens et al., 2015; Horner et al., 2017). Administration of continuous
positive airway pressure (CPAP) is a highly effective treatment for OSA and reported tolerance problems can be mitigated with appropriate patient management (Eastwood et al., 2010); however, a pharmacological treatment option would nevertheless be desirable as a supplement for other treatment strategies or as a primary intervention for patients intolerant of CPAP (Horner et al., 2017). Currently, an effective pharmacotherapy for OSA is not available. Clinical trials to-date have been limited by small sample sizes, short durations, inadequate symptom assessment, a lack of consideration for patient phenotyping, and poor mechanistic rationales in some cases (Mason et al., 2013). Presently, no drugs have been identified which provide large enough improvements in OSA severity to justify their being recommended as an OSA treatment strategy. Of those studies which reported improvements in OSA severity, mean reductions in apnea-hypopnea index (AHI) ranged from 17- 52% (range of mean AHI with placebo = 54-24 events/hour; range of mean AHI with drug treatment = 41-13 events/hour). The vast majority of clinical trials have tested drugs that are proposed to either enhance upper airway muscle activity or increase ventilatory drive (Mason et al., 2013). A small number of studies have trialled drugs topically applied to the upper airway; while they failed to show more than small improvements in AHI, a preclinical study in a porcine model of sleep apnea showed promising improvements in airway collapsibility following topical administration of the potassium channel blocker, AVE0118, with the aim of sensitizing airway mechanoreceptors and enhancing the negative pressure reflex (Wirth et al., 2013).
1.5.3 Mapping potential drug targets in the circuitry controlling upper airway motor output

It is not clear whether the difficulty in treating OSA pharmacologically reflects a deficiency in effective targets or a deficiency in target identification. The pharmacological tractability of a disorder depends, in part, on the differential expression of druggable targets in the underlying physiological systems that can be modulated for clinical benefit whilst limiting toxicity. There is now sufficient data to support genome wide analyses of the differential expression of druggable targets in the circuitry controlling motor output to the upper airway musculature (Horner et al., 2017).

1.5.3.1 The approach used to identify the candidate drug targets

I used the differential search function of the Allen Mouse Brain Atlas’s database of ~25,000 in situ hybridization experiments to isolate genes that differentiate the hypoglossal motor nucleus (HMN) and the primary afferents of the HMN from the rest of the brain (Allen Mouse Brain Atlas, 2004; Lein et al., 2007). Separate differential searches were performed for the HMN and a grouping of HMN pre-motor structures (PRE-HMN). A listing of the primary hypoglossal afferents was taken from previous tracing studies (Dobbins and Feldman, 1995; Fay and Norgren, 1997). Both these studies labelled the circuitry innervating the HMN using injections of pseudorabies viral tracer into the tongue musculature. Importantly, this approach allows for a highly accurate mapping of premotor circuitry since it avoids false positive labeling that occurs when tracer is injected directly into the brain and diffuses beyond target structures. Those studies
identify the primary sources of afferent inputs to the HMN as the central tegmental field, Köllicker-Fuse region, supra-trigeminal, inter-trigeminal, and principal sensory trigeminal nuclei, nucleus subcoeruleus, parvicellular reticular formation, dorsal medullary reticular fields, Probst's region, and the spinal trigeminal nucleus caudalis. I created a database of the genes identified as having at least a 2-fold greater expression in the HMN and/or PRE-HMN relative to the rest of the brain (Database 1; available at, http://onlinelibrary.wiley.com/doi/10.1111/resp.13079/full). The database is separated into four parts on separate spreadsheets: (i) differentially expressed HMN genes, (ii) differentially expressed PRE-HMN genes, (iii) FDA-approved drugs associated with differentially expressed HMN and PRE-HMN genes classified as probable modulators of neuronal activity, and (iv) drugs that have undergone trials for the treatment of OSA and their protein targets. Parts (i) and (ii) include a listing of the target and contrast structures used for the differential searches. The mean fold-change was used in cases where multiple in situ hybridization experiments had been performed for a given gene.

A recent bioinformatics assessment of the ‘druggability’ of the human genome generated a database of all the protein targets for the drugs approved for clinical use by the U.S. Food and Drug Administration (FDA) (Santos et al., 2017). Their analysis confirmed a previously identified trend (Overington et al., 2006) that proteins targeted by approved drugs tend to cluster in four privileged families: G-protein coupled receptors (GPCRs), ligand-gated ion channels, nuclear receptors, and kinases. Because, for the purposes of this analysis, I am interested in potential drug targets capable of modulating cellular activity to increase hypoglossal motor output, I classified the gene products in that database according to their status as modulators of neuronal activity and according to their inclusion in GPCR and ion channel protein families. These classifications were made by screening the differentially expressed mouse genes for those
with human orthologs, followed by cross-referencing the resulting list with the Gene Ontology Consortium’s database of Molecular Function and Biological Process annotations of the human genome (Ashburner et al., 2000; Gene Ontology Consortium, 2015) (AmiGO 2 version: 2.4.24). Final classifications were made using Boolean keyword/phrase searches of the Gene Ontology terms assigned to the genes in our database (the list of the inclusion and exclusion keywords/phrases used are available in Database 1). The database was screened for false-positive results by manually examining the Gene Ontology terms and in situ hybridization results for all differentially expressed genes classified as GPCRs, ion channels, and/or modulators of neuronal activity. Finally, we cross-referenced our database with the database of protein targets and FDA approved drugs (Santos, Ursu et al. 2017) to match FDA approved drugs to genes with enhanced expression in the HMN and/or PRE-HMN. The list of differentially expressed HMN and PRE-HMN drug targets matched to FDA approved drugs is available in Database 1.

1.5.3.2 The identified candidate drug targets

I identified 1,492 genes from the Allen Mouse Brain database of in situ hybridization experiments that showed enhanced expression at the HMN and/or PRE-HMN relative to the rest of the brain; gene expression in the HMN was enhanced as high as 33-fold. Of those 1,492 genes, the expression of 1,168 are specifically enhanced in the HMN, the expression of 88 are specifically enhanced in the PRE-HMN, and 236 exhibit enhanced expression in both the HMN and PRE-HMN. A total of 99 genes were classified as being probable modulators of neuronal activity, of which 18 were classified as GPCRs and 37 were classified as ion channels (i.e., part
of the privileged protein families for drug discovery). These 99 targets are listed in Table 2 and are mapped in Figure 5. Protein products of 26/99 genes are targets of 175 FDA approved drugs. Of those drugs trialled for the treatment of OSA, a total of nine act pharmacologically on ten of the targets (a list drugs trialled for the treatment of OSA (Mason et al., 2013; Smales et al., 2015; Taranto-Montemurro et al., 2016b; Wellman, 2016a, b) annotated with protein targets (Santos, Ursu et al. 2017) is available in Database 1). Importantly, although the identified targets, or combinations thereof, may not ultimately prove effective for OSA pharmacotherapy, our analysis does reveal significant unexplored potential in terms of trialling approved drugs and developing new drugs for differentially expressed targets in the circuitry critical for OSA pathogenesis.

Figure 5 shows that the GPCR group of differentially expressed genes is associated with a greatest number of relatively specific approved drugs (‘specific’ in this case refers to drugs having four or less preferred protein targets). Differentially expressed ion channel genes are associated with a large number of approved drugs; however, many of those drugs are non-specific and exert their clinical effects by acting on large groups of similar ion channels (e.g., dalfampridine (4-aminopyridine)) (Wellman, 2016a) acts on 40 voltage-gated potassium channel targets). Moreover, of the top ten differentially expressed ion channel genes only two are associated with approved drugs (Table 2). Table 2 also includes the inward-rectifying potassium channel 2.4 (Kir2.4) (listed as potassium inwardly-rectifying channel, J, 14 on line 20). Kir2.4, in the brain, is expressed almost exclusively in the cranial motor pools that modulate pharyngeal muscle tone (Topert et al., 1998; Grace et al., 2013b), although there is also expression in the spinal cord and in other non-nervous tissue such as kidney and heart.
Overall, the ion channel class of targets could be a focus of drug discovery efforts for OSA pharmacotherapy. However, the lack of specific ion channel modulators can be taken as an indication of the difficulty in developing specific drugs within this protein target class.

Nevertheless, the fact that Kir2.4 channels exhibit markedly lower barium sensitivity compared to other Kir channels (Töpert, Döring et al. 1998) may reflect some structural uniqueness, which could be exploited in the chemical development of a specific blocker.

1.5.4 A CNS-wide assessment of the relative excitability of hypoglossal motor neurons

In the previous section it was argued that the pharmacological tractability of OSA depends on the differential expression of druggable targets in the underlying physiological systems, i.e., the HMN and the HMN primary pre-motor fields. However, we should not expect those neurons having the highest expression of a given drug target to have the greatest response to systemic target modulation. The reason being that the magnitude of given neuron’s response to drug is a product of target expression and the intrinsic excitability of the neuron. For a given level of differential drug target expression, neuron types with higher intrinsic excitability can be modulated more specifically than less excitable neurons, because, in the former case, there is a greater capacity to limit modulation of off-target neuron types by titrating drug dosage. Therefore, while identification of differentially expressed drug targets at the hypoglossal motor pool is important, it is equally important to assess the excitability of hypoglossal motor neurons relative to other neuron types across the central nervous system. Figure 6 shows recorded
rehobase values — a measure of neuronal excitability related to the minimal current requirement to elicit spiking — for 112 neuron types including hypoglossal motor neurons. Values were taken from the NeuroElectro Database of neuron electrophysiological properties.
Figure 5: A map of potential drug targets differentially expressed in the circuitry controlling upper airway motor output.

This figure shows a mapping of the data listed in Table 2. The numbers associated with individual bars in the radial bar chart refer to the position of the corresponding gene in Table 2. Genes associated with the FDA list of approved drugs are indicated by connections with bubble graphs. Each bubble represents a drug or group of drugs; bubble size is inversely related to drug specificity (i.e., the number of protein targets per drug). The notation in the bubbles is indicated on the figure. ISH; in situ hybridization.
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**Ion channels**

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Table 2. Differential Expression of Neuronal Activity-Related Genes in the HMN/HMN Primary Pre-Motor (PRE) Regions

This table shows the differential expression of neuronal activity-related genes in the hypoglossal motor nucleus (HMN) and primary pre-motor regions (PRE-HMN). A list of genes having at least 2-fold greater expression in the HMN and/or PRE-HMN relative to the brain-at-large are classified as probable modulators of neuronal activity. Numbers under the column identification heading “ID” refer to locations of the corresponding gene information in Figure 5. Gene identifiers under column heading “Gene #” refer to the human orthologs of mouse genes. See Database 1 for a listing of the target structures included in the PRE-HMN group as well as contrast structures used to compute expression fold-changes using the Allen Mouse Brain Atlas differential search function of RNA in situ hybridization experiments. Under the column heading “FDA Drug”, “Y” denotes genes associated with at least one FDA approved drug.
Figure 6: Comparison of hypoglossal motor neuron excitability against neuron types across the CNS

Recorded rehobase values for 112 neuron types including hypoglossal motor neurons taken from the NeuroElectro Database of neuron electrophysiological properties (NeuroElectro.org) (Tripathy, Savitskaya et al. 2014; Tripathy, Burton et al. 2015). For each cell type, the difference in mean rheobase (averages taken across multiple experiments (393 total experiments)) from the median rehobase value across all neuron types. Numbers adjacent to neuron types denote the total number of sampled neurons from all studies making up the average value.
(NeuroElectro.org)(Tripathy et al., 2014; Tripathy et al., 2015). Excitability data from several hypoglossal motor neuron studies, not included in the NeuroElectro compilation, were added for the purposes of this analysis, including measures of hypoglossal motor neuron rheobase made during NREM and REM sleep (Nunez-Abades et al., 1993; Viana et al., 1994; Fung and Chase, 2015). Relative to the broad spectrum of neuron types included in this analysis, motor neurons, including hypoglossal motor neurons, are amongst the least excitable neuron types in the central nervous system. The average hypoglossal motor neuron rheobase was 6.4-fold greater than the median value for all neuron types. This is comparable to reported values for other cranial motor neurons located in the trigeminal motor pool; however, compared to spinal alpha motor neurons, cranial motor neurons of the hypoglossal and trigeminal motor pools are distinctly more excitable. During REM sleep, inhibitory mechanisms further depress the already low excitability of hypoglossal motor neurons. This analysis implies that, relative to most neurons in the central nervous system, the responsiveness of hypoglossal motor neurons to any pharmacological intervention may be dampened by their own intrinsic excitability to such an extent that even a high degree of differential drug target expression will prove to be insufficient compensation. Therefore, the relative inexcitability of hypoglossal motor neurons may make this cell type a poor target for pharmacological interventions. In light of this data, it is particularly important to develop OSA drug targets that, not only have a high degree of differential expression at the HMN, but that also have very low absolute levels of expression in other brain regions, as well as a major capacity to increase neuronal excitability (e.g., Kir2.4).
Chapter 2
Aims and Hypotheses

**Aim 1:** Identify the role of cholinergic input to the pontine SubC region in the generation of REM sleep using a targeted loss-of-function intervention: pharmacological blockade of cholinergic receptors in the SubC region.

**Hypothesis 1:** Contrary to the prevailing hypothesis of cholinergic involvement in REM sleep, I hypothesize that cholinergic input to the SubC is not a source of feedforward inductive REM sleep drive; rather, I expect that cholinergic input to the SubC serve an accessory role in NREM to REM sleep transition dynamics.

**Aim 2:** Determine the involvement of the vlPAG and adjacent DpMe neurons in the generation of REM sleep and the maintenance of sleep bistability using a targeted loss-of-function intervention: pharmacological inactivation of vlPAG and DpMe neurons. Using detailed analysis of NREM/REM intermediary state dynamics and bout lengths, I aim to clarify the relative contributions of the REM sleep-active and REM sleep inactive neuronal subgroups of the vlPAG/DpMe neuronal pool.

**Hypothesis 2:** I hypothesize that reported increases in REM sleep stemming from vlPAG/DpMe inactivation may be more appropriately classified as increases in NREM/REM sleep transitional sleep, indicating that these neurons participate in maintaining the fundamental bistable nature of sleep.
**Aim 3:** Using focal pharmacological manipulations of potential drug targets in the hypoglossal motor pool, establish proof-of-principle that the upper airway can be reactivated throughout sleep by local pharmacological manipulation of targets at the hypoglossal motor pool with the objective of identifying important new translational approaches to the pharmacological treatment of OSA.

**Hypothesis 3:** Upper airway motor activity can be restored to normal waking levels during sleep through the targeted blockade, at the hypoglossal motor pool, of: (i) inwardly rectifying potassium channels, (ii) two-pore domain, TWIK-related acid-sensitive potassium (TASK) channels, and (iii) voltage-gated potassium channels.
3.1 Introduction

REM sleep, characterized by vivid dreaming, motor paralysis, and heightened neural activity, is one of the fundamental states of the mammalian central nervous system. Despite its fundamental nature, central questions remain unanswered regarding the control system responsible for its generation.

Neuroanatomical and electrophysiological studies have delineated the brainstem site most critical to REM sleep generation. REM sleep can be eliminated by lesioning or pharmacological inactivation of the SubC\textsubscript{D/A} (Carli and Zanchetti, 1965; Jones, 1979; Sanford et al., 2003; Lu et al., 2006), a region of the PTF containing neurons that exhibit maximal activity in REM sleep (McCarley and Hobson, 1971; Lu et al., 2006; Sapin et al., 2009). Stimulation of the SubC\textsubscript{D/A} by cholinergic receptor agonists can induce long lasting bouts of REM sleep at short latencies (George et al., 1964; Hobson et al., 1983; Gnadt and Pegram, 1986; Velazquez-Moctezuma et al., 1989; Bourgin et al., 1995; Garzon et al., 1998). Consequently, initial models of REM sleep generation (e.g., the Reciprocal Interaction Hypothesis) claimed that REM sleep initiation is dependent on cholinergic activation of the SubC\textsubscript{D/A} (Pace-Schott and Hobson, 2002). Consistent with this claim, the SubC\textsubscript{D/A} is innervated by the cholinergic PPT/LDT (Quattrochi et al., 1989), which contain subpopulations of REM sleep-active neurons (Maloney et al., 1999). Also, SubC\textsubscript{D/A} neurons are excited by acetylcholine (Shiromani and McGinty, 1986; Yamamoto et al.,...
1990; Sakai and Koyama, 1996) through activation of both pre and post-synaptic muscarinic receptors (Weng et al., 2014).

However, interpretation of these data is cofounded by findings showing that cholinergic stimulation of the SubC\textsubscript{D/A} also induces prolonged bouts of wakefulness characterized by abnormal motor behaviour in cats (Mitler and Dement, 1974; van Dongen, 1980; Baghdoyan et al., 1984a) and rats (Gnadt and Pegram, 1986; Mastrangelo et al., 1994; Deurveilher et al., 1997). Also, PPT or LDT inactivation, which would be expected to reduce REM sleep, often increases or has no effect on REM sleep amounts (Torterolo et al., 2002; Lu et al., 2006; Pal and Mallick, 2009; Grace et al., 2012). Recent evidence supports an alternative claim that a combination of GABAergic disinhibition and tonic glutamatergic excitation initiates SubC\textsubscript{D/A} activation and generates REM sleep; GABA\textsubscript{A} receptor antagonism in the SubC\textsubscript{D/A} induces persistent REM sleep (Boissard et al., 2003; Pollock and Mislberger, 2003) that can be terminated by co-delivery of a glutamate receptor antagonist (Boissard et al., 2002).

It is important to note that, while the capacity of SubC\textsubscript{D/A} cholinergic inputs to contribute to REM sleep generation has been established, existing evidence neither confirms nor refutes the necessity of SubC\textsubscript{D/A} cholinergic neurotransmission in the generation of REM sleep. The contribution, if any, of endogenous SubCD/A acetylcholine in REM sleep generation remains unresolved, because the critical test of its involvement has never been rigorously performed. Here we report the results of that critical test: the focal antagonism of SubC\textsubscript{D/A} muscarinic acetylcholine receptors combined with an analysis of REM sleep transition dynamics.
3.2 Methods

3.2.1 Animal care

Experiments were performed on a total of 32 male Wistar rats (Charles River, Senneville, QC, Canada) (mean body weight = 292.5g ±1.1 (SEM), range 280-301g). Procedures conformed to the recommendations of the Canadian Council on Animal Care, and the University of Toronto Animal Care Committee approved the protocols. Rats were housed individually, maintained on a 12-12h light/dark cycle (lights on at 0700 h), and had free access to food and water.

3.2.2 Surgery

Rats were chronically implanted with electrodes for recording sleep-wake state: EEG and electromyogram (EMG) of the trapezius muscle. Sterile surgery was performed under general anesthesia induced with isoflurane (3.5%). Rats were intraperitoneally injected with buprenorphine (0.03 mg · kg−1) to minimize postoperative pain, atropine sulfate (1 mg · kg−1) to minimize airway secretions, and saline (3 ml, 0.9%) for fluid loading. A surgical plane of anesthesia, as judged by abolition of the pedal withdrawal and corneal blink reflexes, was maintained with isoflurane (2–2.5%) administered with an anesthesia mask placed over the snout. The rats were then implanted with EEG and neck EMG electrodes for the purposes of sleep–wake state recording.

Previous studies have demonstrated that the dorsolateral pontine reticular formation, ventral to the locus coeruleus and LDT, medial to the trigeminal motor nucleus and extending from the caudal part of the ventral tegmental nucleus to the root of the facial nerve, is the region where
lesioning, cholinergic stimulation and GABA_A receptor antagonism produce the greatest effects on REM sleep (i.e., the REM sleep induction zone). The SubC_D/A, as defined by Paxinos and Watson (1998), comprise much of this region. This volume of tissue is inclusive of the sublaterodorsal region as defined by Swanson (2004). Therefore, we stereotaxically (model 962; David Kopf Instruments) targeted microdialysis guides (CXG-9; Eicom, San Diego, CA, USA) bilaterally to positions 3 mm above the SubC_D/A (9.2 mm posterior to bregma, ±1.2 mm lateral to the midline, and 5.5 mm ventral to bregma). In one group of rats (Study 4 – see experimental design section below), an additional guide was positioned above the PPT. This guide was implanted at a 15° angle relative to the sagittal plane and the guide tip was targeted to a point 8.0 mm posterior to bregma, ±3.34 mm lateral to the midline, and 2.82 mm ventral to bregma. Dummy cannulae were placed inside the microdialysis guides to keep them free of debris until the day of the experiment. Microdialysis probes projected an additional 3 mm into either the SubC_D/A or PPT. Following surgery, rats were housed individually and were allowed to recover for at least 6 days before the experiments were performed.

3.2.3 Habituation and recording environment

The evening before the first study day, rats were placed in the recording environment and connected to a lightweight counterbalanced recording cable for habituation purposes. The recording environment consisted of a large open-topped bowl (Rodent Bowl, MD-1514, BAS Inc, West Lafayette, IN, USA) mounted on a turntable (Rat Turn, MD-1404, BAS Inc), housed within an electrically-shielded and soundproofed cubicle (EPC-010, BRS/LVE Inc. Laurel, MD, USA). A video camera located within the cubicle allowed for continuous visual monitoring without disturbing the animal.
3.2.4 Microdialysis

Also the evening before the first study, the internal cannulae were removed from their guides, and microdialysis probes were inserted (CX-I-12-1; 38nL tip volume; 220 μm diameter; 1 mm long membrane; 50,000 Dalton cut-off; Eicom). The probes were connected to FEP Teflon tubing (inside diameter, 0.12 mm) with this tubing connected to 1.0 ml syringes via a zero dead space switch (Uniswitch, BAS, West Lafayette, IN, USA). The probes were perfused with freshly-made artificial cerebral spinal fluid (ACSF) at a flow rate of 2.1μl/min. The composition of the ACSF was as follows (in mM): NaCl (125), KCl (3), CaCl2 (2), MgSO4 (1) NaHCO3 (25) and glucose (10). The ACSF was warmed to 37°C and bubbled with CO2 to a pH of 7.4. It is important to note that fluid was not injected into the tissue, but rather, the semi-permeable cuprophan membrane at the tip of the microdialysis probe permits passive diffusion only.

3.2.5 Experimental design

3.2.5.1 Details common to all studies

All studies had a repeated measure design and consisted of a control period (ACSF) and one or more periods of drug microperfusion. All treatment periods took place on separate but consecutive days, between the hours of 1PM and 4PM to control for potential time-of-day effects. Signals were recorded continuously during these hours. All SubC_{D/A} treatments were administered bilaterally. Treatment order was always alternated, with the exception of studies 1 and 4 where the control period always took place on day 1. All rats were killed on the final treatment day and their brains were removed to determine the position of microperfusion sites.
The locations of these sites were recorded on standard coronal drawings from the stereotaxic atlas of the rat brain prepared by Paxinos and Watson (1998). In experiments targeting the PPT the location of cholinergic PPT neurons was determined using NADPH-Diaphorase histochemical staining.

Rats were perfused with 0.1 m PBS and 4% paraformaldehyde. Subsequently, the brains were removed and fixed overnight in a 1:1 solution of 4% paraformaldehyde and PBS. Brains were then transferred to a 30% sucrose solution for 24 h, after which they were rapidly frozen and cut into 50 μm coronal sections with a cryostat (Leica CM 1850). Slide-mounted sections were incubated in a solution composed of 0.1% NADPH (Sigma N-6505), 0.01% nitro blue tetrazolium (Sigma N-6876), and 0.3% Triton X-100 (Sigma) in 0.05 m Tris-buffered saline, pH 7.4, for 30 min at 37°C. Sections were then counterstained with neutral red.

3.2.5.2 Study 1

In study 1 (n=4; positive control), we sought to confirm the efficacy of muscarinic receptor antagonism at the SubC<sub>D/A</sub>. Study 1 consisted of three microperfusion treatment conditions: (i) ACSF control, (ii) cholinergic receptor agonist alone (carbachol, 0.5mM, Tocris Bioscience, Minneapolis, MN) and (iii) a combination of carbachol (0.5mM) and a muscarinic receptor antagonist (scopolamine hydrobromide, 1mM, Tocris Bioscience). In the third condition scopolamine was pre-applied for one hour (i.e., 1PM to 2PM) before beginning microdialysis perfusion of the carbachol-scopolamine mixture. The concentration of scopolamine needed to counteract the effect of carbachol was determined in pilot studies. A concentration of 0.5mM cabachol delivered at a rate of 2.1μl/min is approximately equivalent to a ~9μg/hour dose of
carbachol (see section 4.2.4 for the method used to estimate drug tissue exposure as a function of time; dose range used in previous microinjection studies is 0.4-9.0 µg).

3.2.5.3 Study 2

As anticipated from previous studies, cholinergic stimulation of the SubC_{D/A} (study 1) produced abnormal motor behaviour and prolonged bouts of wakefulness (see section 3.3 for results). These effects are consistent with our having activated interspersed cholinceptive reticulospinal neurons involved in motor facilitation and locomotion (Lai and Siegel, 1991). I then sought to confirm that, free of the confounding effects of activating supra-spinal motor networks, I was accurately targeting the REM sleep induction zone, and that we could predictably modulate its activity using drug microperfusion. Therefore, I broadly inactivated the region by blocking endogenous excitatory neurotransmission while simultaneously stimulating inhibitory receptors. Glutamatergic drives onto pontine neurons mediating increased muscle tone and locomotion are sensitive to NMDA receptor antagonism. Accordingly, study 2 (n=7; positive control) consisted of two dialysis treatment conditions: (i) ACSF control, and (ii) a N-Methyl-D-aspartic acid (NMDA) receptor antagonist (D-AP5, 200µM, Tocris Bioscience) together with a GABA_{A} receptor agonist (muscimol, 10µM, Tocris Bioscience).

3.2.5.4 Study 3

In study 3 (n=14; positive control), we sought to determine the role of endogenous cholinergic input to the SubC_{D/A} in REM sleep generation. Study 3 consisted of two dialysis treatment
conditions: (i) ACSF control, and (ii) scopolamine alone (1mM; the effective concentration determined in study 1).

3.2.5.5 Study 4

In study 4, we sought to corroborate the role of SubC\textsubscript{D/A} cholinergic input in REM sleep generation suggested by study 3. Study 4 consisted of three microperfusion treatment conditions: (i) ACSF microperfusion at both the SubC\textsubscript{D/A} (bilateral) and PPT (unilateral), (ii) urotensin-II (4pM, Tocris Bioscience, Minneapolis, MN) microperfusion at the PPT along with ACSF microperfusion at the SubC\textsubscript{D/A} and (iii) urotensin-II microperfusion at the PPT and simultaneous scopolamine (1mM) micoperfusion at the SubC\textsubscript{D/A}. Urotensin-II is a neuropeptide that has been shown to selectively excite PPT cholinergic cells in vitro and increase REM sleep when applied locally to the PPT in vivo (Huitron-Resendiz et al., 2005).

3.2.6 Signal analyses

Electrical signals were amplified and filtered (Super-Z head-stage amplifiers and BMA-400 amplifiers/filters, CWE, Ardmore, PA). The EEG was filtered between 1 and 100 Hz, whereas the neck EMG was filtered between 100 and 1,000 Hz. The moving-time average of the neck EMG (time constant = 50 ms) was also obtained (model MA 821, CWE). All signals were digitized at 2,000Hz (Spike 2 software, 1401 interface, CED, Cambridge, UK).
3.2.6.1  EMG and EEG signals

The data were analyzed in consecutive 5s time-bins. Moving-time averages of the neck EMG signals were quantified in arbitrary units relative to electrical zero defined as the voltage recorded with the amplifier inputs grounded. The EEG signal was subjected to a fast-Fourier transform for each 5-sec time bin. The power in frequency bands (2Hz in width) spanning the 1 to 33Hz range was calculated.

Sleep-wake states were identified by visual inspection, and classified into wakefulness, NREM and REM sleep according to standard criteria.

3.2.6.2  NREM to REM sleep transition dynamics

NREM-to-REM sleep transition dynamics were analyzed using a state-space analysis (Gervasoni et al., 2004; Diniz Behn et al., 2010). The objective of a state-space approach is to create a 2-dimensional plot bounded by electroencephalographic variables that correlate with sleep-wake state, in which the position of a single point represents the electroencephalographic state of the forebrain during a single recording epoch (Figure 7A). Spectral power in the 1-19Hz frequency range and the ratio of spectral power in the 7-9Hz and the 1-9Hz frequency ranges were used to create state-space plots. Data was smoothed prior to plotting using a 5-epoch wide moving average (MATLAB R2013b, Natick, MA, USA (function: smooth)). In order to analyze NREM-to-REM sleep transitions, we needed to define standardized boundaries of the NREM and REM sleep state-space clusters. To create the boundaries we: (i) calculated local point densities from state-space plots using Voronoi tessellation, (ii) applied a density threshold to isolate the two high density clusters (i.e., NREM and REM sleep regions) from the surrounding low density
region (Figure 7B), (iii) and lastly plotted the convex envelope (i.e., the minimum convex area enclosing a group of points) for each cluster (Figure 7C). Across the dynamic range of local point densities, only a fraction of the potential density thresholds will yield two non-overlapping cluster boundaries, in contrast to the majority of cases which yield either a single NREM cluster or overlapping NREM and REM sleep clusters. For each animal a density threshold yielding two non-overlapping cluster boundaries was selected. The same density threshold was used for both baseline and drug conditions. Subsequent to identifying cluster boundaries, we were then able to define the duration of NREM-to-REM sleep transitions as the time interval between crossings of the NREM and REM sleep convex envelopes (i.e., transition boundaries). Attempted NREM-to-REM sleep transitions were defined as state-space trajectories: (i) that cross the upper third of the NREM sleep transition boundary (i.e., the boundary region where successful NREM-to-REM sleep transitions enter transition space), (ii) remain in intermediate transition space for at least three epochs, (iii) but never cross the REM sleep transition boundary.

3.2.7 Neural circuit simulations

The wiring diagram of the modelled circuit is shown in the section 3.3: “results”, page 106. The rationale for its topology is detailed as follows. The circuit supports the generation of two states, which we are calling “NREM” and “REM sleep”, separated by a transitional period. The state of “REM sleep” is defined as the period when firing rate of the model “SubC\textsubscript{D/A}” pool is stable at a maximum level. As previously mentioned in the introduction, the SubC\textsubscript{D/A} is the critical site of REM sleep generation in vivo. Also, the SubC\textsubscript{D/A} is the principal source of phenomenological outputs in REM sleep; lesioning SubC\textsubscript{D/A} septal afferents eliminates hippocampal theta
Figure 7. Workflow for generating REM and NREM sleep state-space boundaries
oscillations in REM sleep, while lesioning of more ventrally located SubC_{D/A} neurons projecting to the medullary reticular formation and spinal cord prevent REM sleep motor atonia (Lu et al., 2006). Based on c-fos immunostaining, REM sleep-active SubC_{D/A} neurons are predominately glutamatergic (Clement et al., 2011), and therefore all projections of the “SubC_{D/A}” pool in our model are excitatory. Conversely, the state of “NREM sleep” is defined as the period of “SubC_{D/A}” inactivity (i.e., no spiking). The “NREM-to-REM sleep” transition is defined as the period over which “SubC_{D/A}” firing rate accelerates from the minimum to the maximum level.

As previously mentioned, SubC_{D/A} activation in vivo critically requires the combination of GABAergic disinhibition and tonic glutamtergic excitation (Boissard et al., 2002; Boissard et al., 2003; Pollock and Mistlberger, 2003). Therefore, the “SubC_{D/A}” pool of the model receives a tonic excitatory input and an inactivating inhibitory input (termed REM-off) responsible for initiating “SubC_{D/A}” activity. A hypothesized source of REM-off GABAergic input to the SubC_{D/A} are the GABAergic REM sleep-inactive neurons of the vlPAG and the adjacent DpMe. In the model I adopt the hypothesis of Sapin et al. (2009), that REM-off and REM-on GABAergic vlPAG/DpMe neurons mutually inhibit one another. So, in the model, the REM-off pool inhibits and is inhibited by a REM-on pool. The source of inhibition to the REM-off pool is a graded analog input neuron that represents the build-up of “REM sleep” drive/propensity.

With this arrangement, activation of the REM-on pool is not strictly necessary for “REM sleep” generation; this is consistent with the finding that simultaneous inhibition of overlapping REM-on and REM-off vlPAG/DpMe pools increases REM sleep (Sapin et al., 2009). Nevertheless, mutual inhibition performs a necessary function: the conversion of graded inputs (i.e., REM sleep drive signals) into a two-state output having only a brief transition period (i.e., bistability) (Brandman et al., 2005; Brandman and Meyer, 2008).
In vivo, the SubC\textsubscript{D/A} innervates the vlPAG/DpMe region, and so the model circuit also includes an excitatory projection from the “SubC\textsubscript{D/A}” pool to the REM-on pool (Lu et al., 2006). Since REM sleep-active SubC\textsubscript{D/A} neurons are predominately glutamatergic, we would expect their input to the vlPAG/DpMe region to activate REM-on rather than REM-off neurons.

In vivo, the SubC\textsubscript{D/A} and cholinergic PPT/LDT reciprocally innervate one another and SubC\textsubscript{D/A} neurons are excited by cholinergic receptor agonism (Shiromani and McGinty, 1986; Quattrochi et al., 1989; Semba et al., 1990; Yamamoto et al., 1990; Semba and Fibiger, 1992; Sakai and Koyama, 1996). Accordingly, the model circuit includes a positive feedback loop between the “SubC\textsubscript{D/A}” pool and a “PPT/LDT” pool. I hypothesized that the effects of SubC\textsubscript{D/A} cholinergic receptor antagonism in vivo were due to the interruption of this positive feedback interaction. We therefore expected similar effects when mimicking in vivo cholinergic receptor antagonism in silico, by varying the weight of synaptic inputs from the “SubC\textsubscript{D/A}” pool to the “PPT/LDT” pool.

The model circuit was created and simulated using Circuit SIMulator (Natschlager T, 2003) (CSIM, www.lsm.tugraz.at) interfaced with MATLAB (R14). The circuit is made-up of leaky integrate-and-fire type model neurons (Stein, 1965; Knight, 1972; Burkitt, 2006; Brunel and van Rossum, 2007) for which, membrane potential \( \nu(t) \) is given by:

\[
\tau_m \frac{dv}{dt} = -v(t) + RI_{syn}(t)
\]

where, \( \tau_m \) is the membrane time constant, \( R \) is the membrane resistance, \( I_{syn} \) is the current supplied by the input synapses. As with all integrate and fire neuronal models, spikes are not
explicitly modelled, but rather when membrane potential reaches a set threshold a spike is registered and membrane potential immediately resets to a defined reset potential.

The REM-on, REM-off, “SubC\textsubscript{D/A}”, and “PPT/LDT” pools contain 4, 4, 2, and 1 neuron respectively. Neuron parameters were identical in all cases except for the REM-off pool where the membrane resistance parameter was varied to produce neurons having different intrinsic excitabilities (needed to produce dynamic “NREM-to-REM” transitioning). Synaptic weights were set heuristically. Model parameters were constrained to produce neuronal firing frequencies of 20Hz or less.

3.2.8 Statistics
Data are presented as means ± SEM unless otherwise indicated. The analyses performed for each statistical test are included in the text where appropriate. For all comparisons, differences were considered significant if the null hypothesis was rejected at $p < 0.05$ using a two-tailed test. Where post hoc comparisons were performed after ANOVA-RM, the Bonferroni-corrected p-value was used to test statistical significance. All analyses were performed using SigmaStat (SPSS, Chicago, IL, USA).

3.3 Results
Our main objective was to determine the role of endogenous cholinergic input to the SubC\textsubscript{D/A} in the generation of REM sleep using local muscarinic receptor antagonism. To that end, in Study 1 we determined the concentration of antagonist required to block the effects of exogenous
cholinergic SubC<sub>D/A</sub> stimulation with the expectation that this concentration would be sufficient to effectively block endogenous cholinergic neurotransmission.

Consistent with previous reports, microperfusion of the cholinergic receptor agonist carbachol in the SubC<sub>D/A</sub> produced near-constant wakefulness characterized by abnormal motor behaviour in all rats (Figure 8A-B (example hypnograms) and C (group data); ACSF versus carbachol treatment, t<sub>3</sub> = 14.25, p < 0.001, post hoc paired t test after identification of significant interaction between the factors of treatment and state: F<sub>4,12</sub> = 75.06, p < 0.001, two-way ANOVA-RM). Abnormal motor behaviour included hyperactive circling, forceful kicking of the hind limbs, explosive jumping, and immobility accompanied by tetanic-like muscle contraction. Importantly, the induction of wakefulness and irregular motor behaviour was completely blocked by co-administration of the muscarinic receptor antagonist scopolamine at a concentration of 1mM (Figure 8D (example hypnogram) and C (group data); ACSF versus carbachol/scopolamine mixture, t<sub>3</sub> = 0.33, p = 0.751, post hoc paired t test after identification of significant interaction between the factors of treatment and state (detailed above).

In Study 2, to confirm our ability to effectively target and predictably modulate REM sleep circuitry in the SubC<sub>D/A</sub> free of the confounding effects of motor excitation, we broadly inactivated the region by microperfusing a combination of D-AP5 (NMDA receptor antagonist, 200µM) and muscimol (GABA<sub>A</sub> receptor agonist, 10µM). REM sleep amount, as a percentage of the total recording time, was suppressed 92.2% relative to ACSF controls. (Figure 8E (example hypnogram) and F (group data); t<sub>6</sub> = 2.24, p = 0.045, post hoc paired t test after identification of significant interaction between the factors of treatment and state: F<sub>2,12</sub> = 7.36, p = 0.008, two-way ANOVA-RM) while NREM sleep time increased significantly ( t<sub>6</sub> = 3.37, p = 0.003, post hoc paired t test).
A: ACSF

B: Carbachol (0.5mM)

C: Carbachol (0.5mM) + Scopolamine (1mm)

D: Carbachol + Scopolamine (1mm)

E: D-AP5 (200 μM) + Muscimol (10 μM)

F: ACSF

G: Scopolamine (1mM)

H: ACSF

I: Mean Bout Frequency (bouts/hr)

J: Mean Bout Duration (sec)
Figure 8. Cholinergic input to the SubCD/A is not necessary for REM sleep generation.

Example hyponograms and group data showing the effects of drug microperfusion into the SubCD/A on sleep architecture from studies 1-3. All hypnograms show representative sleep architecture over one hour periods. (A-D) show the results of study 1 (n=4): carbachol (cholinergic receptor agonist; 0.5mM) and carbachol combined with scopolamine (muscarinic receptor antagonist; 1mM). (E-F) show the results of study 2 (n=6): combined microperfusion of D-AP5 (NMDA receptor antagonist; 1mM) and muscimol (GABAA receptor agonist; 10µM). (G-J) show the results of study 3 (n=14): microperfusion of scopolamine alone (1mM). All values are means ± SEM. * indicates significant differences compared to the respective ACSF control (p < 0.05, from paired t-test).
In Study 3 we microperfused scopolamine alone in the SubC$_{D/A}$ at the concentration shown to be effective in Study 1 (1mM). REM sleep amount was not significantly affected in comparison to ACSF controls (Figure 8G (example hypnogram) and H (group data), non-significant main effect of scopolamine treatment: $F_{1,13} = 0.38$, $p = 0.551$, two-way ANOVA-RM). REM sleep bout frequency was also unaffected (Figure 8I, non-significant main effect of scopolamine treatment: $F_{1,13} = 1.11$, $p = 0.311$, two-way ANOVA-RM). Scopolamine microperfusion did, however, produce a modest shortening of REM sleep bout length ($t_{13} = 2.70$, $p = 0.011$, post hoc paired t-test after identification of significant interaction between the factors of treatment and state: $F_{2,26} = 8.44$, $p = 0.002$, two-way ANOVA-RM). These data suggest that SubC$_{D/A}$ cholinergic neurotransmission is unnecessary for REM sleep generation.

While the results of Study 3 indicate that cholinergic neurotransmission in the SubC$_{D/A}$ is not involved in triggering transitions into REM sleep, an analysis of NREM-to-REM sleep transition dynamics shows that cholinergic input may reinforce transitions once initiated. Figure 9A shows the state-space trajectories of successful NREM-to-REM sleep transitions from a single representative animal; each individual plotted line, projecting from NREM to REM sleep transition boundaries, is the state-space trajectory of a successful NREM-to-REM sleep transition. Figures 9B (example) and C (group data) show that scopolamine microperfusion in the SubC$_{D/A}$ increased NREM-to-REM sleep transition duration (significant main effect of treatment: $F_{1,13} = 35.14$, $p < 0.001$, one-way ANOVA-RM).

We also determined NREM-to-REM sleep transition duration using state acceleration plots. While electroencephalographic state is constantly changing: i.e., from one time point to the next an animal’s position in state-space will always change (state speed is always $> 0$), figure 9D shows that, prior to transitions from NREM-to-REM sleep, state speed is nearly constant on
average (i.e., mean state acceleration magnitude \(\approx 0\)). However, NREM-to-REM sleep transitions are marked by significant increases in state acceleration magnitude. Here we define transition onset as the recording epoch where the 95% confidence intervals of the acceleration magnitude versus time plot and the pre-transition acceleration magnitude mean, first separate (i.e., acceleration phase). The time point when these confidence intervals first overlap again after a subsequent deceleration phase denotes transition completion. Comparison of the acceleration magnitude versus time plots for control and scopolamine microperfusion conditions shows that — like with the analysis of state-space trajectories — blocking cholinergic neurotransmission in the SubC\textsubscript{D/A} prolonged NREM-to-REM sleep transitions (in this case by \(\sim 20\) seconds). It is possible that the apparent decrease in mean REM sleep bout length following scopolamine microperfusion was the result of increased NREM-to-REM sleep transition duration.

Figure 9E shows the state-space trajectories of attempted NREM-to-REM sleep transitions recorded during ACSF microperfusion in a single representative animal. Unlike the successful transition trajectories shown in figure 9A, these trajectories fail to cross the REM sleep transition boundary, and instead recoil into waking or NREM state-space. Figures 9F (example) and G (group data) show that scopolamine microperfusion in the SubC\textsubscript{D/A} increased the number of failed transitions, and therefore reduced the efficiency of REM sleep initiation (significant main effect of treatment: \(F_{1,13} = 6.03, p < 0.029\), one-way ANOVA-RM).

Cholinergic neurotransmission in the SubC\textsubscript{D/A} also functions to reinforce the electroencephalographic changes that occur in REM sleep. Figure 10A shows an example EEG spectrogram containing NREM and REM sleep segments. Moving from NREM to REM sleep, relative spectral power in the EEG shifts from the delta frequency band (i.e., 1-4 Hz) towards the theta band, particularly the 7-9 Hz range. Figure 10B shows that, during scopolamine
Figure 9: Cholinergic input to the SubC<sub>D/A</sub> increases the reliability of REM sleep
generation. Shown are the results of a state-space based analysis of NREM-to-REM sleep
transition dynamics. (A-B) Example trajectories of NREM-to-REM sleep transitions in
conditions of ACSF and scopolamine microperfusion into the SubC<sub>D/A</sub> respectively. Each plotted
line projecting from NREM to REM sleep transition boundaries is the state-space trajectory of a
successful NREM-to-REM sleep transition. (C) Group data showing the effect of scopolamine
microperfusion into the SubC<sub>D/A</sub> on the duration of successful NREM-to REM sleep transitions.
(D) Mean state acceleration across NREM-to-REM sleep transitions in ACSF and scopolamine
microperfusion conditions. Note that transition onset is normally marked by the beginning of an
acceleration phase (separation of the 95% confidence interval (CI) of the acceleration mean from
that of the pre-transition acceleration mean). Transition termination is marked by the end of a
subsequent deceleration phase. (E-F) Example trajectories of failed NREM-to-REM sleep
transitions in ACSF and scopolamine microperfusion conditions. (G) Group data showing the
effect of scopolamine microperfusion into the SubC<sub>D/A</sub> on the NREM-to-REM sleep transition
efficiency (# of successful transitions/ (sum of successful and failed transitions). NREM-to REM
sleep transition plots are representative of 1 hour of recording. Values in C and G are means ±
SEM. * indicates significant differences compared to the respective ACSF control (p < 0.05,
from paired t-test).
Figure 10: Cholinergic input to the SubC<sub>D/A</sub> reinforces the activation of theta oscillations in REM sleep. Example spectrograms and group data showing the effects of scopolamine microperfusion into the SubC<sub>D/A</sub> on electroencephalographic activity across sleep-wake states. (A-B) Representative EEG tracings across NREM-to-REM sleep transitions and their corresponding spectrograms taken from ACSF and scopolamine microperfusion conditions respectively. Transition boundaries (vertical dotted lines) were defined using the state-space method described in Figure 2A. (C-E) Group data showing the effects of scopolamine versus ACSF microperfusion on relative EEG power distributions in wakefulness, NREM sleep and REM sleep respectively. (F) Group data showing the absolute changes in relative EEG power in REM sleep with respect to NREM sleep, i.e., the REM sleep-specific effects of scopolamine microperfusion on electroencephalographic activity. All values are means ± SEM (indicated by grey bands). * indicates significant differences compared to the respective ACSF control ($p < 0.05$, from paired t-test).
microperfusion in the SubC<sub>D/A</sub>, this shift in relative power is attenuated relative to control. Group data in figure 10C-E shows that, in REM sleep, microperfusion of scopolamine into the SubC<sub>D/A</sub> significantly increased relative power in the 1-3Hz, 3-5Hz, and 9-11Hz frequency ranges while relative power in the 7-9Hz range decreased significantly (range of t<sub>13</sub> = 7.11 to 1.40, range of p < 0.001 to = 0.042, post hoc paired t tests after identification of significant interaction between the factors treatment and frequency band: F<sub>15,195</sub> = 4.60, p < 0.001, two-way ANOVA-RM). This EEG effect is also evident in the example state space plots shown in figures 9A-B, where in the scopolamine condition the combination of increased delta power and reduced theta power in REM sleep is responsible for the downward-rightward shift of the REM cluster towards the NREM sleep cluster. In figure 10F we plotted the difference of the NREM and REM sleep relative power spectra, which shows the REM sleep-specific effects of scopolamine microperfusion on EEG power. This analysis shows that the major EEG change occurring in REM sleep – increased relative power in the 7-9Hz range – was significantly attenuated by microperfusion of scopolamine into the SubC<sub>D/A</sub> (t<sub>13</sub> = 7.86, p < 0.001, post hoc paired t test after identification of significant interaction between the factors treatment and frequency band: F<sub>15,195</sub> = 5.06, p < 0.001, two-way ANOVA-RM). Also, the normal suppression of relative power in the 9-11Hz band occurring in REM sleep compared to NREM sleep was similarly attenuated (t<sub>13</sub> = 2.35, p < 0.020, post hoc paired t test). Postural muscle tone in REM sleep was unaffected by scopolamine microperfusion in the SubC<sub>D/A</sub> relative to ACSF controls (ACSF control (0.740 ± 0.291 arbitrary units) versus scopolamine (0.741± 0.304); non-significant main effect of treatment: F<sub>1,13</sub> = 0.0018, p = 0.097, one-way ANOVA-RM).

Taken together, the above results suggest that cholinergic neurotransmission in the SubC<sub>D/A</sub> is not a determinant of REM sleep quantity or frequency; rather, cholinergic input functions to
Figure 11: In vivo effects of SubC<sub>D/A</sub> cholinergic receptor blockade can be replicated in silico when cholinergic input to the SubC<sub>D/A</sub> operates as positive feedback.

(A) Wiring diagram of the modelled circuit (the rationale for its topology is detailed in the methods). (B) Normalized (% of maximum) firing frequency versus time plots for model neurons in the SubC<sub>D/A</sub> pool for different weights of the cholinergic PPT/LDT pool input (i.e., W<sub>ACh</sub>). (C) Absolute firing frequency versus time for SubC<sub>D/A</sub> pool neurons at different values of W<sub>ACh</sub>. Time zero equals transition onset and white dotted lines indicate transition endpoints. (D-E) The effect of varying W<sub>ACh</sub> on transition length and maximum SubC<sub>D/A</sub> firing rate respectively. (F) Absolute firing frequency versus time plots for model neurons in the REM-off pool for maximum and inactive W<sub>ACh</sub>. The black dotted line indicates the onset of inhibitory graded input to the REM-off pool. (G) Firing acceleration in the REM-off pool for maximum and inactive W<sub>ACh</sub>; * indicates the occurrence of an acceleration in REM-off neuronal firing. All firing rate data were smoothed by moving average.
reinforce both the transitioning into, and the electroencephalographic features of, REM sleep. We hypothesized that these findings can be explained by a mutually excitatory interaction between the SubC\textsubscript{D/A} and the cholinergic PPT/LDT where, importantly, cholinergic neuron activation is gated by SubC\textsubscript{D/A} activity. We used simulations of a model REM sleep circuit to test the plausibility of this hypothesis (see figure 11A for circuit wiring diagram and refer to the methods section for a detailed rationale). We mimicked in vivo muscarinic receptor antagonism by varying the weight of synaptic input from the “PPT/LDT” pool to the “SubC\textsubscript{D/A}” pool.

Figure 11B shows normalized “SubC\textsubscript{D/A}” firing frequency versus time plots for different levels of cholinergic input to the “SubC\textsubscript{D/A}”. Importantly, reducing or removing cholinergic positive feedback to the “SubC\textsubscript{D/A}” pool did not delay transition onset and therefore did not affect the activation threshold of the SubC\textsubscript{D/A} pool — likely a major determinant of REM sleep quantity in vivo. Nevertheless, reducing cholinergic input to the “SubC\textsubscript{D/A}” pool resulted in transition lengthening and suppression of peak “SubC\textsubscript{D/A}” pool firing rates (figure 11B-E). Recall that in vivo, blockade of muscarinic neurotransmission in the SubC\textsubscript{D/A} likewise increased NREM-to-REM sleep transition duration without a concomitant reduction in REM quantity. Figures 11F-G show REM-off pool firing frequency and acceleration during the “NREM-to-REM sleep” transition. Notice that in the absence of cholinergic positive feedback ($W_{ACH} = 0$), the activity of the mutually inhibitory feedback loop produced alternating periods of firing deceleration and acceleration in the REM-off pool. Transitions are ultimately successful because deceleration amplitude exceeds acceleration amplitude ensuring eventual inactivation of the REM-off pool. While our simple model circuit is not capable of producing failed transitions, accelerations in REM-off (i.e., GABAergic REM-inactive vLPAG/DpMe) neuron firing is a plausible source of the transition failure occurring in vivo. Therefore, of relevance to our in vivo finding that
NREM-to-REM transitions failed less often when muscarinic neurotransmission in the SubC_D/A was intact, the addition of cholinergic positive feedback into the SubC_D/A potentiated REM-off decelerations while reducing the frequency of accelerations.

Our findings can be taken to suggest that cholinergic input to the SubC_D/A plays a minor modulatory role in REM sleep generation: reinforcing transitions into REM sleep through a potential positive feedback interaction between the SubC_D/A and the PPT/LDT. If this interpretation is valid, we would expect selective activation of PPT/LDT cholinergic neurons to produce a reinforcement of REM sleep transitioning that contrasts the effects of cholinergic receptor antagonism in the SubC_D/A. To selectively activate cholinergic cells in the PPT we used microperfusion of urotensin-II. Consistent with a previous report, figure 12A-B shows that microperfusion of urotensin-II in the PPT enhanced REM sleep through an increase in mean bout frequency (t_6 = 4.85 and 6.26, p = 0.009 and < 0.001 respectively, post hoc paired t tests after identification of significant main effect of treatment F_1,6 = 14.61 and 28.51 respectively, all p < 0.001, one-way ANOVA-RM). Opposite the effects of cholinergic receptor antagonism in the SubC_D/A, figure 12 C-D shows that urotensin-II microperfusion in the PPT increased NREM-to-REM sleep transition efficiency and reduced transition length (t_6 = 4.85 and 1.56, p = 0.001 and 0.044 respectively, post hoc paired t tests after identification of significant main effect of treatment: F_1,6 = 29.03 and 20.98 respectively, all p < 0.001, one-way ANOVA-RM).

Importantly, the effects of activating cholinergic PPT neurons with urotensin-II were blocked by simultaneous antagonism of muscarinic acetylcholine receptors in the SubC_D/A. Figure 13 shows the location of the micro-perfusion sites from all experiments in studies 1-4. Figure 13 A-B shows that all microdialysis probes targeted to the SubC are located within the dorsal and alpha parts as defined by Paxinos and Watson (1998). Figure 13C shows an example
Figure 12: Increased REM sleep and reinforcement of NREM-to-REM sleep transitions by PPT cholinergic neurons is prevented by SubC<sub>D/A</sub> cholinergic receptor blockade.

Group data showing the REM sleep effects of urotensin-II microperfusion into the PPT with and without simultaneous microperfusion of scopolamine into the SubC<sub>D/A</sub> (Study 4). All values are means ± SEM. * indicates significant differences compared to the respective ACSF control (p < 0.05, from paired t-test).
Figure 13: (A) Example and (B) group data showing the location of all the SubC<sub>D/A</sub> microperfusion sites from all experiments in Studies 1-4. (C) Example microperfusion lesion located adjacent to NADPH-Diaphorase positive cholinergic PPT neurons (i.e., darkly stained cluster of cell bodies). (D) Group data showing the location of all the PPT microperfusion sites from experiments in Studies 4. Arrows in A and C indicate the location of microperfusion lesion sites. In B and D, red circles indicate the midpoint positions of the permeable membrane portion of the microdialysis probes while the surrounding grey boxes are scale representations of the position of the entire membranes. LDT: laterodorsal tegmental nucleus, SubC<sub>D/A</sub>: subcoeruleus dorsal and alpha parts, Mo5: trigeminal motor pool, CnF: cuneiform nucleus, scp: superior cerebellar peduncle, PPT: pedunculopontine tegmental nucleus, PnO: oral part of the pontine reticular nucleus.
microperfusion lesion located adjacent to NADPH-Diaphorase positive cholinergic PPT neurons (i.e., darkly stained cluster of cell bodies). Figure 13D shows the location of all the PPT microperfusion sites from experiments in Studies 4. The sites of micro-perfusion are within or adjacent to the PPT in all rats.

3.4 Discussion

If cholinergic input to the SubC_D/A were critically involved in the generation of REM sleep, we would expect antagonism of SubC_D/A acetylcholine receptors to reduce REM sleep amounts. However, here we show that local antagonism of SubC_D/A muscarinic acetylcholine receptors did not suppress REM sleep quantity or bout frequency. Since the same concentration of scopolamine was sufficient to block the effects of exogenous cholinergic stimulation, we expect that endogenous cholinergic input to the SubC_D/A was effectively inhibited. Therefore, our data suggests that REM sleep drives to the SubC_D/A are acetylcholine independent – i.e., cholinergic input to the SubC_D/A is not necessary for REM sleep generation.

Our data are consistent with previous preliminary studies reporting the effects of SubC_D/A cholinergic receptor antagonism. George et al., (1964), in their seminal study of carbachol induced REM sleep, reported that while pontine microinjections of either carbachol or oxotremorine (a muscarinic agonist) induced atropine-sensitive REM sleep, atropine alone had no “visible effects”. However, atropine was focally delivered in only two cats and no data were shown. Similar studies in rats by Gnadt and Pegram ((1986) n=5) and Bourgin et al., ((1995) n=3, data were not shown in their study) reported no effects on REM sleep of pontine atropine microinjections at doses sufficient to prevent the wake and REM sleep-inducing effects of carbachol.
Conversely, two studies have reported REM sleep reductions following infusions of cholinergic receptor antagonists. Firstly, Shiromani and Fishbein (1986) reported significant reductions of REM sleep following pontine infusions of scopolamine. However, infusions took place continuously over 5 days using a chronically implanted mini-pump. In this case, scopolamine delivery was therefore more akin to wide-reaching systemic administration and not indicative of blocking muscarinic neurotransmission in the pontine reticular formation per se. Secondly, Imeri et al., (1994) reported reductions in REM sleep following pontine microinjections of methoctramine, a muscarinic receptor antagonist; however, reduced REM sleep may have been a secondary effect of arousal since NREM sleep time was also reduced while wakefulness increased.

Past models of cholinergic involvement in REM sleep generation — e.g., the reciprocal interaction hypothesis — claim that cholinergic input facilitates the initial activation of the SubC_D/A (Pace-Schott and Hobson, 2002). If this were the case, blockade of cholinergic input to the SubC_D/A would have the effect of increasing the activation threshold for REM sleep – i.e., increased levels of GABAergic disinhibition and/or glutamatergic excitation would be needed to initially activate the SubC_D/A and initiate a NREM-to-REM sleep transition. However, our finding that REM sleep amount and bout frequency were unaffected by blockade of muscarinic neurotransmission does not support this mechanism, since we would predict less REM sleep and/or reduced bout frequency when increasing the activation threshold for REM sleep.

Therefore, contrary to some previous models, our data suggest that cholinergic input is not a source of extrinsic feed-forward inductive drive to REM sleep-generating circuitry.

Importantly, our data does indicate that cholinergic input serves to reinforce REM sleep transitions once they are initiated. Our analysis of NREM-to-REM sleep transition dynamics
revealed that blocking muscarinic neurotransmission in the SubC\textsubscript{D/A} increased both the duration and failure rate of transitions into REM sleep. The PPT and LDT nuclei, which contain subsets of neurons that are maximally-active in REM sleep, are the major sources of cholinergic input to the SubC\textsubscript{D/A}. Moreover, the interaction between these two pools is reciprocal, since SubC\textsubscript{D/A} neurons likewise project to the PPT/LDT (Semba and Fibiger, 1992). Our findings show that cholinergic PPT neurons are capable of reinforcing NREM-to-REM sleep transitioning through the SubC\textsubscript{D/A}. Selective activation of PPT cholinergic neurons with urotensin-II decreased the duration and failure rate of transitions into REM sleep, and these effects were blocked by cholinergic receptor antagonism in the SubC\textsubscript{D/A}. Using computational modelling I showed that the in vivo results are consistent with there being a mutually excitatory interaction between the “SubC\textsubscript{D/A}” and “PPT/LDT” pools where, importantly, cholinergic neuron activation is gated by “SubC\textsubscript{D/A}” activity. Modelling showed that with this sequence of activation, reducing or eliminating “PPT/LDT” input to the “SubC\textsubscript{D/A}” pool did not affect the activation threshold of “REM sleep”, i.e., time of transition onset for the “SubC\textsubscript{D/A}” pool was unchanged. However, following transition onset, positive feedback from the “PPT/LDT” pool accelerated firing in the “SubC\textsubscript{D/A}” pool and reduced “NREM-to-REM sleep” transition duration. Our in vivo data also shows that cholinergic input to the SubC\textsubscript{D/A} normally acts to protect against the failure of NREM-to-REM sleep transitions.

Antagonism of muscarinic neurotransmission in the SubC\textsubscript{D/A} also attenuated the increase in relative EEG theta power that characterizes REM sleep. Hippocampal theta oscillations are produced by septo-hippocampal projections (Gerashchenko et al., 2001). The SubC\textsubscript{A} contains glutamatergic neurons projecting to the medial septum and lesioning these SubC\textsubscript{A} cells eliminates theta oscillations during REM sleep (Lu et al., 2006). Therefore, our data would
indicate that cholinergic input to the SubC<sub>D/A</sub> contributes to the generation of theta oscillations in REM sleep through facilitation of a SubC<sub>D/A</sub>-septum-hippocampus pathway. Simulations showed that “cholinergic” positive feedback into the “SubC<sub>D/A</sub>” pool produces amplification of “SubC<sub>D/A</sub>” firing, which could underlie reinforcement of theta oscillations by SubC<sub>D/A</sub> acetylcholine in vivo. Conversely, despite evidence that carbachol activates spinally projecting SubC<sub>D/A</sub> neurons via pre and post-synaptic muscarinic mechanisms in vitro (Weng et al., 2014), we observed no reduction in motor atonia during REM sleep following antagonism of SubC<sub>D/A</sub> muscarinic neurotransmission.

In summary, cholinergic input to the SubC<sub>D/A</sub> is not a determinant of REM sleep quantity, but does function to enhance the reliability of REM sleep generation by reinforcing NREM-to-REM sleep transitions: making them shorter and less prone to failure. Cholinergic input to the SubC<sub>D/A</sub> also reinforces the activation of theta oscillations in REM sleep. We argue that these results do not support the claim that cholinergic input provides a feed forward inductive drive to REM sleep generating circuitry. Rather, these data are more consistent with the interpretation that cholinergic inputs to the SubC<sub>D/A</sub> are a source of positive feedback.

The construction of the model REM sleep circuit that we present in this study can be described as three interlocking positive feedback loops. The first loop is the mutually inhibitory interaction between GABAergic REM-on and REM-off neurons. The second is the mutually excitatory interaction between the GABAergic switch and the SubC<sub>D/A</sub>, while the third and final loop is the mutually excitatory interaction between the SubC<sub>D/A</sub> and the cholinergic PPT/LDT. Note that while only a single positive feedback loop is required to create bistability in a signaling pathway, nested feedback loops are nevertheless common in biology. For instance, the polarization of cell growth in yeast (Wedlich-Soldner and Li, 2004), mammalian calcium signaling (Lewis, 2001)
and the maturation of xenopus oocytes (Abrieu et al., 2001) all utilize interlocking positive feedback loops. Brandman et al. (2005) has shown that an advantage of using multiple interlocking feedback loops over single loops is that a multiple-loop construction can produce more reliable switches that are rapidly inducible and more resistant against sources of extrinsic noise.

A positive feedback mechanism can also account for why modulating endogenous SubC_D/A acetylcholine has little effect on REM sleep while exogenous cholinergic stimulation can potently induce REM sleep. Our own data show that exogenously activating cholinergic PPT input to the SubC_D/A increased REM sleep, however, cholinergic receptor antagonism at the SubC_D/A did not decrease REM sleep amounts below baseline levels. Exogenous cholinergic stimulation of the SubC_D/A effectively mimics the action of an extrinsic feed-forward inductive drive. However, in the event that endogenous cholinergic input provides positive feedback, the effects of exogenous stimulation would not be indicative of the normal function of SubC_D/A cholinergic inputs. In this case, exogenous stimulation would unphysiologically reverse the normal sequence of SubC_D/A and cholinergic neuron activity. Therefore, in the case that cholinergic input is providing a positive feedback to the SubC_D/A, cholinergic receptor antagonism could have little or no effect on REM sleep quantity despite there being a major capacity for exogenous cholinergic stimulation to initiate REM sleep or its component parts. Consistent with this interpretation, the increase in firing rates of REM sleep-active GABAergic and glutamatergic neurons in the pontomesencephalic tegmentum that occurs during the NREM to REM sleep transition period precedes that of cholinergic LDT/PPT neurons (Boucetta et al., 2014).
I have previously shown that, the predominately GABAergic and glutamatergic, REM sleep-active neurons of the PPT prevent REM sleep initiation, particularly during periods of low REM sleep drive propensity (Grace et al., 2012). It is possible that, PPT subpopulations exert opposing yet complementary influences on REM sleep. Non-cholinerigc REM sleep-max active PPT neurons may function to raise the drive threshold for REM sleep induction in order to limit REM sleep episodes to periods of high propensity; following the onset of a transitioning into REM sleep, cholinergic PPT neurons may be recruited to reinforce switching and increase the probability of transition success.

While cholinergic agonism of the SubC_D/A can trigger REM sleep, it also induces prolonged bouts of wakefulness characterized by abnormal motor behaviour in both cats (Mitler and Dement, 1974; van Dongen, 1980; Baghdoyan et al., 1984b) and rodents (Gnadt and Pegram, 1986; Mastrangelo et al., 1994; Deurveilher et al., 1997). Consistent with previous rodent studies, the major effect of carbachol delivery in our study was induction of wakefulness. We hypothesized that the induction of wakefulness was an epiphenomenon of motor activation. REM sleep generating neurons in SubC_D/A overlap with pools of cholinceptive reticulospinal cells that facilitate muscle activity and locomotion (Lai and Siegel, 1991). These pools are innervated by the mesencephalic locomotory region, stimulation of which produces motor activity like that seen in this and other studies – i.e., hyperactive circling, forceful kicking of the hind limbs, explosive jumping and freezing (Depoortere et al., 1990). Importantly, we confirmed that we had effectively targeted REM sleep circuitry and that we could predictably modulate REM sleep generation by avoiding the confounding activation of locomotion circuitry. Reticulospinal cells responsible for motor activation are driven by NMDA receptor-dependent glutamatergic inputs (Lai and Siegel, 1991). Simultaneous delivery of a NMDA receptor
antagonist and a GABA<sub>A</sub> receptor agonist effectively prevented motor activation while strongly suppressing REM sleep generation. Moreover, indirect enhancement of SubC<sub>D/A</sub> acetylcholine release through activation of cholinergic PPT neurons successfully increased REM sleep. These findings support the interpretation that wakefulness induced by direct cholinergic stimulation of the SubC<sub>D/A</sub> is the result of motor activation that effectively conceals any REM sleep effects.

3.4.1 Summary

(1) The capacity of SubC<sub>D/A</sub> cholinergic afferents to generate REM sleep has been firmly established by gain-of-function experiments. (2) The function of endogenous SubC<sub>D/A</sub> cholinergic input in REM sleep generation cannot be determined by gain-of-function experiments; rather, loss-of-function studies are required. (3) Loss-of-function studies show that endogenous cholinergic input to the SubC<sub>D/A</sub> is not required for REM sleep generation. (4) Cholinergic input to the SubC<sub>D/A</sub> serves an accessory role in REM sleep generation: reinforcing NREM-to-REM sleep transitions making them quicker and less likely to fail.
4.1 Introduction

One of the fundamental features of mammalian sleep is its separation into two distinct states: REM sleep and NREM sleep. While many neuronal populations have been implicated in the control of these sleep states, the circuit logic underlying sleep bistability is poorly understood. The question of how sleep bistability arises is of particular salience when considered in the context of prevalent flip-flop hypotheses of sleep-state switching (Lu et al., 2006b; Sapin et al., 2009). Flip-flop hypotheses posit that sharp transitioning between sleep-states is the product of a mutual inhibition between independent NREM and REM sleep generative networks. Flip-flop switches are common in biology, and primarily serve to convert graded input signals, which change slowly over time, into binary outputs that limit the occurrence of intermediate states that would dominate in their absence (Brandman et al., 2005; Brandman and Meyer, 2008).

Neuronal populations of the vlPAG and the adjacent DpMe have been implicated in the control of sleep-wake state and are hypothesized components of REM sleep generating flip-flop circuitry (Luppi et al., 2007; Saper et al., 2010; Fraigne et al., 2015). There is significant potential for functional complexity in the vlPAG/DpMe region. Based on measures of c-fos expression following REM sleep deprivation and recovery, the vlPAG/DpMe region contains some of the largest populations of REM sleep-inactive and REM sleep-active neurons in the brainstem (24% of the total REM sleep-active population)(Sapin et al., 2009). Extracellular single unit recordings show that the REM sleep-active pool can be further subdivided on the basis of activity profile.
(wake/REM sleep active neurons versus neurons specifically active in REM sleep) and on the basis of firing pattern (phasic firing versus tonic firing neurons) (Crochet et al., 2006).

Inactivation of the vlPAG/DpMe reliably increases REM sleep amounts in rat (Lu et al., 2006b; Sapin et al., 2009), mouse (Weber et al., 2015), cat (Petitjean et al., 1975; Sastre et al., 1996; Crochet et al., 2006), and guinea pig (Vanini et al., 2007) by means of lesioning, focal muscimol delivery, and optogenetic stimulation of ventral medullary GABAergic axons in the vlPAG/DpMe. Reported increases in REM sleep time ranged from 130-380% of baseline and, in most cases, resulted from increases in both the duration and frequency of episodes. Based on these findings and neuroanatomical evidence of vlPAG/DpMe connectivity with groups that are majorly involved in the control of sleep state, current hypotheses of vlPAG/DpMe involvement in REM sleep control are focused on the role of REM sleep-inactive GABAergic neurons in gating NREM-to-REM sleep transitioning, possibly through participation in a mutually inhibitory flip-flop switch (Lu et al., 2006b; Sapin et al., 2009; Saper et al., 2010).

However, there is, as yet, little direct evidence that neurons within the vlPAG/DpMe enforce sleep bistability. In order to measure changes in sleep stability, we must quantify NREM-REM sleep intermediate states. Such states develop naturally during normal transitioning from NREM to REM sleep (Benington et al., 1994; Boucetta et al., 2014). We have previously quantified changes in NREM-to-REM sleep transition dynamics to show that cholinergic inputs to the SubC make transitions quicker and less likely to fail (Grace et al., 2014a).

Here the results of inactivating vlPAG/DpMe neurons is presented and it is shown that a detailed analysis of bout statistics and NREM/REM transitionary sleep does not support major vlPAG/DpMe involvement in gating NREM-to-REM sleep transitions; the data better supports a major vlPAG/DpMe involvement in preventing breakdowns in bistability during REM sleep.
4.2 Methods

4.2.1 Animal care
Experiments were performed on a total of 25 male Wistar rats (Charles River, Senneville, QC, Canada) (mean body weight = 285.2 g ±1.6 (SEM), range 276-300g). Procedures conformed to the recommendations of the Canadian Council on Animal Care, and the University of Toronto Animal Care Committee approved the protocols. Rats were housed individually, maintained on a 12-12h light/dark cycle (lights on at 0700 h), and had free access to food and water.

4.2.2 Surgery
Rats were chronically implanted with electrodes for recording sleep-wake state: EEG and EMG of the trapezius muscle. Sterile stereotaxic (model 962; David Kopf Instruments) surgery was performed as previously described in chapter 3.

Microdialysis guides (CXG-6; Eicom, San Diego, CA, USA) were targeted to a position 0.8 mm anterior to lambda (as redefined by Paxinos and Watson (1998) as the midpoint of the curve of best fit along the lambdoidal suture), 0.6 mm lateral to the midline, and 3.7 mm ventral to lambda. These coordinates correspond to a position 2.5 mm above the targeted region of drug delivery in the vlPAG/DpMe (Paxinos and Watson, 1998). Dummy cannulas were placed inside the microdialysis guides to keep them free of debris until the day of the experiment. Microdialysis probes projected an additional 2.5 mm from the guide and so targeted the
Following surgery, rats were housed individually and were allowed to recover for at least 6 days before the experiments were performed.

4.2.3 Microdialysis

Habituation of rats to the recording environment was performed as described in Chapter 3. At the time of recording cable attachment, the internal cannula was removed from the guide, and the microdialysis probe was inserted (CX-I-8-005; 19nL tip volume; 220 µm diameter, 500 µm long membrane; 50,000 Dalton cut-off; Eicom). The probes were connected to FEP Teflon tubing (inside diameter, 0.12 mm) with this tubing connected to 1.0 ml syringes via a zero dead space switch (Uniswitch, BAS, West Lafayette, IN, USA). The probes were perfused with freshly-made artificial cerebral spinal fluid (ACSF) at a flow rate of 2.1µl/min.

4.2.4 Experimental design

The study consists of separate time-control (n=13) and drug groups (n=12). Experiments took place between 1000 and 1430 hours. Signals were recorded continuously during this time. Between 1000 and 1200 hours, ACSF was microperfused in all animals. Between 1200 and 1430 hours, ACSF microperfusion was maintained in the time-control group, while the drug-group received microperfusion of the GABA<sub>A</sub> receptor agonist muscimol (Tocris Bioscience; 85µM in ACSF (microdialyzed solution); 1µg/hour (expected rate of drug delivery into the brain). Muscimol concentrations of 100µM, 500µM and 1mM, administered by reverse microdialysis (Eicom, probe model: A-L-50-01; 2 hour microperfusion), were used previously in cats to inactivate the vlPAG/DpMe (Crochet et al., 2006). In that study, muscimol inactivation of the
vlPAG/DpMe had a REM sleep enhancing effect stemming from an increase in the duration and frequency of REM sleep episodes. I found, in preliminary experiments, that muscimol concentrations above 200uM consistently resulted in prolonged wakefulness characterized by hyperactive motor behaviour. Data collected between 1200 and 1230 hours was excluded from analysis to allow microperfused muscimol to suffuse the vlPAG/DpMe region (i.e., the first 30 minutes following the switch from ACSF to muscimol).

Following completion of the experiments, the rats were killed and their brains were removed to determine the position of microperfusion sites. The locations of these sites were recorded on standard coronal drawings from the stereotaxic atlas of the rat brain prepared by Paxinos and Watson (1998). From the positions of the microperfusion sites in our experiments, we predicted the approximate tissue volumes where we expect muscimol mediated suppression of neuronal activity to have occurred — based on a previous study by Arikan et al. (2002). That study measured changes in spontaneous neural activity surrounding microinjections of titrated muscimol (20µg in 1ul) prior to quantifying tissue levels of bound muscimol using autoradiography. Over a 2.5 hour period post injection, the maximum tissue volumes where muscimol produced any observable suppression or complete suppression of neuronal activity, had radii of 1 mm and 0.4mm respectively. Over a 2.5 hour time period, the volume of tissue where muscimol produces neuronal suppression in the case of a 20µg microinjection, can be considered a conservative indicator of the volume likewise modulated by reverse microdialysis of an 85µM solution. In support of this claim, I performed diffusion simulations to determine the relative muscimol tissue exposure for a 20µg microinjection versus microdialysis of an 85µM solution (MATLAB smooth3 function; matrix size = 250x250x250; convolution kernel size = 3; Gaussian filter with a standard deviation of 1.2; microinjection, initial value = 1.75x10^{-7} moles; microdialysis, initial value = 1.75x10^{-10} moles, reset after every smoothing iteration (equivalent
to the number of moles in 2.1μl of 85μM muscimol solution, perfusing the microdialysis probe tip every minute). The standard deviation of the gaussian filter was set such that the maximum concentration adjacent to the diffusion site approaches a value 15-20% of the concentration of the microdialyzed solution. This range is taken from measurements in agarose tissue phantoms, reported in section 5.2.3, of permanganate concentrations adjacent to a microdialysis diffusion source (molecular weight (MW) permanganate =119g/mol versus MW muscimol = 114g/mol). Here we ignore the extracellular bulk flow induced by pressure microinjection and that the initial volume in the case of microinjection (1ul/extracellular volume fraction) is much greater than that of microdialysis (19nl).

4.2.5 Signal analyses

The details of signals processing are as described in chapter 3.

4.2.5.1 EMG and EEG signals

The data were analyzed in consecutive 5s time-bins. Moving-time averages of the neck EMG signals were quantified in arbitrary units relative to electrical zero defined as the voltage recorded with the amplifier inputs grounded. The EEG signal was subjected to a fast-Fourier transform for each 5-sec time bin. The power in frequency bands (2Hz in width) spanning the 1 to 33Hz range was calculated.

Sleep-wake states were initially identified by visual inspection, and classified into wakefulness, NREM and REM sleep according to standard criteria. Subsequent to visual scoring we used the method described in section 4.2.5.2 (NREM to REM sleep transition dynamics) to perform additional scoring of NREM/REM sleep transition (NRt) epochs.
4.2.5.2  NREM to REM sleep transition dynamics

NREM to REM sleep transition dynamics were analyzed using a state-space analysis (Gervasoni et al., 2004; Diniz Behn et al., 2010; Grace et al., 2014a). The objective of a state-space approach is to create a 2-dimensional plot bounded by electroencephalographic variables that correlate with sleep-wake state, in which the position of a single point represents the electroencephalographic state of the forebrain during a single recording epoch. The log transform of spectral power in the 1-19Hz frequency range and the log transform of the ratio of spectral power in the 7-9Hz and the 1-9Hz frequency ranges were used to create state-space plots. Data was smoothed prior to plotting using a 5-epoch wide moving average (MATLAB R2013b, Natick, MA, USA (function: smooth)). In order to analyze transitions between NREM and REM sleep states, standardized boundaries of the NREM and REM sleep state-space clusters needed to be defined. To create the boundaries I used an alternative method to the one reported previously (Section 3.2.6.2; Grace et al., 2014). Previously, I defined the position of state boundaries, demarcating NREM and REM sleep space from transitional space, by thresholding plotted points according to density. Also, with the previous method, separate state boundaries were drawn for control and drug conditions. The alternative method employed here uses a less arbitrary rule to define the boundary positions and uses only the boundaries drawn using control data, which allows for detection of breakdowns in sleep bistability. The rule used to draw state boundaries in this study is a product of our assumptions regarding the mechanisms underlying sleep state generation. We assume that the occurrence of distinct states of NREM and REM sleep is a result of distinct generating circuits imposing distinct boundary conditions on brain state over time. State dynamics observed across transitions between NREM and REM sleep are assumed to be a consequence of competition between NREM and REM sleep
generating circuits for control over brain state (i.e., a period with no prevalent set of boundary conditions). Based on these assumptions we should expect NREM and REM sleep regions of state space, in contrast to transitionary space, to contain bounded trajectories. Where a trajectory is constrained by a boundary we should expect that trajectory to undergo reversals in direction resulting in trajectory intersections (i.e., loops). Where a trajectory is unbounded and has a directional preference, looping should occur much less or not at all. For each two-hour ACSF control period, I calculated the position of trajectory intersections occurring within a one minute (12-epoch) moving window. Each intersection is bounded by four data points; fitting two separate convex envelopes to all such points visually scored as epochs of NREM and REM sleep respectively reliably produced pairs of non-overlapping state boundaries.

Not all data points located in transitionary space were classified as transitionary. To score epochs as transitionary sleep we used the following criteria, adapted from the American Sleep Disorders Association’s EEG scoring rules for EEG arousals from sleep (American Sleep Disorders Association, 1992): (i) transitionary epochs must be preceded by two consecutive epochs of NREM or REM sleep (i.e., two consecutive points located within a sleep state boundary), (ii) following a period of transitionary sleep, two consecutive epochs located within a sleep state boundary are required to score NREM-to-REM sleep or REM-to-NREM sleep transitions. (iii) where an excursion into transitionary space is terminated by an arousal or by re-entry into the state of origin, the excursion into transitionary space must be at least two epochs in duration, (iv) bouts of transitionary sleep interrupted by visually scored arousals, lasting three epochs or less, are not considered separate bouts, (v) excursions into transitionary space are scored as transitionary epochs only when such trajectories pass through the region of transitional space demarcated by NREM-to-REM sleep transition trajectories, (vi) for excursions from
NREM state space into transitionary space, only trajectories traversing transitionary space from right-to-left (i.e., towards REM sleep state space) were scored as transitionary sleep.

In addition to quantifying the frequency of individual REM sleep bouts, we quantified the frequency of REM sleep clusters (Amici et al., 2000). Any group of 2 or more REM sleep bouts occurring within 90 seconds of each other is considered a cluster. REM sleep clustering is most commonly the result of transitioning between REM sleep and NRt or brief arousal periods.

4.2.6 Statistics
For all comparisons, differences were considered significant if the null hypothesis was rejected at \( p < 0.05 \) using a two-tailed test. Where post hoc comparisons were performed after ANOVA-RM, the Bonferroni-corrected p-value was used to test statistical significance. All analyses were performed using SigmaStat (SPSS, Chicago, IL, USA).

4.3 Results
4.3.1 Evidence supporting the reliability of the NRt scoring method
To test the involvement of vIPAG/DpMe neurons in the control of sleep bistability it is necessary to quantify NREM/REM sleep transitionary states and to that end I must first validate my quantification method. I used the NREM and REM sleep state-space boundaries that were defined from data collected in period one (i.e., control period) to score NRt bouts in both periods one and two (i.e., the period of muscimol microperfusion in the drug treatment group). Given that I hypothesized that vIPAG/DpMe inhibition would compromise sleep bistability — resulting in increased time spent in transitionary sleep — I tested whether or not our NRt scoring method
is predisposed to potential false-positive NRt identification. In the group of control rats ((n=13), Figure 14) shows that there was no effect of scoring method on the measured amounts of REM sleep or wakefulness (non-significant main effect of scoring method: range of $F_{1,12} = 0.76$ and 1.02, $p = 0.400$ and 0.332 respectively, two-way ANOVA-RM). Figure 14 also shows that when NRt scoring is performed, NRt levels are offset by reduced levels of NREM sleep independent of the period ($t_{12} = 7.24$, $p < 0.001$, post hoc paired t test after identification of significant main effect of scoring method: $F_{1,12} = 52.40$, $p < 0.001$, two-way ANOVA-RM). However, NRt scoring did not result in any changes to NREM sleep levels from period one to period two that were not detected by conventional three-stage scoring (non-significant main effect of period and non-significant interaction between the factors period and scoring method: $F_{1,12} = 0.01$ and 0.002, $p = 0.926$ and 0.963 respectively, two-way ANOVA-RM). Most importantly, we found that in the control group, NRt amounts did not change, as a percentage of the total recording time, from period one to period two (non-significant effect of scoring method: $F_{1,12} = 1.85$, $p = 0.199$, one-way ANOVA-RM). In summary, the method of defining transitional sleep did not produce spurious changes in the amounts of wake, NREM, NRt, or REM sleep.

4.3.2 Effects of vIPAG/DpMe inhibition on NREM/REM transitionary sleep

Figure 15A shows that time spent in NREM sleep, as a proportion of the total recording time, was not significantly affected by muscimol microperfusion (non-significant effect of drug treatment: $F_{1,11} = 0.54$, $p = 0.480$, one-way ANOVA-RM). In contrast, inhibition of the
Figure 14. Control group validation of the NRt scoring method.

Values are means ± standard error of the mean (n=13). * indicates significant effect of NRt scoring on levels of NREM sleep independent of the period (P < 0.05, from analysis of variance).
vlPAG/DpMe increased time spent in REM sleep, as a percentage of the total recording time, relative to 3-stage and NRt baseline scoring \( (t_{11} = 4.72 \text{ and } 2.60, \ p < 0.001 \text{ and } p=0.023) \) respectively, post hoc paired t tests after identification of a significant main effect of drug treatment: \( F_{1,11} = 13.91, \ p = 0.003, \text{ two-way ANOVA-RM} \). Also, we found that inhibition of the vlPAG/DpMe increased time spent in transitionary sleep \( (t_{11} = 6.53, \ p < 0.001, \text{ post hoc paired t test after identification of significant effect of drug treatment: } F_{1,11} = 42.66, \ p < 0.001, \text{ one-way ANOVA-RM}) \). However, figure 15A shows that significant increases in REM sleep time and NRt time were accompanied by an increase in sleep opportunity, as muscimol microperfusion reduced the time rats spent awake \( (t_{11} = 2.86, \ p = 0.016, \text{ post hoc paired t test after identification of significant effect of drug treatment: } F_{1,11} = 8.17, \ p = 0.016, \text{ one-way ANOVA-RM}) \). Therefore, changes in REM sleep and NRt levels should be considered as proportions of the total sleep time.

As a proportion of the total sleep time, REM sleep time increased when using conventional 3-stage scoring; however, figure 15B shows that much of this increase in REM sleep may be better classified as transitionary sleep. We found that 59.6\% of the increase in REM sleep detected using conventional three-stage scoring was more appropriately scored as transitionary sleep using NRt scoring. The mean increases in REM sleep levels, as percentages of total sleep time, detected with conventional and NRt scoring were 6.79\% and 2.74\% respectively \( (t_{11} = 5.91 \text{ and } 2.39, \ p < 0.001 \text{ and } p=0.032 \) respectively, post hoc paired t tests after identification of a significant main effect of drug treatment: \( F_{1,11} = 19.09, \ p = 0.001, \text{ two-way ANOVA-RM} \).

Figure 15B shows that the major sleep effect of vlPAG/DpMe inhibition was a 134.9\% increase in NRt relative to its baseline proportion of the total sleep time \( (t_{11} = 9.02, \ p < 0.001, \text{ post hoc} \)
Figure 15. Effects of vlPAG/DpMe inhibition on sleep macroarchitecture and bistability.

(A) Effects of vlPAG/DpMe inhibition, by 85μM muscimol, on the levels of sleep-wake states as a proportion of the total recording time. Levels of REM sleep are reported in the case of NRt scoring and in the case of conventional three-stage scoring. (B) Effects of vlPAG/DpMe inhibition on the levels of REM sleep and NRt states as a proportion of the total sleep time. REM sleep levels with and without transition scoring are shown. NRt levels are shown for the ACSF/muscimol group (n=12) and the control (ACSF/ACSF) group (n=13). Values are means ± standard error of the mean (n=13). * indicates a significant effect of muscimol inhibition of the vlPAG/DpMe relative to the ACSF control (P < 0.05, from analysis of variance).
paired t test). Figure 15B also provides further validation that the increase in NRt, as a proportion of the total sleep time, is not an effect of the scoring method or time of day given that NRt levels were not different between periods one and two in the control group ($t_{11} = 1.34, p = 0.193$, post hoc paired t test after identification of a significant interaction between animal group and period: $F_{1,23} = 31.08, p < 0.001$, two-way ANOVA-RM).

### 4.3.3 Effects of vlPAG/DpMe inhibition on REM sleep stability

Figure 16A-B shows that inhibition of the vlPAG/DpMe produced a fragmentation of REM sleep; the frequency of short ($\leq 15$ epochs in duration) REM sleep bouts increased by 86% relative to baseline, while the frequency of longer REM sleep bouts ($>15$ epochs in duration) was not significantly changed ($t_{11} = 3.78$ and $0.97, p = 0.001$ and $p=0.345$ respectively, post hoc paired t tests after identification of a significant main effect of drug treatment: $F_{1,11} = 9.00, p = 0.012$, two-way ANOVA-RM). Figure 16 C-D shows that, during muscimol microperfusion rats entered transitional sleep more frequently and remained in NRt longer relative to baseline. The frequency of long ($\geq 3$ epochs in duration) NRt bouts increased by 108%, while the frequency of shorter NRt bouts was not significantly changed ($t_{11} = 4.01$ and $1.06, p = 0.001$ and $p=0.308$ respectively, post hoc paired t tests after identification of a significant main effect of drug treatment: $F_{1,11} = 7.71, p = 0.018$, two-way ANOVA-RM). Importantly, in contrast to REM sleep, NREM sleep was not fragmented as a result of vlPAG/DpMe inhibition as evidenced by our observation that the distribution of NREM sleep bout lengths was unchanged by muscimol microperfusion (Figure 16 E-F; non-significant main effect of drug treatment and non-significant interaction between drug treatment and bout length: $F_{1,11} = 0.09$ and $1.01, p = 0.770$ and
Figure 16. Selective fragmentation of REM sleep by vlPAG/DpMe inhibition

Mean numbers of short versus long bout lengths and corresponding bout length histograms for REM sleep (A-B), NRt (C-D), NREM sleep (E-F) and wake (G-H). Bout length histograms show cumulative bout numbers across the all 12 rats in the muscimol group. Numbers of bouts shown in short-versus-long bout plot are the average number over two hour periods. Error bars indicate the standard error of the mean. * indicates a significant effect of muscimol inhibition of the vlPAG/DpMe relative to the ACSF control (P < 0.05, from analysis of variance)
Figure 17. Hypnograms for all transitions into REM sleep

Shown are the hypnograms for all transitions into REM sleep from all rats numbered 1-12, inclusive of REM sleep episodes and the preceding ninety seconds. All transitions are aligned according to the epoch where the transition into REM sleep takes place (denoted as time 0). Transitions from ACSF control period 1 (left); transitions from muscimol period 2 (right).
0.337 respectively, two-way ANOVA-RM). Figure 16 G-H shows that the decrease in waking time, induced by vIPAG/DpMe, stemmed from a reduced number of sustained waking bouts (>3 epochs in duration); however, the overall number of waking bouts was unchanged by muscimol microperfusion, owing to an increase in the frequency of brief arousals (<3 epochs in duration) ($t_{11} = 2.71$ and $3.51$, $p = 0.014$ and 0.002 respectively, post hoc paired t tests after identification of a significant interaction between drug treatment and bout length: $F_{1,11} = 31.09$, $p < 0.001$, two-way ANOVA-RM).

That NREM sleep was not fragmented by muscimol microperfusion indicates that the increased number of NRt bouts induced by vIPAG/DpMe inhibition originated during periods of REM sleep. Figure 17 shows the hypnograms for all transitions into REM sleep from all rats in the muscimol group, inclusive of REM sleep episodes and the preceding ninety seconds. The figure shows that, under normal conditions, entries into NRt from REM sleep were rare, occurring only five times in twenty-four hours of baseline recording in 12 rats. In contrast, during vIPAG/DpMe inhibition there was increased emergence of NRt from REM sleep episodes. While the proportion of sleep time accounted for by NRt bouts originating from NREM sleep increased during muscimol microperfusion (Figure 18A-B; $t_{11} = 5.962$, $F_{1,11} = 35.551$, $p < 0.001$, one-way ANOVA-RM), the frequency of NRt bouts stemming from NREM sleep did not change significantly ($9.25 \pm 0.69$ bouts at baseline versus $13.17 \pm 1.80$ with muscimol; $p = 0.075$, one-way ANOVA-RM). Figure 18B shows an example of one rat where the frequency of NREM-to-REM sleep transitioning increased along with an increase in the number of NRt episodes originating in REM sleep. It should be noted that the largest muscimol-induced hypersomnia also occurred in this animal (time spent awake was reduced by 60%). Nevertheless, the mean increase in NRt time originating in NREM sleep was driven by a 51% increase in the length of NRt bouts originating in NREM sleep ($40.20 \pm 3.22$ seconds at baseline versus $60.89 \pm 7.34$ with muscimol).
In other words, NREM-to-REM sleep transitioning propensity was not markedly increased during vIPAG/DpMe inhibition. Figure 18C-D shows that vIPAG/DpMe inhibition produced a disproportionate increase in NRt time originating from REM sleep periods (876.3% increase relative to baseline; \( t_{11} = 5.49, F_{1,11} = 30.16, p < 0.001 \), one-way ANOVA-RM) such that the fraction of total NRt time originating from bouts of REM sleep increased by 249% during muscimol microperfusion of the vIPAG/DpMe (\( t_{11} = 5.647, F_{1,11} = 31.884, p < 0.001 \), one-way ANOVA-RM). Figure 18D shows the trajectories of individual excursions into NRt space, originating in REM sleep, from a single rat. The deterioration of REM sleep bistability that occurred during vIPAG/DpMe inhibition is evident as an increased number and duration of NRt trajectories originating in REM sleep state space.

The increased time spent in NRt stemming from REM sleep came as a result of an increased mean duration and frequency of NRt bouts. The mean frequency of NRt bouts originating in REM sleep rose from \( 0.67 \pm 0.188 \) to \( 4.583 \pm 0.874 \) and the mean duration of NRt bouts rose from \( 14.17 \pm 4.165 \) seconds to \( 44.78 \pm 5.27 \) seconds (\( t_{11} = 4.67 \) and \( 4.04 \), \( p < 0.001 \) and \( p = 0.002 \) respectively, post hoc paired t-test after identification of significant effect of drug treatment: \( F_{1,11} = 21.79 \), one-way ANOVA-RM). Taken together, these results show that the increase in NRt sleep following inhibition of vIPAG/DpMe is not primarily a result of increased transitioning from NREM-to-REM sleep, but rather is a consequence of diminished REM sleep stability. During vIPAG/DpMe inhibition, the fragmentation of REM sleep episodes by NRt bouts was also accompanied by increased REM sleep clustering; therefore, while the number of REM sleep bouts increased, they tended to cluster together and they were often separated by NRt bouts.
Figure 18. Increased NRt originates disproportionally from REM sleep

(A) Group data showing the effect of muscimol inhibition of the vlPAG/DpMe on the proportion of sleep time occupied by NRt originating in NREM sleep, while (B) shows example state-space plots, for ACSF and muscimol conditions, depicting the NRt episodes originating in NREM sleep from a single rat (corresponds to number 7 in figure 17). (C) Group data showing the proportion of sleep time occupied by NRt originating in REM sleep, while (D) shows example state-space plots, for ACSF and muscimol conditions, depicting the NRt episodes originating in REM sleep from a single rat. The inset of the bottom panel in (D), shows an individual example of sleep instability as a trajectory through state space (numbering indicates the epoch sequence). (E) Shows the effect of muscimol on the frequency of individual REM sleep bouts versus the frequency of REM sleep clusters. Values are means ± standard error of the mean.* indicates a significant effect of muscimol inhibition of the vlPAG/DpMe relative to the ACSF control (P < 0.05, from analysis of variance).
Figure 19. Examples of normal and unstable EEG dynamics across transitions between NREM and REM sleep

(A) Example spectrogram and corresponding state-space plot depicting normal spectral changes in the EEG across a NREM-to-REM sleep transition during ACSF microperfusion of the vlPAG/DpMe. (B) Example spectrogram and corresponding state-space plot depicting a period of unstable sleep during muscimol mediated inhibition of the vlPAG/DpMe. In (B), numbering in the state-space plot and corresponding spectrogram indicate the sequence of epochs.
Figure 20. EEG effects of vlPAG/DpMe inhibition

Group data showing the effect of muscimol on relative EEG power (in 2Hz-wide bins from 1-19Hz) in (A) REM sleep classified with conventional scoring, (B) NRt, (C) REM sleep classified as a part of the NRt scoring method, (D) wakefulness and (E) NREM sleep. Values are means ± standard error of the mean (n=13). * indicates a significant effect of muscimol inhibition of the vlPAG/DpMe relative to the ACSF control (P < 0.05, from analysis of variance).
and/or brief arousals rather than periods of NREM sleep. Figure 18E shows that, while the frequency of REM sleep bouts increased during vlPAG/DpMe inhibition, the frequency of REM sleep clusters did not significantly change ($t_{11} = 0.98$, $p = 0.343$, post hoc paired t test after identification of a significant interaction between the factors REM bout grouping method and drug treatment: $F_{1,11} = 6.79$, $p = 0.024$, two-way ANOVA-RM).

### 4.3.4 Effects of vlPAG/DpMe on EEG power

Figure 19 shows an example of spectral changes in the EEG across a normal NREM-to-REM sleep transition compared to the spectral changes across a period of sleep instability during vlPAG/DpMe inhibition. That much of the increase in REM sleep induced by vlPAG/DpMe inhibition is better classified as transitionary sleep, is evident in the power analysis of the REM sleep EEG. When using conventional three-stage scoring, REM sleep during vlPAG/DpMe inhibition appears electroencephalographically distinct relative to baseline REM sleep (Figure 20A). EEG power in frequency bins from 5-9hz was reduced, while EEG power in frequency bins from 9-15hz was elevated relative to baseline REM sleep (range of $t_{11} = 9.68$-$2.89$, range of $p < 0.001$-$0.004$, post hoc paired t tests after identification of a significant interaction between the factors EEG band and drug treatment: $F_{14,154} = 9.78$, $p < 0.001$, two-way ANOVA-RM). These EEG power changes are characteristic of the difference in EEG power between REM sleep and NRt (Figure 20B) and reflect the tendency of brain state to drift from REM sleep into intermediate sleep when the vlPAG/DpMe is inhibited. The same REM sleep EEG changes were largely absent upon repetition of the analysis with NRt scoring included. The reduction in 7-9Hz power detected with conventional scoring was attenuated, but still statistically significant with NRt scoring (Figure 20C; $t_{11} = 4.55$, $p < 0.001$, post hoc paired t test after identification of a
significant interaction between the factors EEG band and drug treatment: $F_{14,154} = 1.945$, $p = 0.026$, two-way ANOVA-RM). We did not find any other EEG power changes in wakefulness, NREM sleep or NRt due to vlPAG/DpMe inhibition (Figure 20B,D-E; non-significant effect of drug treatment: range of $F_{1,11} = 0.192$-3.988, range of $p = 0.999$-0.071, two-way ANOVA-RM).

### 4.3.5 Anatomical results

Figure 21 shows the location of the micro-perfusion sites from both experimental groups. Figure 21A shows an example microperfusion lesion located at the boundary of the vlPAG and the dorsal aspect of the DpMe. Figure 21B shows that for all 25 rats, probe locations were in the combined vlPAG/DpMe region, from the anteriopterior level defined by the caudal pole of the paratrochlear nucleus to the caudal extent of the superior cerebellar peduncle decussation. We have previously shown that, using microdialysis, the outward diffusion of permanganate approaches concentrations between fifteen and twenty percent of the concentration of the microdialyzed solution over a 2 hour period (for a flow rate of $2.1\mu l/min$) (Grace et al., 2014b). This value is related to the diffusion coefficient of permanganate, which we assume is comparable to that of muscimol given their similarity in terms of size and molecular weight.

Figure 21C shows the predicted level of muscimol in an area adjacent to the diffusion source over a 2 hour time period relative to that of a microinjection of an equivalent quantity of muscimol. The predicted levels of muscimol as a function of distance with microdialysis versus microinjection at different time points are shown in Figure 21D and E respectively. Figure 21F shows a comparison of muscimol level as a function of diffusion distance for microdialysis (internal probe concentration = $85\mu M$; corresponding tissue exposure over 2 hours = $2\mu g$) relative to a microinjection of $20\mu g$. This amount was used by Arikan et al. (2002) to determine
the extent of activity suppression surrounding focal injections of muscimol. Combining electrophysiological measurement of neuronal inhibition with autoradiography-based determination of the extent of receptor bound muscimol, this study found that over a 2.5 hour period post injection that the maximum tissue volumes, where muscimol produced any observable suppression and complete suppression of neuronal activity, had radii of 1000 and 400 µm respectively. Figure 21F shows, that for a given distance from the diffusion source, that the muscimol concentration is generally lower in the case of microdialysis relative to a 20µg muscimol microinjection. However, Figure 21G shows that at distant points from the source of diffusion, that muscimol levels for microdialysis and microinjection converge. Therefore, we conservatively assumed that the volume of tissue where muscimol produced partial and complete inhibition of neuronal activity in our study was comparable to that of Arikan et al. (2002). Figure 21H, shows the predicted regions of maximal neuronal inhibition from each experiment and the degree of overlap across all experiments. The area of greatest overlap is located at the boundary between the vlPAG and the DpMe. Figure 21I shows the predicted region, common to all experiments, where muscimol produced any level of activity suppression. The majority of this region is comprised of the DpMe and the PAG including the lateral and ventrolateral aspects. The suppression region also includes the dorsal raphe, but excludes the PPT located adjacent to the DpMe.
Figure 21. Location of microperfusion sites and prediction of the anatomical extent of muscimol-mediated inhibition.

(A) Shows an example microperfusion lesion located at the boundary of the vIPAG and the DpMe. The black arrow indicates the most ventral point of the lesion (B) Probe locations sites for all 25 rats (blue rectangles = muscimol group; red rectangles = control group) located between the anterioposterior level defined by the caudal pole of the paratrichlear nucleus to the caudal extent of the superior cerebellar peduncle decussation. (C) Simulated level of muscimol in an area adjacent to the diffusion source over a 2 hour time period (microinjection (2μg) versus microdialysis (85μM; 2μg)). Corresponding plots showing the predicted levels of muscimol as a function of distance with microdialysis (D) versus microinjection (E) at different time points. (F) Shows a comparison of muscimol level as a function of diffusion distance for microdialysis (85μM; 2μg) versus microinjection (20μg; Arikan et al. (2002)). (G) shows that at distant points from the source of diffusion, that muscimol levels for microdialysis and microinjection converge. Based on electrophysiological measurements from Arikan et al. (2002), (H) shows the predicted regions of maximal neuronal inhibition from each experiment and the degree of overlap across all experiments; (I) shows the predicted region, common to all experiments, where muscimol produced any level of activity suppression.
4.4 Discussion

The results of inactivating the vIPAG/DpMe, by focal muscimol administration, are consistent with the REM sleep enhancement reported by previous inactivation studies (Lu, Sherman et al. 2006; Sapin, Lapray et al. 2009; Weber, Chung et al. 2015; Petitjean, Sakai et al. 1975; Sastre, Buda et al. 1996; Crochet, Onoe et al. 2006; Vanini, Torterolo et al. 2007); however, I will argue that my analysis of bout statistics and NREM-REM sleep intermediate states does not support the prevailing hypothesis of vIPAG/DpMe functional involvement in REM sleep generation, namely that its REM sleep-inactive subpopulation is a critical regulator of NREM-to-REM sleep transition.

Previous studies showing that vIPAG/DpMe inactivation reliably increases REM sleep time have been taken to suggest that vIPAG/DpMe neurons are important inhibitory regulators of NREM-to-REM sleep transitioning (Lu et al., 2006b; Saper et al., 2010). Consistent with this hypothesis, the vIPAG and DpMe are amongst the principal sources of afferent input to the pontine SubC region, an important brain region for REM sleep generation (Boissard et al., 2003), where GABA_A receptor antagonism has a REM sleep enhancing effect (Xi, Morales et al. 1999; Boissard, Gervasoni et al. 2002; Pollock and Mistlberger 2003; Sanford, Tang et al. 2003). Furthermore, the DpMe is a major source of GABergic input to the SubC (Boissard et al., 2003). It ought to be noted, however, that the vast majority of DpMe projections to the SubC were found to be non-GABAergic and that no GABergic projections from the PAG to the SubC were detected (Boissard et al., 2003). Nevertheless, consistent with a vIPAG/DpMe role in gating REM sleep transitions, the vIPAG/DpMe contains a large number of REM sleep-inactive GABAergic neurons in addition to its abundant REM sleep-active neuronal populations (Sapin et al., 2009). However, it should be emphasized that the vIPAG/DpMe REM sleep-off population was identified by measuring c-Fos expression, where the size of the REM sleep-inactive
population is taken as the increase in the number of c-Fos expressing neurons under REM sleep deprivation conditions compared to control. Importantly, the deprivation condition is characterized not only by reduced REM sleep, but by increased wakefulness (with the levels of NREM sleep being unchanged). As a result, a proportion of those neurons identified as REM sleep-inactive according to this method are likely to be strictly arousal related. The vlPAG contains a dopaminergic wake-active neuronal population, the selective ablation of which increases sleep time (Lu et al., 2006a). Consistent with these findings, the present study showed that vlPAG/DpMe inhibition also reduced time spent awake. Therefore, based on c-Fos expression following REM sleep deprivation, we cannot determine what proportion of cells is also active in NREM sleep with the potential to play a role in NREM-to-REM sleep transitioning. Direct electrophysiological recording of vlPAG/DpMe neurons has mainly revealed REM sleep-active cell types: wake/REM sleep-active neurons and neurons specifically active in REM sleep (Crochet et al., 2006). Based on these data, the hypothesized role of the REM sleep-inactive vlPAG/DpMe pool in gating NREM-to-REM sleep transitions is not unjustified; however, there also exists significant potential for the involvement of other vlPAG/DpMe pools in the control of REM sleep.

The present results show that much of the reported increase in REM sleep following vlPAG/DpMe inhibition may be more appropriately classified as a NREM/REM sleep transitionary state. The increases in time spent in NRt were the result of increases in both the frequency and duration of intermediary sleep bouts. Therefore, our data indicate that some population(s) of vlPAG/DpMe neurons contributes to the production of sleep bistability.

NREM/REM transitionary sleep is a feature of normal sleep cycling (Benington et al., 1994). Episodes of REM sleep are commonly preceded by transitionary periods where the EEG
signatures of NREM sleep evolve into that of REM sleep. The EEG transition from NREM-to-REM sleep is mirrored by accelerations in the discharge rate of REM sleep-active brainstem neurons from low rates in NREM sleep to maximal firing rates in REM sleep (Steriade and McCarley, 2005b; Boucetta et al., 2014). These transitionary periods account for only a small proportion — less than five percent — of the total sleep-wake record in rodents. The small proportion of sleep time that is spent in NREM/REM sleep intermediate states is evidence of the relative instability of NRt in contrast to the stability of NREM sleep and REM sleep states. Theoretical modeling supports the involvement of a mutually inhibitory flip-flop mechanism in the generation of sleep bistability (Dunmyre et al., 2014). Evidence consistent with vlPAG/DpMe involvement in such a switching mechanism is limited to the finding that vlPAG/DpMe inactivation increases the frequency of REM sleep episodes (Lu, Sherman et al. 2006; Sapin, Lapray et al. 2009; Weber, Chung et al. 2015; Petitjean, Sakai et al. 1975; Sastre, Buda et al. 1996; Crochet, Onoe et al. 2006; Vanini, Torterolo et al. 2007). Inhibiting a component of a flip-flop switch weakens inhibitory positive feedback within the circuit, causing the switch to operate nearer to its transition threshold, leading to more frequent state transitions for a given external input (Rempe et al., 2010). Another possible consequence of inhibiting a flip-flop switch component is a loss of sleep bistability, as evidenced by increased time spent in intermediary states. A loss of sleep bistability would occur when: (i) reduced inhibitory positive feedback extends the time needed for the REM sleep-active and REM sleep-inactive components of the switch to transition between steady-state firing rates, and (ii) steady-state firing rates of the REM sleep-active and REM sleep-inactive components of the switch converge — i.e., intermediate firing rates may give rise to intermediate/blended states due to inappropriate co-activation of NREM and REM sleep generative networks; vlPAG/DpMe neurons may promote sleep bistability by limiting such co-activation.
If vlPAG/DpMe neurons contributed to negatively regulating the initiation of REM sleep, then inhibition of the vlPAG/DpMe would be expected to increase the probability of transitioning from NREM-to-REM sleep as evidenced by a shortening of NREM sleep bout lengths and an increase in the number of transitions from NREM-to-REM sleep and/or NRt. However, our results show that the increased time spent in REM sleep and NRt following vlPAG/DpMe inhibition was not a product of an increased propensity for NREM-to-REM sleep transitioning. Namely, we did not observe a shortening of NREM sleep bout lengths as a result of vlPAG/DpMe inhibition, nor did we find there to be a general increase in the frequency of entries into NRt space from NREM sleep. While the frequency of entries into NRt space from NREM sleep did not significantly change, we did find that the length of NRt bouts originating in NREM sleep was increased. These data are not consistent with their being a major vlPAG/DpMe contribution to NREM sleep processes that gate transitions into REM sleep (e.g., possible integration of a REM sleep homeostatic drive). Nevertheless, the dynamics of NREM-to-REM sleep transitions, once initiated, are impacted by vlPAG/DpMe inhibition: vlPAG/DpMe neurons normally enforce NRt state instability by accelerating transitions between NREM and REM sleep.

Rather than having a major role in gating entry into REM sleep, our data indicates that vlPAG/DpMe neurons are major contributors to the stability of the REM sleep state. Under normal conditions, entries into NRt from REM sleep were rare. However, when inhibiting the vlPAG/DpMe, bouts of REM sleep were frequently interrupted by state excursions into NRt space. This fragmentation of REM sleep bouts by NRt was the most consistently observed effect of vlPAG/DpMe inhibition. Unlike NRt bouts originating in NREM sleep which were lengthened but no more frequent — indicating greater NRt stability, whilst the stability of the NREM state is unchanged — NRt bouts originating in REM sleep were longer and more
frequent, indicating a loss of REM sleep state stability. Further evidence for reduced REM sleep stability during vlPAG/DpMe inhibition includes the increased clustering of REM sleep episodes. Clustering, which was more pronounced in some animals relative to others, is often the result of REM sleep fragmentation by brief arousals. This is important because previous vlPAG/DpMe inactivation studies have reported increased cycling, back and forth, between wake and REM sleep as well as more frequent sleep onset REM episodes (Lu et al., 2006b; Vanini et al., 2007; Sapin et al., 2009). REM sleep instability is also evidenced by the occurrence of transitions from REM sleep to NREM sleep in some animals, which were never observed under baseline conditions. These data implicate PAG/DpMe neurons that are active during REM sleep in the maintenance of REM sleep stability and the prevention of reversals in state trajectory: i.e., transitions from REM sleep into NRt and NREM state-space. Sapin et al, detected REM sleep-active neurons, not only in the vlPAG/DpMe, but also more dorsally in the lateral, dorsolateral and dorsomedial aspects of the PAG. We expect that muscimol-mediated inhibition of neural activity likely extended into the dorsal compartments of the PAG and therefore REM sleep-active cells maintaining REM sleep stability may be spread across this region. Nevertheless, fragmentation of REM sleep by excursions into NRt cannot be explained by an inhibition of REM sleep-inactive vlPAG/DpMe neurons that negatively regulate REM sleep: the vlPAG/DpMe subpopulation which is the focus of current theories of REM sleep switching involving the vlPAG/DpMe.

4.4.1 On the need for quantification of intermediary sleep and improved scoring standardization

The fragmentation of REM sleep episodes by NRt bouts resulted in an increase in the number of short REM sleep bouts. With conventional sleep staging, an episode of REM sleep fragmented
by NRt can be scored either as a continuous bout or as multiple transitions between REM sleep and NREM sleep depending on the scorer. In the former case, frequent transitioning between REM sleep and NRt will present as a shift in the distribution of REM sleep bout lengths towards longer episodes, rather than a fragmentation of the REM sleep state. This is important because previous vlPAG/DpMe inactivation studies, which did not quantify NRt periods, have reported lengthening of REM sleep bouts (Sastre et al., 1996; Crochet et al., 2006; Lu et al., 2006b; Weber et al., 2015). In the latter case, frequent transitioning between REM sleep and NRt will present as a fragmentation of both REM sleep and NREM sleep bout lengths. These scoring variations can produce major differences in functional interpretation from the same data. Therefore, in cases where experimental manipulations may compromise sleep bistability — an important experimental outcome considered in the context of flip-flop theories of REM sleep generation — transitionary states should be quantified. To that end, state-space analysis can be a useful tool for editing the results of manual sleep scoring so as to reduce inter- and intra-scorer variability.
Chapter 5
Identification of a Pharmacological Target for Genioglossus Reactivation Throughout Sleep

5.1 Introduction

Obstructive sleep apnea (OSA) is a serious clinical problem due to its high prevalence and association with adverse cardiovascular, metabolic, and cognitive outcomes (Brooks, Horner et al. 1997; Teran-Santos, Jimenez-Gomez et al. 1999; Peppard, Young et al. 2000; Malhotra and White 2002) OSA is characterized by repeated episodes of upper airway narrowing and closure during sleep (Malhotra and White 2002). Although the causative factors for OSA are variable and complex within individuals,(Eastwood, Malhotra et al. 2010) that the periods of upper airway closure occur only during sleep highlights the necessary role of sleep-induced changes in pharyngeal motor control in the pathogenesis of OSA (Remmers et al., 1978).

Because pharyngeal motor activity in wakefulness is sufficient to maintain airway patency even in patients with severe OSA, an effective pharmacotherapy should aim to restore pharyngeal motor activity in sleep to at least a physiological pattern of activity present when awake (Eastwood, Malhotra et al. 2010). Pharmacological attempts (Smith and Quinell, 2004; Veasey, 2005; Dempsey et al., 2010; Lin et al., 2012) to reverse pharyngeal hypotonia in sleep have essentially used two major strategies: increasing central respiratory drive and attempting to potentiate excitatory neurotransmission onto the pharyngeal motor neurons that drive the upper airway musculature. These strategies have been largely unsuccessful, likely because they fail to address the root mechanisms of pharyngeal hypotonia in sleep and the varying contributions of different neural mechanisms across sleep-wake states.
The root cause of pharyngeal hypotonia in sleep is suppression of cranial motor neuron activity by state-dependent changes in synaptic input (Horner 2009; Dempsey, Veasey et al. 2010). In NREM sleep, hypoglossal motor neurons are disfacilitated by withdrawal of excitatory inputs, with noradrenergic, serotonergic, and glutamatergic influences being a particular focus of previous studies (Fenik, Davies et al. 2005; Chan, Steenland et al. 2006; Burgess, Lai et al. 2008; Steenland, Liu et al. 2008). In periods of REM sleep, motor neuron activity can be abolished by additional recruitment of muscarinic receptor-mediated cholinergic inhibition (Grace, Hughes et al. 2013). This REM sleep-specific mechanism is powerful, such that concomitant local stimulation of the hypoglossal motor pool with supraphysiological concentrations of excitatory neurotransmitters, or increased central respiratory inputs produced by hypercapnic respiratory stimulation, are insufficient to restore pharyngeal motor activity. (Horner, Liu et al. 2002; Burgess, Lai et al. 2008; Horner 2008) In summary, the changing neurochemical environment at the hypoglossal motor pool from wakefulness to sleep is such that motor neuron activity and responsiveness to excitatory inputs can be reduced or virtually abolished, especially in REM sleep. Based on these basic observations, it should not be expected that pharmacological agents that simply increase respiratory drive or increase excitatory neurotransmission onto hypoglossal motor neurons would be effective in the treatment of OSA.

To effectively reactivate pharyngeal motor activity in sleep, pharmacological strategies are needed that counteract the state-dependent suppression of hypoglossal motor excitability. The multiple state-dependent neurotransmitter systems identified previously (Fenik, Davies et al. 2005; Chan, Steenland et al. 2006; Burgess, Lai et al. 2008; Steenland, Liu et al. 2008; Grace, Hughes et al. 2013) potentially produce such suppression via a convergent ionic mechanism: increased potassium conductance. The inhibitory effect of monoaminergic disfacilitation of the hypoglossal motor pool may be mediated by an increase in potassium conductance, for example
via TWIK-related acid-sensitive potassium (TASK) channels (Bayliss et al., 1997; Talley et al., 2000). Furthermore, the periods of atonia of the pharyngeal musculature during REM sleep are produced by cholinergic activation of G-protein coupled inwardly rectifying potassium channels. (Grace, Hughes et al. 2013) Considering the pivotal role of potassium conductance in the state-dependent modulation of hypoglossal motor neuron excitability, we tested the hypothesis that potassium channel blockade would be an effective pharmacological strategy capable of reactivating the pharyngeal musculature throughout sleep.

To this end, we locally microperfused pharmacological agents into the hypoglossal motor pool to modulate potassium channels of three major classes: inwardly rectifying (Kir), two-pore domain, and voltage-gated (Kv). In this respect, the inwardly rectifying family of potassium channels are important mediators of neuronal excitability (Hibino et al., 2010). Significantly, the Kir2.4 channel subunit, a member of the constitutively active Kir2 channel subfamily, is the most restricted of all Kir subunits in the brain, being expressed mainly on cranial motor neurons such as the hypoglossal (Topert, Doring et al. 1998). Given their highly restricted expression, Kir2.4 is potentially a significant pharmacological target for the manipulation of upper airway muscle activity and OSA. There are currently no available blockers for this class of Kir channels. However, they would be of potential high priority for development and subsequent testing if their possible efficacy in reactivating the pharyngeal musculature can be identified or implicated. Kir channels are blocked with relative specificity by micromolar concentrations of barium (Hibino, Inanobe et al. 2010) whereas millimolar concentrations block many other potassium channel subtypes including notable regulators of neuronal excitability such as two-pore domain TASK channels (Bayliss, Viana et al. 1992; Fisher and Nistri 1993) and voltage sensitive Kv7.2 (KCNQ2) channels (Halliwell and Adams, 1982; Constanti and Galvan, 1983). We aimed to
achieve tissue concentrations of barium at the hypoglossal motor pool that would predominately affect Kir channels, and likewise for blockers of the other channels of interest.

5.2 Methods

5.2.1 Animal care

Experiments were performed on 33 male Wistar rats (Charles River, Senneville, QC, Canada). Procedures conformed to the recommendations of the Canadian Council on Animal Care, and the University of Toronto Animal Care Committee approved the protocols.

5.2.2 Surgery

Sterile surgery was performed to implant electrodes for chronic recording of the EEG and EMGs of the trapezius (neck), diaphragm, and genioglossus muscles (Grace et al., 2012; Grace et al., 2013a). Sterile surgery was performed as in section 3.2.2. Genioglossus muscle electrodes were considered correctly positioned if their electrical stimulation produced apical tongue contraction. It has been shown previously using sections of the medial branches of the hypoglossal nerves that genioglossus activity is recorded with such electrode placements (Morrison et al., 2002). Microdialysis guides were placed stereotaxically 3 mm above the hypoglossal motor pool (anterioposterior, -14.0 mm; mediolateral, -0.3 mm; dorsoventral, -6.7 mm with respect to bregma). The day before the experiments a microdialysis probe (45nL tip volume; 240-µm-wide by 1-mm-long membrane, 6000 Dalton cutoff; CMA/11-14/01, Chromatography Sciences Company, Montreal, Canada) was inserted into the guide. The probes projected 3 mm from the tip of the guide and targeted the hypoglossal motor pool. At the end of surgery, all the electrodes
were connected to pins and inserted into a miniature plug (STC-89PI-220ABS, Carleton University, Ottawa, ON, Canada). The plug and microdialysis guides were then affixed to the skull with dental acrylic and anchor screws. The rats were given 7–8 d to recover before any experiments were performed.

5.2.3 Microdialysis and experimental design

Habituation of rats to the recording environment was performed as described in Chapter 2. Microdialysis probes were perfused with ACSF for 2 hrs (control) followed by 2 hrs of drug. The first hour following the switch to drug was excluded from data analysis to allow for diffusion and stabilization of responses. In separate experiments we applied 4-aminopyridine (4-AP, 500 µM, Tocris Bioscience, Minneapolis, MN, USA) in nine rats, tetraethylammonium chloride (TEA, 10 mM, Tocris) in nine rats, barium chloride (2.5 mM, Sigma- Aldrich, St. Louis, MO, USA) in six rats, and the cannabinoid receptor agonist/TASK channel blocker, (R)-(+)—methanandamide (Tocris) in nine rats. When adding TEA or barium chloride to the ACSF, the concentration of sodium chloride was reduced by 10 or 2.5 mM, respectively, to maintain the osmolarity of the perfusate. Stock solutions of methanandamide dissolved in a water-soluble emulsion (1:4 ratio of soya oil/water and the block copolymer Pluronic F68 - Tocrisolve™100, Tocris Bioscience) were diluted ~140-fold in ACSF to a final concentration of 100 µM.

When microperfusing agents via reverse microdialysis, the tissue concentrations of drugs are only a fraction of the prepared concentrations. Tissue concentration and drug distribution was estimated ex situ by imaging the microperfusion of dye (2.5 mM potassium permanganate) into 0.6% agarose tissue phantoms, which mimics the diffusion characteristics of brain tissue (Chen et al., 2004). Permanganate was chosen because its molecular weight (119 g/mol) is similar to
the lightest (i.e., most diffusible) compounds microperfused in vivo, i.e., barium, TEA, and 4-AP (range, 94–137 g/mol). For these studies, the color intensity of microperfused potassium permanganate dye surrounding the probe tip in agarose tissue phantoms was captured and analyzed using light microscopy (Olympus BX41, Olympus Canada Inc., Markham, Canada) and MATLAB software (Mathworks, Natick, MA, USA). Dye concentration was calculated by comparing color intensity values in the perfused tissue phantoms against intensity values from a standard agarose phantom prepared with the same concentration of dye used in the microperfusion (2.5 mM). Agarose tissue phantoms were incubated at 37°C prior to use.

Probe placements in the hypoglossal motor pool were confirmed by histology (Grace, Liu et al. 2012; Grace, Hughes et al. 2013) and referenced to a stereotaxic atlas of the rat brain (Paxinos and Watson 1998).

5.2.4 Signal analyses

The electrophysiological signals were analyzed as previously described in Chapter 2 (Grace, Liu et al. 2012; Grace, Hughes et al. 2013). For these analyses, sleep-wake states were identified by visual inspection of the neck EMG and the EEG and classified into wakefulness, NREM, and REM sleep according to standard criteria (Grace, Liu et al. 2012; Grace, Hughes et al. 2013). Specifically, each 5-sec epoch was scored as wake, NREM sleep, REM sleep without transient muscle activations, i.e., “twitches” (REM sleep ([−])), or REM sleep with transient muscle activations (REM sleep ([+])).

As previously described, (Grace, Liu et al. 2012; Grace, Hughes et al. 2013) because changes between behavioral states are sometimes associated with difficulty in determining sleep-wake
states and accompanied by unstable breathing, the following exclusion criteria were adopted to help ensure that all data included in the analysis of sleep-wake state phenomenology were obtained from temporally enduring periods within unequivocally defined sleep-wake states. Periods of wakefulness or sleep lasting < 30 sec were excluded from analysis. If a state transition occurred during a scoring epoch, then that epoch was also excluded from the analysis. All periods of wakefulness in which rats were eating, drinking, or grooming were excluded from the analysis. During such waking periods, the diaphragm EMG recordings can become contaminated by movement-related artefact, and so periods of active wakefulness were excluded from analysis because an inability to identify the peak and trough of each diaphragm breath prevents a meaningful analysis of the principal variables being measured (i.e., diaphragm amplitude, respiratory rate, and respiratory-related genioglossus activity).

When reporting levels of genioglossus muscle activity in REM sleep we differentiated between periods of REM sleep without and with muscle twitching (REM sleep ([−]) and REM sleep ([+]), respectively). In order to distinguish genioglossus muscle twitching from respiratory modulation (i.e., in the event that atonia is prevented and respiratory activity emerges) we defined thresholds to differentiate between these two manifestations of motor activity. Muscle twitching occurs with a shorter interburst interval (i.e., a faster frequency) than respiratory modulation, and therefore an epoch of REM sleep was scored as having muscle twitching if the frequency of phasic muscle activation exceeded 125% of the respiratory rate for the same epoch (Grace, Liu et al. 2012; Grace, Hughes et al. 2013). Twitch events were analyzed as follows: muscle twitching activity was analyzed from the rectified raw genioglossus and neck muscle signals followed by derivation of the moving time average signal using a shorter time constant of 30 msec. Using an impulse function, the background tonic activity was filtered out, thereby isolating muscle twitches from the other components of the signal. Upon isolating the muscle twitches, their
frequency and peak amplitude were calculated. In all cases, epochs of REM sleep identified as having twitching activity were confirmed via visual inspection of the sleep record (Grace, Liu et al. 2012; Grace, Hughes et al. 2013).

Even in the absence of motor activity during REM sleep without twitching, low levels of genioglossus muscle activity can always be measured from high-gain EMG recordings. Therefore, in each animal we zeroed baseline tonic and respiratory-related genioglossus activity in REM sleep without muscle twitching (i.e., biological zero) and equally adjusted levels in all other states/conditions.(Grace, Liu et al. 2012; Grace, Hughes et al. 2013)

For each breath, the analysis of the genioglossus EMG was time-locked to breathing as defined by the peak and trough of the diaphragm signal. Genioglossus activity was quantified as mean tonic activity (i.e., basal activity in expiration) and respiratory-related activity (i.e. peak inspiratory activity – tonic activity).(Grace, Liu et al. 2012; Grace, Hughes et al. 2013) As with previous studies,(Grace, Liu et al. 2012; Grace, Hughes et al. 2013) it is noted that measures of tonic genioglossus activity will inevitably include both basal tone and any background, spontaneous, continuous activity that may occur during certain behaviors, e.g., the sporadic twitches of REM sleep. Likewise, although clear sporadic genioglossus muscle twitches can occasionally occur in REM sleep, whether such sporadic activity that occurs in synchrony with the diaphragm is truly “respiratory” or random activation due to behavioral or REM sleep processes cannot be determined with this or other methods. However, such activations do not affect the measurements in quiet wakefulness, NREM sleep, and REM sleep without such sporadic genioglossus muscle twitches, i.e., REM(-), that are a major focus of this study.

In some cases, changes in genioglossus muscle activity were also analyzed as a function of brain arousal state. We used a ratio of electroencephalographic power in two frequency bands (power,
0-20Hz/power, 0-55Hz) to define the spectrum of arousal according to the method of “state-space analysis” (Gervasoni, Lin et al. 2004).

Data are presented as means ± standard error of the mean. For the two-way analyses of variance with repeated measures (ANOVA-RM), the two factors were Treatment (i.e., ACSF versus drug applied to the hypoglossal motor pool) and State (i.e., wakefulness, NREM sleep, and REM sleep). For all comparisons, differences were considered significant if the null hypothesis was rejected at $P < 0.05$ using a two-tailed test. Where post hoc comparisons were performed after ANOVA-RM, the Holm-Sidak test was used to test statistical significance. All analyses were performed using Sigmastat (SPSS, Chicago, IL, USA).

### 5.3 Results

Figure 22A and B shows that after 2 h of microperfusion, the maximum concentration of drug in the phantom tissue surrounding the microdialysis probe tip was ≤ 18% of the concentration in the perfusion medium. We therefore microperfused 2.5 mM barium into the hypoglossal motor pool, yielding an estimated peak tissue concentration of ≤ 450 µM. This concentration is reasonable given the barium sensitivity of Kir2.4 (IC50=390µM) (Topert, Doring et al. 1998). Under these conditions, microperfusion of barium into the hypoglossal motor pool reactivated genioglossus throughout sleep (Figure 22C and D). Figure 22D also shows that microperfusion of barium into the hypoglossal motor pool completely prevented the periods of motor atonia that normally accompany REM sleep, such that tonic and respiratory genioglossus muscle activities were preserved throughout the REM sleep episodes. Given that waking pharyngeal motor activity is sufficient to prevent airway collapse even in patients with severe OSA, this targeted manipulation of the hypoglossal motor pool reveals a mechanism that is capable of increasing
genioglossus activity throughout both NREM and REM sleep to levels that even exceed those during baseline wakefulness.

Group data from six rats (Figure 22E and F) identified that microperfusion of barium into the hypoglossal motor pool significantly increased tonic and respiratory-related genioglossus muscle activities, relative to ACSF controls, independent of the prevailing sleep-wake state (range of \( t_5 = 4.44 \) to 4.03, range of \( P = 0.010 \) to 0.008, post hoc paired t-tests following identification of: (1) a significant main effect of treatment, range of \( F_{1,5} = 16.22 \) to 18.13, range of \( P = 0.008 \) to 0.010, and (2) a nonsignificant interaction between treatment and state, \( F_{3,15} = 0.84 \) to 2.58, \( P = 0.517 \) to 0.069, two-way ANOVA-RM). To our knowledge, such a robust effect in activating pharyngeal motor activity throughout sleep has not been achieved with any other blocking agent that targets a mechanism that appears largely restricted to the pharyngeal motor nuclei (Topert, Doring et al. 1998). This observation identifies proof-of-principle and opens up the possibility of targeting a small molecule to Kir2.4 channels to reactivate respiratory and tonic pharyngeal muscle activity throughout sleep.

The effects of microperfusion of barium into the hypoglossal motor pool were specific to genioglossus muscle as other variables were not significantly affected. The lack of effects on other variables included the amplitude of diaphragm activity (Figure 22G, \( F_{1,5} = 0.11, \ P = 0.750 \), two-way ANOVA-RM), respiratory rate (Figure 22H, \( F_{1,5} = 0.64, \ P = 0.461 \)), neck muscle activity (Figure 22I, \( F=0.40, \ P = 0.461 \)), and electroencephalographic power within any frequency band (\( F_{1,5} = 1.29 \) to 0.042, \( P = 0.308 \) to 0.812). Figure 22J shows that the sites of microperfusion of barium were within the hypoglossal motor pool in all rats.
Figure 22. Microperfusion of barium into the hypoglossal motor pool restores genioglossus muscle activity throughout sleep to waking levels.

*Ex situ* determination of tissue drug concentration relative to internal probe concentration is shown in A and B. (A) An aerial view of a 0.6% agarose slab microperfused with 2.5 mM permanganate dye (i), with the bottom inset (ii) showing an agarose standard prepared with the same dye. The black distortion in the middle of the agarose is where the microdialysis probe was placed. (B) Histogram showing the average decay profile of drug concentration along multiple radii stemming from the origin of drug diffusion: two agarose slabs with eight radii per experiment. (C) Example in one rat showing genioglossus muscle activation with microperfusion of 2.5 mM barium into the hypoglossal motor pool across sleep-wake states, with longer rapid eye movement (REM) episodes shown in (D). Note that in the presence of barium at the hypoglossal motor pool the motor suppression during REM sleep is completely reversed. Group data (n = 6) showing the effects of barium on respiratory-related (E) and tonic (F) genioglossus muscle activities during wakefulness, non-REM, and REM sleep with ([-]) and without ([+]) muscle twitching. The lack of effects of barium in sleep and wakefulness on other control variables are shown in G-I: diaphragm muscle amplitude, respiratory rate, and tonic neck muscle activity. (J) Example and group data showing the location of microdialysis probes. The example shows a coronal section of tissue with the site of microdialysis within the hypoglossal motor pool. The location of the ventral tip of the probe site is indicated by the black arrow, and the approximate position of the entire microdialysis probe membrane is denoted by the white dotted section. The coronal diagrams of the rat medulla show the locations of all the sites of microdialysis. Grey rectangles represent the space occupied by the membrane portion of the microdialysis probes. Values are means ± standard error of the mean. # indicates significant
effect of barium relative to artificial cerebrospinal fluid (ACSF) controls independent of sleep-wake state (P < 0.05, from analysis of variance).
Two-pore domain TASK channels produce ‘background’ or ‘leak’ potassium currents that maintain membrane potential at hyperpolarized levels (Enyedi and Czirjak). TASK channels are responsible for most of the resting potassium conductance of hypoglossal motor neurons in vitro, and can be inhibited by arousal-related excitatory neurotransmitters such as serotonin, noradrenaline, and glutamate (via group I metabotropic glutamate receptors) (Bayliss et al., 1997; Talley et al., 2000; Berg et al., 2004). Accordingly, modulation of TASK channel activity may be a potentially significant pharmacological target for the manipulation of upper airway muscle activity. As for Kir channels, however, there are few available blockers for TASK channels.

Based on the estimates of the maximum concentration of drug in the tissue phantoms surrounding the microdialysis probe tip being ≤ 18% of the concentration in the perfusion medium (Figure 22A-C), we microperfused 100 µM methanandamide for an estimated tissue concentration of ≤ 18 µM. TASK-1 currents are nearly abolished by 10 µM methanandamide in vitro (Maingret et al., 2001). The example shown in Figure 23A shows that microperfusion of methanandamide into the hypoglossal motor pool did not reactivate pharyngeal motor activity in sleep, but did produce a motor activation that was present only in wakefulness. Group data from nine rats (Figure 23B and C) shows a significant activating effect of methanandamide at the hypoglossal motor pool on both respiratory and tonic genioglossus motor activities compared to both the ACSF and emulsificant controls, with this activation effect being present only during wakefulness (range of \( t_8 = 4.13 \) to 6.41, all \( P < 0.001 \), post hoc paired t-tests following identification of: (1) a significant main effect of treatment, \( F_{2,16} = 6.56 \) to 6.95, \( P = 0.007 \) to 0.008; and (2) a significant interaction between treatment and state, \( F_{3,16} = 4.57 \) to 6.03, \( P \leq 0.001 \), two-way ANOVA-RM). The emulsificant used to solubilize methanandamide did not affect genioglossus activity relative to ACSF controls (\( t_8 = 0.71 \) to 1.92, \( P = 0.479 \) to 0.059, post
Figure 23. Microperfusion of methanandamide into the hypoglossal motor pool activates genioglossus muscle activity exclusively in wakefulness.

(A) Example in one rat showing genioglossus muscle activity during microperfusion of: artificial cerebrospinal fluid (ACSF) (control), a water soluble emulsificant (control) and 100 µM emulsified methanandamide into the hypoglossal motor pool across sleep-wake states. Note the selective genioglossus muscle activation by methanandamide compared to controls. Group data (n = 9) showing the effects of methanandamide on respiratory-related (B) and tonic (C) genioglossus muscle activities during wakefulness, non-REM, and REM sleep with ([-]) and without ([+]) muscle twitching, compared with ACSF and emulsificant controls. (D) A scatterplot bounded by two electroencephalographic ratios showing the position of 5-sec segments of wakefulness and non-REM sleep in a two-dimensional state-space. (E) A density plot of the points in D. Note that in D and E, separate wake and non-REM sleep clusters are visible along the y-axis, meaning that the spectral power ratio (0-20Hz/0-55Hz) is an effective indicator of the arousal spectrum (high in wake to low in non-REM sleep). (F and G) Group data showing the effects of methanandamide on respiratory and tonic genioglossus muscle activities during waking epochs as a function of waking arousal level identified previously. Note the relationship between increased level of arousal and increased methanandamide-mediated genioglossus muscle activation. The lack of effect of methanandamide across sleep-wake states on the control variables are shown in H-J for diaphragm amplitude, respiratory rate and tonic neck muscle activity. (K) Group data showing the location of microdialysis probes from coronal sections. The example from a single animal indicates the site of microdialysis within the hypoglossal motor pool. Grey rectangles represent the space occupied by the semi-permeable membrane portion of the microdialysis probes. Values are means ± standard error of the mean.
Asterisk indicates significant effect of methanandamide compared with controls (P < 0.05, from analysis of variance). mANA, methanandamide.
hoc paired t-tests), and therefore was not responsible for the wakefulness-dependent
genioglossus activation. To further characterize this wakefulness-dependent motor activation
with methanandamide at the hypoglossal motor pool, we used an electroencephalographic
frequency ratio that reflects moment-to-moment shifts along the spectrum of arousal from active
wakefulness through to deep NREM sleep (Figure 23D and E). Here we show that the activating
effects of methanandamide on respiratory and tonic genioglossus motor activities increased with
increasing levels of waking electrocortical arousal but was absent at lower levels of waking
arousal (Figure 23F and G; range of $t_8 = 2.06$ to $5.36$, $P = 0.043$ to $< 0.001$, post hoc paired t-
tests following identification of: (1) a significant main effect of treatment, $F_{1,8} = 9.07$ to $21.32$, $P$
$= 0.017$ to $0.002$, and, (2) a significant interaction between treatment and level of
electroencephalographic arousal, $F_{9,8} = 2.99$ to $4.83$, $P = 0.005$ to $< 0.001$, two-way ANOVA-
RM). For these studies, the ACSF and emulsificant control groups were pooled due to the
absence of significant differences between them in the previous analysis. Overall, these data
show that the activating effect of methanandamide at the hypoglossal motor pool is restricted to
periods of high-waking electrocortical arousal. This result suggests that other such agents with
the pharmacological profile of methanandamide may not be an effective strategy for direct
reactivation of the upper airway musculature during sleep if their primary targeted site of action
was the hypoglossal motor pool. However, such a definitive result can only be confirmed with
more specific TASK channel blockers but these are not currently commercially available.
Overall, these findings are significant because modulating these channels is cited as a possible
pharmacotherapeutic target for OSA (Brendel et al., 2007; Coburn et al., 2011a; Coburn et al.,
2011b).

The effects of microperfusion of methanandamide into the hypoglossal motor pool were also
specific to the genioglossus because other variables were not significantly affected. The lack of
effects on other variables included diaphragm activation (Figure 23H; \( F_{2,16} = 9.83, P = 0.002 \), two-way ANOVA-RM), respiratory rate (Figure 23I; \( F_{2,16} = 2.31, P = 0.131 \)), and neck muscle activity (Figure 23J; \( F_{2,16} = 1.16, P = 0.339 \)). Microperfusion of methanandamide also did not affect electroencephalographic power within any frequency band (range of \( F_{2,16} = 0.32 \) to \( 0.88, P = 0.718 \) to \( 0.131 \), two-way ANOVA-RM). The absence of changes in these indices of brain electrocortical arousal state and postural (neck) motor activity indicate that the wakefulness-specific genioglossus activation produced by microperfusion of methanandamide into the hypoglossal motor pool was likely not the indirect result of increased motor drive related to a change in behavioural state. Figure 23K shows that the sites of microperfusion of methanandamide were within the hypoglossal motor pool in all rats.

Control of neuronal activity by Kv channels is complex. High-threshold Kv channels act to reduce action potential duration thus permitting high rates of repetitive firing, whereas low-threshold Kv channels oppose neuronal depolarization to action potential threshold, thereby restraining presynaptic and postsynaptic hyperexcitability (Dodson and Forsythe, 2004; Wulff et al., 2009). Consistent with a predominant effect on the latter group, microperfusion of the Kv channel blockers TEA and 4-AP into the hypoglossal motor pool strongly activated genioglossus muscle activity throughout sleep.

Figure 24A shows examples of increased genioglossus muscle activity across sleep-wake states during local microperfusion of 4-AP and TEA into the hypoglossal motor pool. Estimates based on the maximum concentration of drug in the phantom tissue surrounding the microdialysis probe tip being \( \leq 18\% \) of the concentration in the perfusion medium (Figure 22A-C) suggest peak tissue concentrations of \( \leq 90 \mu M \) and 2.1 mM for 4-AP and TEA, respectively. In Figure 24A, note that 4-AP and TEA increased genioglossus activity throughout both NREM and REM
sleep to levels exceeding those in normal wakefulness. Figure 24B shows that local microperfusion of 4-AP and TEA into the hypoglossal both completely prevented the periods of motor atonia that normally accompany REM sleep, such that tonic and respiratory genioglossus muscle activities were preserved throughout the REM sleep episodes.

Group data from nine rats with microperfusion of 4-AP into the hypoglossal motor pool, and the other nine rats with microperfusion of TEA, identified a significant increase in respiratory-related and tonic genioglossus muscle activities relative to the ACSF controls that occurred independently of the prevailing sleep-wake state (Figure 24C and D; range of $t \_8 = 2.39$ to $3.35$, $P = 0.044$ to $0.010$, post hoc paired t-tests following identification of: (1) a significant main effect of treatment, $F \_1,8 = 7.97$ to $15.27$, $P = 0.022$ to $< 0.001$, and (2) an insignificant interaction between treatment and state, $F \_3,24 = 0.44$ to $2.21$, $P = 0.728$ to $0.113$, two-way ANOVA-RM).

The effects of microperfusion of TEA and 4-AP into the hypoglossal motor pool were specific to the genioglossus muscle as other variables were not significantly affected. The lack of effects on other variables included the amplitude of diaphragm activity (Figure 24E, range of $F \_1,8 = 0.48$ to $2.80$, $P = 0.508$ to $0.133$, two-way ANOVA-RM), respiratory rate (Figure 24F, $F \_1,8 = 1.25$ to $1.60$, $P = 0.296$ to $0.240$), neck muscle activity (Figure 24G, $F \_1,8 = 3.88$ to $1.59$, $P = 0.700$ to $0.084$) or electroencephalographic power within any frequency band ($F \_1,8 = 0.008$ to $4.99$, $P = 0.932$ to $0.056$). Figure 24H shows that the sites of microperfusion of TEA and 4-AP were within the hypoglossal motor pool in all rats.
A.  
Wakfulness  
ACSF  
4-AP  
TEA  
Non-REM sleep  
ACSF  
4-AP  
TEA  
REM sleep  
ACSF  
4-AP  
TEA  

Genioglossus  
Genioglossus EMG  
Diaphragm  
EEG  

B.  
ACSF  
5 sec  
4-AP  
5 sec  
TEA  
5 sec  

Non-REM sleep  
REM sleep  

C.  
Respiratory-Related Genioglossus Muscle Activity  

D.  
Tonic Genioglossus Muscle Activity  

E.  
Diaphragm Muscle Amplitude  

F.  
Respiratory Rate  

G.  
Tonic Neck Muscle Activity  

H.  
Pyriform Motor Nucleus  

Legend:  
ACSF  
4-AP (Probe concentration = 500µM; Maximum tissue concentration = 90µM)  
TEA (Probe concentration = 10mM; Maximum tissue concentration = 1.8mM)
Figure 24. Blockade of voltage-gated potassium channels at the hypoglossal motor pool produces suprawaking levels of genioglossus muscle activity throughout sleep.

(A) Examples from individual rats showing genioglossus muscle activation by microperfusion of 500 µM 4-AP (n = 9) and 10 mM TEA (n = 9) into the hypoglossal motor pool across sleep-wake states (ACSF sections taken from rat administered 4-AP), with longer REM episodes shown in (B; ACSF sections taken from rat-administered TEA). Group data showing the effects of 4-AP and TEA on respiratory-related (C) and tonic (D) genioglossus muscle activities during wakefulness, non-REM, and REM sleep with ([−]) and without ([+]) muscle twitching. E-H shows the effects of 4-AP/TEA across sleep-wake states on the control variables: diaphragm amplitude, respiratory rate, tonic neck muscle activity, and the amplitude of REM sleep-specific muscle twitching. (I) Example and group data showing the location of microdialysis probes. The example from a single animal indicates the site of microdialysis within the hypoglossal motor pool. The location of the ventral tip of the probe site is indicated by the black arrow and the approximate position of the entire microdialysis probe membrane is denoted by the white dotted section. The locations of all the sites of microdialysis are also shown. Grey rectangles represent the space occupied by the semipermeable membrane portion of the microdialysis probes. Values are means ± standard error of the mean. # indicates significant effect of either 4-AP or TEA relative to ACSF controls independent of sleep-wake state (P < 0.05, from analysis of variance). ACSF, artificial cerebrospinal fluid; REM, rapid eye movement; TEA, tetraethylammonium chloride; 4-AP, 4-aminopyridine.
5.4 Discussion

Here we show that targeted modulation of potassium channel conductance at the hypoglossal motor pool is, in principle, an effective strategy to reactivate genioglossus muscle throughout sleep to at least normal waking levels. It is important to note that the restoration and sustained reactivation of both tonic and respiratory components of pharyngeal muscle activity, throughout both NREM and REM sleep, has not been achieved by other blocking agents at this motor pool. These results, therefore, establish an important new direction for translational sleep science by identifying potassium channel modulation as an appropriate target for developing a pharmacotherapy for OSA. As discussed in the next paragraphs, manipulation of such a target is viable because certain potassium channel subunits show highly restricted expression in the brain, mainly on cranial motor neurons such as the hypoglossal (Topert, Doring et al. 1998). Given the highly restricted expression, such channels provide an appropriate and high-priority molecular target for drug development to selectively manipulate upper airway muscle activity for OSA.

Kir channels significantly influence the excitability of hypoglossal motor neurons. Barium-mediated blockade of hypoglossal Kir channels in vitro produces motor neuron depolarization, tonic spike firing, and increased spiking frequency in response to suprathreshold current injections (Topert, Doring et al. 1998). Here we show that putative barium-mediated blockade of Kir channels at the hypoglossal motor pool in vivo produces supra-waking levels of pharyngeal motor activity during both NREM and REM sleep. We have previously shown that targeted blockade of G-protein coupled Kir3 channels restores respiratory-related genioglossus activity specifically in REM sleep (Grace, Hughes et al. 2013). That study provided evidence of a powerful and REM sleep-specific motor inhibitory mechanism that can be viewed as a genuine and significant mediator of REM sleep pharyngeal motor inhibition because its blockade has its largest influence in REM sleep and trivial, or no, effect in other sleep-wake states (Grace,
Hughes et al. 2013). In contrast and of significance, the relatively nonspecific blockade of Kir channels in the current study produced activation of pharyngeal motor activity across all sleep-wake states, an effect consistent with a state-independent increase in motor neuronal gain stemming from the additional blockade of constitutively open Kir channels. Importantly, hypoglossal motor neurons together with other cranial motor pools express the Kir2.4 channel, whereas evidence of its expression is lacking elsewhere (Topert, Doring et al. 1998). Kir2.4 is a member of the constitutively open Kir2 channel subfamily, and appears to be the most abundantly expressed Kir2 family subunit at the hypoglossal motor pool (Topert, Doring et al. 1998). The near-exclusive expression of Kir2.4 to cranial motor pools together with the efficacy of Kir channel blockade in restoring waking levels of pharyngeal motor activity throughout sleep suggest that Kir2.4 is a rational and high-priority target for the future development of a pharmacological treatment for OSA. There are currently no available agents to specifically modulate Kir2.4 channel function.

Inhibition of TASK channels is cited as a possible pharmacotherapeutic target for OSA (Brendel, Goegelein et al. 2007; Coburn, Wang et al. 2011; Coburn, Luo et al. 2011). TASK channels are strongly expressed at motor nuclei including the cranial motor pools, are major determinants of hypoglossal motor neuron resting potassium conductance in vitro, and are inhibited by neuromodulators arising from neuronal groups that are active in wakefulness but less active in sleep (Bayliss, Viana et al. 1997; Talley, Lei et al. 2000; Berg, Talley et al. 2004). Together, these observations suggest that withdrawal of endogenous wake-active neuromodulators from wakefulness to sleep may lead to TASK channel opening and reduced motor neuronal excitability, whereas targeted inhibition of such channels would prevent this endogenous mechanism and reactivate pharyngeal motor activity in sleep. Here we show that local microperfusion of the TASK channel blocker/cannabinoid receptor agonist methanandamide into
the hypoglossal motor pool does activate pharyngeal motor activity, but this effect is apparent only in wakefulness and not in sleep. Further analysis confirmed that the magnitude of the methanandamide-mediated increase in waking genioglossus activity was related to the level of waking arousal as judged by frequency analysis of the EEG.

The wakefulness-only genioglossus activating effect of microperfusion of methanandamide into hypoglossal motor pool is consistent with exaggerated nocturnal motor activity in TASK-3 knockout mice (Linden et al., 2007). However, the lack of other observable motor or neurological differences in TASK channel knockout mice may be due to a compensatory increase in inhibitory GABA$_A$ receptor function that can occur independently of changes in GABA$_A$ receptor expression (Brickley et al., 2001; Linden et al., 2008). It is possible, therefore, that in our study the physiological significance of TASK channel modulation is masked by a rapid homeostatic regulation of membrane potential mediated by GABA$_A$ receptors. Even if this scenario of compensation for the intervention is correct, however, TASK channel blockade at the hypoglossal motor pool may not be an effective strategy to reactivate pharyngeal motor activity in sleep (as the effects are confined to wakefulness) such that targeting these channels may not be effective for OSA. Such a definitive conclusion can only be confirmed, however, with more specific TASK channel blockers.

In addition to blocking TASK channels, methanandamide is also an agonist of cannabinoid receptors. A function of cannabinoid receptors throughout the nervous system, including the hypoglossal motor pool (Mukhtarov et al., 2005) is presynaptic depression of inhibitory neurotransmitter release. Accordingly, the motor-activating effect of microperfusion of methanandamide into the hypoglossal motor pool could be explained by such a mechanism. If such a scenario is correct, however, we would expect a priori that depression of inhibitory
neurotransmission at the hypoglossal motor pool would produce motor activation in sleep as well as wakefulness, and this did not occur. Methanandamide can also inhibit voltage-gated sodium channels (Kennard et al., 2005), N-type/T-type calcium channels (Mackie et al., 1993; Chemin et al., 2001), and shaker-type voltage-gated potassium channels (Poling et al., 1996). With the exception of the potassium channels, however, such non-TASK channel effects potentially mediated by methanandamide would be expected to inhibit hypoglossal motor neurons.

We also showed that blockade of either TEA or 4-AP sensitive potassium conductances at the hypoglossal motor pool were sufficient to activate pharyngeal muscle activity throughout sleep to waking levels. TEA and 4-AP sensitive Kv channels having high voltage thresholds mediate the fast component of the after-hyperpolarization in hypoglossal motor neurons; blockade of such channels increases action potential duration, reduces firing frequency, and would therefore tend to suppress downstream motor activity (Viana, Bayliss et al. 1993). As a consequence, the TEA and 4-AP-mediated activation of pharyngeal muscle activity is likely mediated by blockade of low threshold Kv channels, which normally act to suppress presynaptic and postsynaptic membrane excitability (Dodson and Forsythe 2004).

Low-threshold Kv channels operate below the threshold for action potential generation, producing outward currents in response to depolarizations from resting membrane potential (Wulff, Castle et al. 2009). The relatively low concentration of 4-AP in this study (estimated maximum tissue concentration of ~90 µM) would be expected to broadly inactivate the low threshold Kv1 subfamily whereas TEA (estimated maximum tissue concentration of ~1.8mM) would be expected to more specifically block Kv1.1 and 4-AP insensitive Kv7.2 (KCNQ2) channels (Enyedi and Czirjak; Kavanaugh et al., 1991; Fedida et al., 1993; Grissmer et al., 1994; Stephens et al., 1994; Fedida et al., 1996; Kalman et al., 1998; Wang et al., 1998; Yang et al.,
Blockade of these channels likely contributes to the increased pharyngeal motor activity observed during sleep in the presence of microperfusion of TEA or 4-AP into the hypoglossal motor pool. Inactivation of low threshold Kv channels would be expected to increase the probability of action potential firing via sensitization of the postsynaptic membrane to depolarizing inputs. Low threshold Kv channels, specifically Kv1.1, are also located presynaptically where they prevent hyperexcitability and spontaneous action potential generation. Presynaptic actions of TEA and 4-AP also likely contribute to the observed pharyngeal motor activation in sleep, particularly REM sleep, when significant facilitatory input is required to overcome motor neuron inhibition. Ultimately, the combination of presynaptic and postsynaptic effects of 4-AP and TEA may underlie their ability to activate motor activity in REM sleep.

5.4.1 Summary

In summary, these findings establish proof-of-principle that targeted blockade of certain potassium channels at the hypoglossal motor pool is an effective strategy to produce sustained activation of tonic and respiratory components of pharyngeal muscle activity throughout NREM and REM sleep. Such responses have not been observed with other blocking agents at this motor pool. Certain potassium channels show highly restricted expression to the cranial motor pools, particularly the hypoglossal. (Topert, Doring et al. 1998) Accordingly, development of specific agents (currently lacking) to manipulate such channels – aiming to restore physiological patterns of activity previously present in wakefulness—may provide a viable and tractable pharmacological target of benefit, for example, to patients with OSA. Further studies may also identify the broad applicability of the current findings to other cranial motor pools (as would be suggested by the regionalized distribution of channel expression (Topert, Doring et al. 1998)), and the type of motor units preferentially affected by the interventions and the mechanical
consequences on airway stability (e.g., tonic and/or inspiratory-modulated units (Saboisky, Butler et al. 2006; Bailey, Fridel et al. 2007)).
Chapter 6
Additional Discussion and Future Directions

6.1 Compensatory changes in hypoglossal motor neuron excitability in response to changes in background conductances

Chapter 5 detailed attempts to determine whether potassium channel blockade at the hypoglossal motor pool can restore activity in tongue musculature to waking levels during sleep. We targeted three major classes of potassium channels in that study including TASK channels of the tandem-pore domain leak channel family. Unlike the blockade of voltage-dependent and inwardly rectifying potassium channels, the blockade of TASK channels had minimal effects on hypoglossal motor output. Increases in tongue muscle activity following TASK channel blockade were constrained to periods of active wakefulness. This was contrary to our hypothesis that the effects of TASK channel blockade would be greatest during sleep, when whole cell TASK conductance is likely maximal due to the withdrawal of arousal-related neurotransmitters that normally suppress TASK leak currents. Here I will argue that the robustness of hypoglossal motor output against TASK channel blockade is suggestive of homeostatic regulation of excitability in the hypoglossal motor pool.

Members of the two-pore domain channel family produce potassium leak currents that dictate resting membrane potential, input resistance, and overall neuronal excitability (Viana et al., 1993; Duprat et al., 1997). Neurotransmitter modulation of two-pore domain potassium channels may be involved in the short-term dynamic control of neuronal activity, while over the long-term, two-pore domain channels may participate in the homeostatic maintenance of neuronal excitability (Talley et al., 2000; Brickley et al., 2001). In motor neurons, a resting potassium
current attributed to the two-pore domain TASK channels is modulated by several excitatory neurotransmitters including serotonin, norepinephrine, thyrotropin-releasing hormone, substance P, and glutamate (Bayliss et al., 1997; Talley et al., 2000). These neurotransmitters inhibit an instantaneous, barium-sensitive, open-rectifier potassium current with a pH-sensitivity of approximately 7.4 — these characteristics are known only for TASK among all potassium channels cloned to date (Bayliss et al., 2001). The action of these neurotransmitters on TASK channels has been verified using occlusion experiments and heterologous expression systems (Talley et al., 2000). The association between TASK channels and arousal-related neurotransmitters in motor pools suggests that withdrawal of wake-active neuromodulators from wakefulness to sleep may lead to TASK channel opening and reduced motor neuronal excitability that accounts for muscle tone suppression during sleep.

In contrast to our hypothesis, the normal suppression of genioglossus motor activity that occurs in transition from wakefulness to sleep was not attenuated by TASK channel blockade. TASK channel blockade only increased genioglossus activity during periods of behavioural arousal — when, presumably, hypoglossal motor output is being dynamically modulated. The absence of an effect of TASK channel blockade in quiet wakefulness and sleep could be interpreted as evidence of a minimal contribution of TASK channels to neuronal excitability in the hypoglossal motor pool. However, this is unlikely given the major contribution of TASK channels to the resting potassium conductance of hypoglossal motor neurons in vitro — acidification-mediated TASK Channel blockade results in membrane depolarization above the threshold for repetitive action potential generation (Bayliss et al., 2001). Alternatively, the robustness of hypoglossal motor output against TASK channel blockade might indicate the presence of compensatory mechanisms acting to homeostatically correct disturbances in hypoglossal motor neuronal excitability.
The hypothesis that TASK channels participate in the homeostatic regulation of neuronal excitability is supported by data from cerebellar granule cells. A common feature of these cells is a strong potassium leak conductance, which emerges during development in parallel with increasing TASK channel expression (Millar et al., 2000). Also contributing to the resting excitability of cerebellar granule cells are tonically active populations of extrasynaptic GABA_A receptors (Rossi and Hamann, 1998). These receptors are asynchronously activated by low levels of ambient GABA, producing a tonic inhibition of neuronal activity. However, cerebellar granule cells from transgenic mice lacking these extrasynaptic GABA_A receptors do not exhibit differences in excitability — membrane properties, responses to current injection, or excitatory post synaptic potential properties — relative to cells from wild-type mice (Brickley et al., 2001). Moreover, mice lacking extrasynaptic GABA_A receptors have normal motor function (Jones et al., 1997; Nusser et al., 1999) (Jones et al., 1997; Nusser et al., 1999). Normal excitability in cerebellar granule cells lacking extrasynaptic GABA_A receptors may be explained by the compensatory increase in potassium leak conductance observed in these cells (Brickley et al., 2001). Consistent with TASK channel involvement, this resting potassium current can be modulated by physiological changes in pH (Brickley et al., 2001).

Like transgenic mice lacking extrasynaptic GABA_A receptors, TASK knock-out mice do not exhibit significant motor deficits beyond exaggerated nocturnal motor activity (Linden et al., 2007), this despite the normally high levels of TASK channel expression in motor pools and the cerebellum (Duprat et al., 1997; Leonoudakis et al., 1998). However, consistent with there being a compensatory relationship between potassium leak current mediated by TASK channels and tonic inhibition mediated by extrasynaptic GABA_A receptors, TASK-1 knockout mice show enhanced sensitivities to the ataxic effects of gaboxadol (a selective extrasynaptic GABA_A receptor agonist) (Linden et al., 2008). This result may reflect an upregulation of extrasynaptic
GABA<sub>A</sub> receptor function that compensates for diminished potassium leak current. This upregulation would be expected to be even greater when both TASK-1 and TASK-3 channel subunits are knocked out given that compensatory increases in TASK-3 homodimer expression have been observed in TASK-1 knockout mice (Aller et al., 2005). Importantly, autoradiography showed that GABA<sub>A</sub> receptor expression levels were not increased in TASK knock out mice indicating that increased drug sensitivity may be the result of rapid conductance changes (Linden et al., 2008).

As previously mentioned, there is currently a lack of commercially available and specific TASK channel blockers. Merck and Co. have developed a novel series of TASK channel antagonists: derivatives of 5,6,7,8-tetrahydropyrido[4,3-d]pyrimidine. One such derivative, referred to here as ‘C23’, is a potent blocker of TASK-3 (IC<sub>50</sub>=35nM) and TASK-1 (IC<sub>50</sub>=300nM) channels. Another pharmacological option for inactivating TASK channels is doxapram hydrochloride, which, when administered systemically, acts as a respiratory stimulant by blocking potassium leak currents in the carotid bodies and medullary respiratory neurons (Yost, 2006). Based on previous studies we would expect C23 to attenuate the activation of hypoglossal motor neurons caused by stimulation of Gα<sub>q</sub> linked receptors for the neurotransmitters serotonin, norepinephrine, thyrotropin-releasing hormone, substance P, and glutamate (Chen et al., 2006). Additionally, we would expect the genioglossus motor activating effects of C23 to be absent in cholinergic neuron-specific TASK-1/3 channel knock out mice. The lack of effect in cholinergic neuron-specific TASK knock out mice would confirm that the effects of these compounds are: (i) TASK channel specific and (ii) specific for cholinergic neurons (i.e., presumably motor neuron specific).
After validating the conditions under which C23 and/or doxapram can be used to specifically block TASK channels in the hypoglossal motor pool, we can further test whether TASK channel activation in the HM pool is responsible for the pharyngeal muscle suppression that occurs in transition from wakefulness to sleep. Where local pharmacological blockade of TASK channels at the hypoglossal motor pool eliminates or attenuates suppression of pharyngeal motor activity in sleep, we can conclude that TASK channel activation in the hypoglossal motor pool is a non-redundant mechanism responsible for the suppression of pharyngeal motor activity in sleep. Where local pharmacological blockade of TASK channels at HM pool has no effect, we would have to conclude that TASK channel activation at the hypoglossal motor pool is not involved in the mechanism of pharyngeal motor suppression in sleep or that it is a redundant mechanism of motor suppression. In the latter case, where such compensatory regulation is present, it is possible that diminished motor tone in sleep may not be the exclusive consequence of the passive response of motor neurons to state-dependent changes in neuromodulatory tone. Motor neurons may be programmed with multiple strategies to homeostatically maintain state-specific levels of excitability. We might hypothesize that state-specific levels of hypoglossal motor neuron excitability are homeostatically maintained by TASK channels and extrasynaptic GABA\textsubscript{A} receptors acting redundantly. To evaluate this hypothesis we could test if the focal administration of C23 and/or doxapram at the hypoglossal motor pool attenuates genioglossus muscle hypotonia occurring in sleep to a greater degree in mice lacking extrasynaptic GABA\textsubscript{A} receptors. Also, we could test if the focal administration of gaboxadol (Brickley and Mody, 2012) at the hypoglossal motor pool has a greater effect on hypoglossal motor output in TASK KO mice compared to controls.
6.2 Using the database of candidate HMN and HMN primary pre-motor field drug targets

There are several factors that ought to be considered when using the database, detailed in section 1.5.3, as a tool for the identification of potential drug targets for OSA. Firstly, reported differences in RNA expression determined by in situ hybridization experiments are not strictly indicative of differences in protein expression. Secondly, we did not screen the list of FDA approved drugs associated with genes in our database according to the likelihood of their having clinically beneficial effects on hypoglossal motor output. For example, expression of the alpha 1a adrenergic receptor gene is enhanced at the HMN, and noradrenergic inputs to the HMN mediate an important component of the state-dependent modulation of tongue muscle activity (Chan, Steenland et al. 2006). We list a total of 36 FDA approved drugs capable of targeting this receptor, fourteen of which are antagonists whose direct effects on the HMN would be expected to negatively impact upper airway motor tone. Nevertheless, the number and selectivity of drugs associated with a given target is useful information, not least because it can be taken as an indicator of the relative druggability of that target. Moreover, initial studies have shown that desipramine has beneficial effects on genioglossus muscle tone, upper airway collapsibility and OSA severity (Taranto-Montemurro et al., 2016a; Taranto-Montemurro et al., 2016b).

Desipramine is a tricyclic anti-depressant with strong noradrenergic and some anti-muscarinic effects. It was selected for those studies with the aim of preserving the excitatory noradrenergic tone to the pharyngeal motor pools that is normally withdrawn in sleep (Chan, Steenland et al. 2006; Burgess and Peever 2013), and to counter the strong muscarinic cholinergic receptor-mediated inhibition of the tongue musculature that occurs in REM sleep (Grace, Hughes et al. 2013).
This database is also an important resource for investigators working in pre-clinical animal models, because in the case of several targets, pre-clinical studies will be required to evaluate their potential clinical utility. For instance, expression of the gene for 5-hydroxytryptamine 2c (5-HT2c) receptors is enhanced in the PRE-HMN but not in the HMN (Table 1), yet we do not know what effect the selective agonism of these receptors would have on hypoglossal motor output, or if 5-HT2c receptor-positive neurons innervate the HMN. Although, preliminary studies have shown that focal stimulation of 5-HT2c receptors in the parvicellular reticular formation and dorsal medullary reticular fields with 25CN-NBOH strongly increases hypoglossal motor output in anesthetized rats (data not shown). This illustrates the potential usefulness of targeting select sources of premotor input to the HMN, rather than the HMN itself, for OSA pharmacotherapy. It should be noted that, in the analysis presented in section 1.5.3, differential searching was performed for the grouping of HMN pre-motor structures relative to the rest of the brain. By grouping the pre-motor structures together, we biased our analysis towards the identification of genes whose expression is relatively high across multiple pre-motor structures (i.e., this method is less sensitive when compared to performing differential searches, relative to the brain at large, for each pre-motor field individually). This diminished sensitivity is likely responsible for the overall levels of differential gene expression in the pre-motor grouping being much less than that for the hypoglossal motor pool. In section 1.5.4, I argued that the relative inexcitability of hypoglossal motor neurons necessarily limits their potential as a target for OSA pharmacotherapy. In theory, pharmacologically targeting a HMN pre-motor group with high relative excitability would permit greater activation of hypoglossal motor neurons than would be possible with direct modulation of the motor pool with an equivalent dose of drug. In other words, pre-motor groups with high relative excitability can be thought of as amplifiers of drug
action on the hypoglossal motor pool. However, indirect reactivation of the motor pool may not be possible in REM sleep.

The analysis focused specifically on identifying targets for the activation of upper airway motor tone, one of only several possible pharmacological strategies for the treatment of OSA. Nevertheless, consulting databases of this kind will be important regardless of the ultimate stratagem employed, because when screening candidate drugs for OSA pharmacotherapy it is prudent to consider the potential positive or negative effects of drug action on the targets present in the HMN and PRE-HMN.

6.3 The problem of deriving higher order circuit logic from reductionist experimental data

If a principal aim of interrogating brain circuits is to develop a cause and effect account of how different network states arise from dynamic sets of logical interactions between sub-groups of network nodes, then the future of neuroscience faces a very significant problem. In taking a reductionist approach to understanding brain networks — by selectively interrogating neuronal groups one at a time — we create the problem of how to derive the higher order network logic from the collection of data pertaining to individual nodes. Doing so is not simply a matter of collecting large amounts of data, because complex network logic cannot be solved by a brute force approach. In section 1.3.3, it was argued that gain-of-function studies assume that we can effectively understand complex networks by deemphasizing the importance of endogenous initial conditions and attributing functional significance to the nodes themselves, rather than their dynamic interactions, and that this approach, relative to loss-of-function studies and the measures of nodal embeddedness that they produce, provide little basis for deriving higher order network
logic. While determining the functional significance of all the node-to-node interactions in network may be useful towards assembling a holistic model of network function, it is not practically possible. For a network of a given size, evaluating the functional significance of the entire set of network interactions requires the modulation of each node individually in addition to every permutation of the possible subsets of network nodes (Kumar et al., 2013). Consider for instance the hypothesized mechanism of action accounting for the experimental results reported in Chapter 2: “Endogenous Cholinergic Input to the Pontine REM Sleep Generator Reinforces, But Does Not Initiate, Transitioning into REM Sleep” (Grace et al., 2014a). It was hypothesized that, activation of the SubC initiates NREM-to-REM sleep transitioning and gates activity in PPT/LDT cholinergic neurons which then provide cholinergic positive feedback to SubC neurons, thereby reinforcing transitioning. To fully test this mechanism, it is necessary to:

(i) Inhibit the PPT/LDT (for the purposes of this argument, I will treat the PPT/LDT as a single entity denoted by, ACh) followed by simultaneous inhibition of the SubC (S)

(ii) Inhibit S followed by simultaneous inhibition of ACh.

These experiments give four experimental states that need to be compared:

(i) Inactivation of the cholinergic neurons per se (ACh-OFF),

(ii) Inactivation of the SubC neurons per se (S-OFF),

(iii) Inactivation of cholinergic neurons with previous inactivation of SubC neurons (ACh-OFFS-OFF),
(iv) Inactivation of SubC neurons with previous inactivation of ACh neurons (S-OFF \(_{ACh-OFF}\)).

To be consistent with the hypothesis above, the following statements regarding the experimental data would have to be true:

(i) effect size of S-OFF > ACh-OFF,

(ii) effect size of ACh-OFF \(_s\)-OFF = 0,

(iii) effect size of S-OFF \(_{ACh-OFF}\) = (S-OFF) – (ACh-OFF)

This experimental analysis is only valid when ignoring the surrounding network. Consideration of the surrounding network further complicates the analysis due to the probable existence of additional parallel pathways connecting the SubC and the PPT/LDT. Therefore, brute force interrogation of neural circuitry is not practical because for a network having \(N\) interacting elements, the number of possible node-subsets grows faster than exponentially with \(N\) (Kumar et al., 2013). For a network of size \(N\), the number of node subsets is given by Bell’s number:

\[
B_N = \sum_{k=0}^{N-1} \left( \frac{(N - 1)!}{k! (N - 1 - k)!} B_k \right)
\]

For example, a network with ten interacting nodes contains 115,975 subgroups. Therefore, reliable determination of higher order circuit logic will require the development of an epistemological framework for synthesizing reductionist experimental data so as to permit modelling of complex networks without a combinatorial explosion in the number of required experiments.
6.4 Identifying the logical motifs that comprise the REM sleep generating circuit

Experimental investigation of sleep circuitry has revealed a complex executive network of neuronal groups that are capable of controlling REM sleep; however, little consensus has been reached regarding the logical structure of the REM sleep generating network. The validity of proposed REM sleep generating models must be judged according to how well they account for the statistical features of sleep architecture. In order to effectively model circuitry underlying REM sleep generation we need to identify features of REM sleep that constrain the possible logical configurations. One such feature is the rebound of REM sleep following selective deprivation (Dement, Greenberg et al. 1966; Morden, Mitchell et al. 1967; Brunner, Dijk et al. 1990; Endo, Roth et al. 1998). The sensitivity of REM sleep generation to previous sleep history — over long time scales — is important. This sensitivity implies that the nervous system has a memory of the REM sleep interval (Benington and Heller, 1994). Therefore, we should expect that some system parameter(s) varies as a function of time spent out of REM sleep. Furthermore, the REM sleep generating circuit must be sensitive to this parameter(s), but must not respond to changes in this parameter(s) continuously, otherwise undesired blending of sleep states would occur. The potential requirement of the REM sleep generative circuit to convert graded external drive signals into a binary output is the strongest rationale for invoking a flip-flop logic in REM sleep generation mechanisms. Future studies should aim to identify the neural substrate(s) of REM sleep ‘memory’. Such studies may involve the identification of neuronal pools whose activity is predictive of the time elapsed since a previous REM sleep episode. Consideration will have to be given to whether such pools are intrinsic detectors of the REM sleep interval or whether they are simply modulated by such detectors. Experiments that impair REM sleep
'memory’, or interrupt its transmission to executive structures in the brainstem, could address whether or not REM sleep generation is dependent on a memory-related drive or if this drive simply modulates a central pattern generator. While such an oscillation could result from the actions of a pattern generating circuit (e.g., reciprocal interaction hypothesis), it is also possible that REM sleep generating circuitry is driven by stochastic processes (Stephenson et al., 2013) with a sensitivity determined by a memory-related drive. By mapping the efferent pathways of identified REM sleep memory-related neuronal pools, we will be able to identify neuronal groups responsible for the integration of REM sleep ‘memory’.
References


Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, Harris MA, Hill DP, Issel-Tarver L, Kasarskis A, Lewis S, Matei...


Clement O, Sapin E, Berod A, Fort P, Luppi PH (2011) Evidence that neurons of the sublateralodorsal tegmental nucleus triggering paradoxical (REM) sleep are glutamatergic. Sleep 34:419-423.


Enyedi P, Czirjak G Molecular background of leak K+ currents: two-pore domain potassium channels. Physiological reviews 90:559-605.


Jouvet M (1962) [Research on the neural structures and responsible mechanisms in different phases of physiological sleep.]. Arch Ital Biol 100:125-206.


Jouvet M, Michel F (1960a) [New research on the structures responsible for the "paradoxical phase" of sleep.]. J Physiol (Paris) 52:130-131.
Jouvet M, Mounier D (1960) [Effect of lesions of the pontile reticular formation on sleep in the cat.]. Comptes rendus des seances de la Societe de biologie et de ses filiales 154:2301-2305.

Jouvet M, Michel F (1960b) [Release of the "paradoxal phase" of sleep by stimulation of the brain stem in the intact and chronic mesencephalic cat]. Comptes rendus des seances de la Societe de biologie et de ses filiales 154:636-641.


Shouse MN, Siegel JM (1992) Pontine regulation of REM sleep components in cats: integrity of the pedunculopontine tegmentum (PPT) is important for phasic events but unnecessary for atonia during REM sleep. Brain research 571:50-63.


Van Dongen PA (1980) Locus ceruleus region: effects on behavior of cholinergic, noradrenergic, and opiate drugs injected intracerebrally into freely moving cats. Exp Neurol 67:52-78.


Wirth KJ, Steinmeyer K, Ruetten H (2013) Sensitization of upper airway mechanoreceptors as a
new pharmacologic principle to treat obstructive sleep apnea: investigations with


Xi MC, Morales FR, Chase MH (1999) Evidence that wakefulness and REM sleep are controlled

Yamamoto K, Mamelak AN, Quattrochi JJ, Hobson JA (1990) A cholinceptive desynchronized
sleep induction zone in the anterodorsal pontine tegmentum: locus of the sensitive region.
Neuroscience 39:279-293.

Yang WP, Levesque PC, Little WA, Conder ML, Ramakrishnan P, Neubauer MG, Blanar MA
(1998) Functional expression of two KvLQT1-related potassium channels responsible for

Yost CS (2006) A new look at the respiratory stimulant doxapram. CNS drug reviews 12:236-
249.
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Text in Chapter 3, and figures 7-13, are adapted from the content of an original research article published by Kevin P. Grace, Lindsay E. Vanstone, and Richard L. Horner (Grace KP, Vanstone LE, Horner RL (2014) Endogenous Cholinergic Input to the Pontine REM Sleep Generator Is Not Required for REM Sleep to Occur. The Journal of Neuroscience 34(43)). Permission is given under the terms of the Creative Commons Attribution-Noncommercial-Share Alike 3.0 Unported License (http://creativecommons.org/licenses/by-nc-sa/3.0).

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