CHARACTERIZING A ROLE FOR DOPAMINE ON SLEEP AND CATAPLEXY IN NARCOLEPTIC MICE

by

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

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Narcolepsy is a disabling sleep disorder that is characterized by persistent sleepiness, and cataplexy – an involuntary loss of waking muscle tone. Cataplexy and narcolepsy are caused by the loss of hypocretin containing neurons in the hypothalamus. However, it is hypothesized that dopamine is also involved in sleep and motor control and plays a role in cataplexy. This study investigated how manipulating dopamine affected sleep and cataplexy in narcoleptic mice devoid of hypocretin. We used d-amphetamine to increase endogenous dopamine levels and quinpirole (D2 agonist) to agonize D2 receptor sites. Amphetamine promoted wakefulness while decreasing sleep in wild-type mice, but was less effective in narcoleptic mice. Amphetamine also reduced cataplexy as well as sleep attacks (an indicator of sleepiness) in narcoleptic mice. Quinpirole had no effect on sleep or wakefulness; however, it potently increased cataplexy without affecting sleep attacks in narcoleptic mice.
ACKNOWLEDGEMENTS

There are a number of people that I would like to thank because without their guidance and support, this thesis would not have been possible.

Firstly, I would like to express my utmost appreciations to Drs. John Yeomans and John Peever for investing their time and confidence in me as a graduate student. Their guidance and support has been instrumental in developing me as a young and confident scientist.

Much of my final research was conducted in Dr. John Peever’s lab where I was fortunate enough to work with a very enthusiastic and knowledgeable supervisor. In addition, I was privileged to have worked with amazing people in the lab from which I have forged new friendships. Thanks a lot guys for the good times and moral support. I will always remember those days in my graduate years.

I would also like to thank to Dr. Melanie Woodin for being my graduate advisor over the years. Your kindness and support was much appreciated.

I would also like to acknowledge my best friend Alex Drew for being supportive over the years in addition to my tennis partner, workout partner, and buddy since our days in preschool.

Finally, I would like to most importantly thank my family. I want to thank my dad for being there when I was growing up and for supporting me over the years, in addition to being my sponsor (racing). Also, I want to acknowledge my very special Aunt CY, little cousin Vincent, and grandmother who I love very much and always will for everything they have done for me. To my brother Ter and sister Jocelyn, thanks for the support and encouragement over the years.
To the most important people in my life:

My family: Dad (Uncle Ben), Ter, Jocelyn, Aunt CY, Vincent, and Grandma
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<td>AP</td>
<td>Anterior-Posterior</td>
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<tr>
<td>cm</td>
<td>Centimeter</td>
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<td>CNS</td>
<td>Central Nervous System</td>
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<td>CSF</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<td>DR</td>
<td>Dorsal Raphe Nucleus</td>
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<tr>
<td>DV</td>
<td>Dorsal-Ventral</td>
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<td>EDS</td>
<td>Excessive Daytime Sleepiness</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>EEG</td>
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<td>EOG</td>
<td>Electrooculogram</td>
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<td>Excitatory Postsynaptic Potential</td>
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<td>GHB</td>
<td>γ-hydroxybutyrate</td>
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<td>HcrtR</td>
<td>Hypocretin Receptor</td>
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<td>HLA</td>
<td>Human Leukocyte Antigen</td>
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<td>I.P</td>
<td>Intraperitoneal</td>
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<td>Knockout</td>
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<td>Liter</td>
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<tr>
<td>LC</td>
<td>Locus Coeruleus</td>
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<td>LDT</td>
<td>Laterodorsal Tegmental Nucleus</td>
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<tr>
<td>LH</td>
<td>Lateral hypothalamus</td>
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<tr>
<td>M</td>
<td>Molar</td>
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<td>mm</td>
<td>Millimeter</td>
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<td>mM</td>
<td>Millimolar</td>
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<td>mg</td>
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<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>mL</td>
<td>Milliliter</td>
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<tr>
<td>ML</td>
<td>Medial-Lateral</td>
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<tr>
<td>MW</td>
<td>Molecular weight</td>
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<tr>
<td>NA</td>
<td>Noradrenaline</td>
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<tr>
<td>NaCl</td>
<td>Sodium Chloride</td>
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<tr>
<td>NREM</td>
<td>Non-rapid eye movement</td>
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<tr>
<td>P</td>
<td>Probability</td>
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<td>PAG</td>
<td>Periaqueductal gray</td>
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<td>PPT</td>
<td>Pedunculopontine Tegmental Nucleus</td>
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<td>REM</td>
<td>Rapid-eye movement</td>
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<td>RM</td>
<td>Repeated Measures</td>
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<td>SEC</td>
<td>Seconds</td>
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<td>SNr</td>
<td>Substantia Nigra</td>
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<td>SOREMP</td>
<td>Sleep onset REM periods</td>
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<tr>
<td>TAE</td>
<td>Tris Acetate</td>
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<td>TN</td>
<td>Tuberomammillary nucleus</td>
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<tr>
<td>U.V</td>
<td>Ultraviolet</td>
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<tr>
<td>V</td>
<td>Volts</td>
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<td>VLPO</td>
<td>Ventral lateral preoptic area</td>
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<td>VTA</td>
<td>Ventral tegmental area</td>
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Chapter One:

INTRODUCTION
1. INTRODUCTION

I. Rationale of Research

Narcolepsy is a disabling sleep disorder that affects 0.03-0.16% of the population (Nishino et al. 2000). Individuals with this disorder are plagued by a series of debilitating symptoms; two of the most common symptoms are excessive sleepiness during the day (EDS), and cataplexy (involuntary loss of skeletal muscle tone). Although there are therapies that manage this disorder, there are no definitive treatments that cure it. The pathophysiology of narcolepsy has been linked to hypocretin, and it has been suggested that the dysregulation or loss of this neuropeptide is responsible for the disorder (Siegel et al. 2001). However, the mechanisms that govern the symptoms of narcolepsy/cataplexy and how they interact with the hypocretin system, still remain unknown.

II. Experimental Aims and Approach

Hypocretin cells project to monoaminergic brain areas (e.g. dopamine cells) that regulate sleep and motor control. Since manipulating dopamine affects sleep and wakefulness, suggests that the hypocretin and dopamine systems are an integral part of the neurocircuitry controlling sleep and motor functions. Hypocretin neurons excite dopaminergic neurons of the midbrain that participate in regulating sleep and muscle activity. When hypocretin is disrupted, it can no longer orchestrate normal dopaminergic control of sleep and motor activity and as a result, narcolepsy/cataplexy develops.

The role for dopamine in regulating sleep and motor control is still in its infancy. It has been suggested that dopamine neurons do not change their neuronal activity across sleep and wakefulness (Monti and Monti 2007), however others have found that dopaminergic cells of the ventral periaqueductal gray (vPAG) fire during wakefulness but cease to fire during sleep (Lu et
Dopamine has a significant influence on sleep and wakefulness since inhibiting dopamine uptake in canines increases wakefulness (Kanbayashi et al. 2000) while facilitating the increase of endogenous dopamine levels by amphetamines, increases hyperactivity and wakefulness (Kitahama and Valatx 1979). In addition, lesioning dopamine neurons increase total daily sleep (Lu et al. 2006).

Dopamine is also implicated in regulating muscle activity because the agonism of dopamine D2 receptors increases cataplexy while antagonizing these same dopamine D2 receptors reduces cataplexy (Reid et al. 1996).

Hypocretin is important because it interacts with dopamine to regulate sleep and motor control. We therefore examine dopaminergic control of sleep and cataplexy in null-mutant hypocretin mice that exhibit many symptoms of narcolepsy. We used behavioural electrophysiology and pharmacology to determine the following two goals. *Aim 1*: To determine the effects of d-amphetamine (to increase endogenous dopamine levels) and quinpirole (to agonize dopamine D2 receptors) on sleep and wakefulness in narcoleptic mice. *Aim 2*: To determine the effects of these same drugs on cataplexy in narcoleptic mice.

Using pharmacological manipulations of dopamine in a hypocretin null-mutant mouse model will elucidate the neurocircuitry that regulates sleep and narcolepsy. The following sections will review the regulation of sleep and motor control, in addition to an overview of the hypocretin system and how the disruption of this system results in narcolepsy.
III. The Sleep-Wake Cycle

In mammals, sleep is characterized by a cascade of behavioural traits such as: 1) state reversibility (unlike death or a coma), 2) recumbent posture, 3) stereotypical reduction in muscle tone, and 4) an elevated arousal threshold to stimuli (Peever and McGinty 2007). The most important brain areas controlling sleep are the hypothalamus and brainstem (Jones 2005; Siegel 2004). The pathophysiology of sleep disorders often result from the disruption of important sleep regulating areas and neurochemicals, and can lead to disorders such as insomnia (inability to sleep), hypersomnia (inability to maintain wakefulness), and narcolepsy.

Identifying States of Sleep and Wakefulness

Sleep and waking are categorically defined into 3 states: wakefulness, NREM sleep (non rapid-eye movement sleep), and REM sleep (rapid-eye movement sleep). Wakefulness, is behaviorally defined as a state that is alert and responsive to the environment and its stimuli (Carskadon and Dement 2000). NREM sleep (also known as slow-wave sleep) and REM sleep (also known as paradoxical sleep or dreaming sleep), are behaviorally similar in that skeletal muscle tone is reduced or abolished, and response to the environment is greatly attenuated (Carskadon and Dement 2000). It is difficult to distinguish NREM and REM sleep behaviorally, without examining the underlying electrophysiology.

Electrophysiology is employed using a variety of polysomnographic techniques such as: electroencephalogram (EEG), electromyogram (EMG), and electrooculogram (EOG). The EEG is an electrical signal generated from the summated potential of cortical neurons. This is typically measured using recording electrodes trans-cranially or intra-cranially. The EMG is an electrical signal that is measured from the surface of a muscle (ie. postural muscle such as the neck muscle). The EOG is an electrical signal generated from the change in electrical potential by the movement of the dipoles with the eye movements (Carskadon and Rechtschaffen 2000).
Rodent Electroencephalogram (EEG) and Electromyogram (EMG)

Waking EEG and EMG: The sleep architecture of wakefulness, is generally defined in rodents by an EEG of high frequency and low amplitude. The typical trend of waking EEG is marked by beta rhythms. Beta rhythms are characterized by sleep waves that are desynchronized and of low amplitude in the frequency range 11.25-25 Hz (Tobler et al. 1997). Accompanying the waking EEG, the EMG of wakefulness can be associated with high postural muscle activity (Carskadon and Dement 2000). The following diagram illustrates the waking behaviour of a mouse.

![Waking EEG and EMG Diagram]

Figure 1.1: The electroencephalogram (EEG) and electromyogram (EMG) representing the waking behaviour of a mouse. The waking EEG is characterized by high frequency and low amplitude waveforms. The waking EMG is characterized by high muscle activity.

NREM sleep EEG and EMG: The sleep architecture of NREM sleep is typically the opposite of wakefulness and is defined by an EEG of low frequency and high amplitude. NREM sleep or slow-wave sleep (SWS), is named because of the large delta sleep waves that encompass this stage of sleep. These delta waveforms are composed of frequencies of 0.5-4.0 Hz in the EEG (Tobler et al. 1997). The EMG that accompanies NREM sleep is generally characterized by a
decrease in muscle activity (hypotonia). The following diagram illustrates the NREM sleep behaviour of a mouse.

![Diagram of NREM sleep EEG and EMG](image)

**Figure 1.2: The electroencephalogram (EEG) and electromyogram (EMG) representing the NREM sleep behaviour of a mouse.** The NREM sleep EEG is characterized by low frequency and high amplitude waveforms. The NREM sleep EMG is characterized by low muscle activity (hypotonia).

**REM sleep EEG and EMG:** The sleep architecture of REM sleep is similar to wakefulness in that it is also defined by an EEG of high frequency and low amplitude. REM sleep EEG however is accompanied by theta activity in the frequency range 6-9 Hz (Tobler et al. 1997). Although wakefulness and REM sleep exhibit similar EEG patterns, the EMG in REM sleep is immediately distinguishable from wakefulness in that there is virtually complete loss of muscle activity (atonia) that is occasionally accompanied by muscle twitches known as myoclonic jerks (Carskadon and Rechtschaffen 2000; Rechtschaffen and Kales 1968). The following diagram illustrates the REM sleep behaviour of a mouse.
The Neural Mechanisms Regulating Sleep and Wakefulness

Several brain areas regulate sleep and wakefulness through widespread projections in the brain. Neurophysiological approaches have been paramount in elucidating the neurocircuitry that regulates sleep and wakefulness by using various techniques such as stimulation studies of the brain, lesion studies, transection studies, and pharmacological manipulation studies. As such, the brain regions that appear to be involved in regulating sleep and awake states include the laterodorsal tegmental nuclei (LDT) and pedunculopontine tegmental nuclei (PPT), dorsal raphe nuclei (DR), tuberomammillary nucleus (TN), locus coeruleus (LC), ventral tegmental area (VTA), substantia nigra, ventrolateral preoptic area (VLPO), hypothalamus, basal forebrain, and the perifornical nucleus (Saper et al. 2005b). The interactions of the aforementioned brain areas with one another, regulate patterns of wakefulness, NREM and REM sleep.
Wake generating systems:

The area of the brain that exerts the greatest effects on promoting waking behaviour is the reticular formation that extends throughout the medulla, pons, and the midbrain (Jones 2005). Studies have demonstrated that the stimulation of this region promotes cortical activation and wakefulness (Jones 2005) while bilateral lesioning of the area causes loss of cortical activity accompanied by a state of coma (Steriade 1996). The reticular formation has ascending projections to the forebrain by two major routes: dorsally and ventrally. The dorsal route projects to the thalamus that in turn sends projections to the cerebral cortex. The ventral route projects to the basal forebrain and septum while crossing through the posterior hypothalamus and subthalamus. From the basal forebrain and septum, neurons then project to the cerebral cortex (Jones 2005).

Glutamate:

The principal excitatory neurotransmitter in the central nervous system is glutamate with the abundance of glutamatergic neurons residing from the reticular formation, thalamus, and hippocampus (Jones 2005) (refer to fig 1.4). Also, glutamate is produced by hypocretin neurons that project to many of the brainstem arousal systems (Saper et al. 2005a). Glutamatergic inputs are important for the majority of cortical activation and wakefulness in the brain.

Acetylcholine:

Cholinergic neurons in the reticular formation are one of the main forebrain arousal systems (Siegel 2004). The principal areas that contain acetylcholine in the reticular formation are the laterodorsal tegmental nucleus (LDT) and the pedunculopontine tegmental nucleus (PPT), that project to the thalamus (Hallanger and Wainer 1988). Cholinergic neurons that are also found in the basal forebrain, fire rapidly during wakefulness and project directly to the cerebral cortex and hippocampus to elicit cortical activation (Saper et al. 2005a) (refer to fig 1.4).
Serotonin - 5-HT:

Serotonin (5-HT) is involved in wakefulness and is principally found in the dorsal and median raphe nucleus within the brainstem and have various projections to the basal forebrain, thalamus, hypothalamus, and cortical regions (Dahlstrom and Fuxe 1964). Serotonergic cells fire most rapidly in periods of waking and reduce firing during NREM sleep and virtually cease firing in REM sleep (Saper et al. 2005a). Although implicated in generating wakefulness, serotoninergic neurons are usually associated with periods of less active tasks such as grooming or novel tasks that do not require active thinking (Jones, 2005).

Histamine:

Histamine is also involved in wakefulness and is exclusively produced by histaminergic neurons of the tuberomammillary nucleus (TN) (refer to fig 1.4), where it projects throughout the hypothalamus, basal forebrain, and the amygdala (Brown et al. 2001). Like serotonin, histaminergic neurons fire maximally during waking and are progressively reduced in sleep (Saper et al. 2005a). Studies have demonstrated using knockout mouse models of histidine decarboxylase (an enzyme that plays a role in histamine synthesis), that these mice exhibit chronic hypersomnia (Parmentier et al. 2002). Furthermore, during cataplexy, studies show that while other brain regions fall silent during periods of cataplexy, histaminergic activity is remarkably elevated and is hypothesized in maintaining the consciousness that is preserved in cataplexy (John et al. 2004). Lesioning studies of histaminergic neurons also result in decreased arousal (Jones, 2005).

Noradrenaline:

Noradrenaline is principally derived from noradrenergic neurons located in the LC (refer to fig 1.4) of the dorsal pontine tegmentum, and have been shown to have widespread connections with the cortex, thalamus, hypothalamus, and basal forebrain (Jones and Moore 1977). The noradrenergic system is typically more active in periods of mental alertness (Berridge
and Waterhouse 2003). Furthermore, noradrenergic neurons discharge most in waking, reduce discharge in NREM sleep, and become quiescent in REM sleep (Hobson et al. 1975).

**Dopamine:**

The dopaminergic influence on wakefulness is unclear because unlike the aforementioned neurotransmitters and that they fire maximally during wakefulness, dopamine neuron activity in the VTA and substantia nigra does not change across sleep-wake cycle. The major abundance of dopamine neurons in the CNS are principally located in the substantia nigra and VTA (Bjorklund and Dunnett 2007; Siegel 2004). Studies that demonstrate lesioning in these areas are accompanied by a decrease in behavioural arousal (Jones 2005), while studies in canines using dopamine uptake inhibitors promote wakefulness (Kanbayashi et al. 2000).

It has been suggested that the patterns of dopamine release from these two areas do not change across the sleep-wake cycle (Monti and Monti 2007), however, other studies have revealed that there is a predictable pattern of dopamine release from the ventral periaqueductal gray area (vPAG) during wakefulness, and when these dopamine neurons are killed, there is an increase in total sleep (Lu et al. 2006). As such, the mechanisms of dopamine release are not yet clear. Dopamine with be further discussed later in this thesis (p17).

**Hypocretin:**

Hypocretin is one of the more recent neuropeptides found to be involved in many physiological processes and especially important in modulating wakefulness. Hypocretin neurons are exclusively produced in the lateral and perifornical hypothalamus (refer to fig 1.4), and have widespread projections to the cerebral cortex, basal forebrain, and brainstem arousal systems as mentioned above (Saper et al. 2005a) in addition to spinal motor neurons (Siegel and Boehmer 2006). Hypocretin neurons are active during wakefulness. Microdialysis experiments in cats, demonstrate that hypocretin levels are high in wakefulness as well as REM sleep (Kiyashchenko et al. 2002). The loss of hypocretin results in the sleep disorder narcolepsy, which is
predominantly characterized by excessive sleepiness or inability to maintain wakefulness (Siegel and Boehmer 2006). These neurons appear essential for regulating wakefulness and arousal since they affect many cell groups that promote wakefulness (Siegel 2004).

**NREM sleep generating systems:**

Sleep is elicited when there is a reduction of cortical activity and arousal. Inhibitory influences on wake promoting brain areas cause the shift to NREM sleep. This is achieved by the principal inhibitory neurotransmitter, γ-aminobutyric acid (GABA), which is actively released during sleep (Jones 2005). The wake inhibitory neurons are principally located in the anterior hypothalamus, the ventral lateral preoptic area (VLPO) and the basal forebrain (Sterman and Clemente 1962). GABAergic neurons project from the VLPO as well as the basal forebrain to the cerebral cortex and promote NREM sleep (Jones 2005). Lesions to the VLPO that disrupt the GABAergic influences on sleep lead to insomnia (Jones 2005). Other studies that stimulate the basal forebrain produce states of drowsiness as well as EEG patterns that resemble NREM sleep (Sterman and Clemente 1962).

Sleep and wakefulness are remarkably intertwined in that there is a reciprocal inhibition of cell groups during each state. For example, VLPO neurons send projections to inhibit the major cell groups of the hypothalamus, basal forebrain, and brainstem structures that actively engage in arousal and wakefulness (Gallopin et al. 2000). This is hypothesized to generate sleep. During wakefulness, the same cell groups that are inhibited during sleep, inhibit the GABAergic sleep-promoting cells. This generates wakefulness. These cyclical patterns of wakefulness to sleep, and sleep to wakefulness, are termed the “flip-flop” switch and disruption of this switch can commonly lead to dysregulation of sleep and awake states (Saper et al. 2005b).
REM sleep generating systems:

Cholinergic influence is the principal mechanism that induces REM sleep (Szymusiak et al. 2000). REM sleep generation is coupled to the reduction in the activity of wake promoting neurons that inhibit the cholinergic neurons of the LDT/PPT (Pace-Schott and Hobson 2002). Noradrenaline from the locus coeruleus is responsible for the majority of inhibition of LDT/PPT cholinergic neurons during wakefulness (Jones 2005). Studies in cats demonstrate that carbachol (a cholinergic receptor agonist) induced REM sleep and caused increased activation of REM-like states (Torterolo et al. 2001). Furthermore, studies in mice using neostigmine (anticholinesterase inhibitor) (Coleman et al. 2004), as well as studies in humans using physostigmine (another anticholinesterase inhibitor) (Sitaram et al. 1978) promote REM sleep. These studies demonstrate that cholinergic inputs are important for the generation of REM sleep and that the disinhibition from noradrenergic systems (since NA activity is silent during REM sleep) (Hobson et al. 1975) is important for generating REM sleep. The regulation of REM sleep is also influenced by hypocretin in the context that narcoleptics (lacking hypocretin), exhibit more REM sleep than normal individuals (Mochizuki et al. 2004).
Figure 1.4: A figure illustrating the important neurotransmitters involved in sleep and wakefulness in addition to the neural pathways that regulate these states: wakefulness, NREM, and REM sleep. Electroencephalogram activity (EEG) is depicted in the top left hand corner of this figure while electromyogram (EMG) (muscle activity) is depicted in the bottom right hand corner of this figure. Waking behaviour (W) is characterized by high frequency, low amplitude EEG waves accompanied by high EMG (muscle) activity. NREM sleep behaviour or slow wave sleep behaviour (SWS) is characterized by low frequency, high amplitude EEG waves accompanied by a reduced level of EMG (muscle) activity (hypotonia). REM sleep behaviour or paradoxical sleep (PS) is characterized by high frequency, low amplitude EEG waves (similar to waking behaviour) however, REM sleep is also accompanied by EMG activity that is nearly quiescent (atonia). The neural systems involved in eliciting arousal and wakefulness include: cholinergic projections (ACh) from the laterodorsal and pedunculopontine tegmental nucleus (LDT/PPT); noradrenergic projections (NA) from the locus coeruleus (LC); Histaminergic projections (H) from the tuberomammillary nucleus (TM); orexinergic projections (Orx or hypocretin) from the posterior hypothalamus (PH); and glutamatergic projections from the reticular formation, thalamus, and hippocampus. The neural systems involved in eliciting slow EEG sleep activity include: GABAergic projections from the basal forebrain, preoptic area (POA), and anterior hypothalamus. Arousal generating systems project to sleep generating systems and vice versa, to reciprocally inhibit the neuronal systems that govern wakefulness and sleep. This figure was taken from (Jones 2005).
IV. Muscle Control During Sleep and Wakefulness

Muscle activity has a decreasing pattern across the sleep-wake cycle. Muscle activity is typically highest during periods of wakefulness, is reduced in NREM sleep, and is further reduced in REM sleep (Chase and Morales 2005). In order to understand the decreasing patterns of muscle activity during sleep, one must understand the neural pathways as well as neurochemicals that impart control on muscles during sleep; however, the mechanisms of motor control are still unclear.

In the somatic motor system, the basic unit that connects the central nervous system to skeletal muscle fibers is the α motor neuron, a nerve cell with an axon that terminates on muscle fibers (Chase and Morales 2000; Rye 2002). Movement of muscles results from the excitation of motor neurons (ie. glutamate) that produce muscle contractions, while the inhibition (ie. GABA and glycine) of these same motor neurons, result in muscle atonia (Chase and Morales 2005).

There are two mechanisms suggested in the control of muscle activity through motor neurons. One mechanism is postsynaptic inhibition while the other mechanism involves postsynaptic disfacilitation (Chase and Morales 2005). Postsynaptic inhibition involves the release of inhibitory neurotransmitters (ie. GABA and glycine from inhibitory brain regions) onto motor neurons (Houghton et al. 2004). Postsynaptic disfacilitation involves a decrease in the release of excitatory neurotransmitters (ie. glutamate, noradrenaline, serotonin, and dopamine from excitatory brain regions) onto motor neurons thereby reducing the level of excitation on motor neurons and hence bringing about a reduction in motor activity (Garraway and Hochman 2001; Siegel and Boehmer 2006; Wu et al. 1999).

Studies have reinforced the mechanism of postsynaptic inhibition where the agonism of glycine or GABA inhibitory receptors at the level of spinal motor neurons reduce muscle activity (Morrison et al. 2003; Morrison et al. 2002). Furthermore, similar studies reinforce the
mechanism of postsynaptic disfacilitation in that the antagonism of excitatory neurotransmitters (ie. noradrenaline) at motor neurons reduces muscle activity (Chan et al. 2006).

Cataplexy is the symptom of narcolepsy where there is improper regulation of muscle activity when an individual is awake. Muscle activity is lost during wakefulness uncontrollably, suggesting that the atonia found in REM sleep, is improperly gated and intrudes during wakefulness. It has been suggested that the regulation of muscle control during cataplexy involves the loss of excitation onto motor neurons by noradrenergic cells in the LC (Siegel and Boehmer 2006). However, a role for dopamine in regulating cataplexy has been suggested by studies using D2 receptor agonists and antagonists, but the dopaminergic mechanism still remain unclear. Hence, this thesis focuses more closely on dopamine, and how it regulates the muscle activity during cataplexy. Consider the following figure (Fig 1.5) regarding a proposed mechanism of the neural pathways of muscle control during sleep, wakefulness, and cataplexy. The roles for hypocretin and dopamine in regulating sleep, wakefulness, and cataplexy are still in their infancy and their roles for regulating these behavioural states are still under investigation.

**Figure 1.5:** This figure illustrates brain regions that modulate muscle control during sleep and wakefulness. During wakefulness, primary monoaminergic brain region (that contain serotonin and noradrenaline) exert inhibition on the LDT and PPT that release ACh. Locus coeruleus project directly to spinal motor neurons and maintain postural muscle tone by excitation. During REM sleep, monoaminergic activity is silent which in turn removes the inhibition from LDT/PPT brain regions. This allows the release of acetylcholine to the medial medulla causing a direct inhibition of spinal motor neurons (via GABA and glycine) and the loss of muscle tone (postsynaptic inhibition). The medial medulla also has ascending projections to inhibit brain regions that excite motor neurons such as the LC (postsynaptic disfacilitation). In cataplexy, the amygdala has a role during strong emotions, that result in the loss of muscle activity. Figure adopted from (Houghton et al. 2004).
The Neurotransmitters involved in Muscle Control

It is still unclear which neurotransmitters are involved in the control of motoneurons at the level of the CNS. Certain neurotransmitters inhibit motor neurons while others excite them. Certain neurotransmitters are neuromodulatory. It is important to discuss the neurotransmitters that are suggested to be involved in muscle control, because in cataplexy, neurotransmitters may be improperly regulated predisposing the atonia during wakefulness. The examination of neurotransmitters that enhance muscle activity may unravel the “broken” pathway of neurotransmitter control in cataplexy.

Inhibitory neurotransmitters:

The primary neurotransmitter that is suggested to inhibit muscle activity is glycine since studies have demonstrated that applications of strychnine (a glycineric antagonist) on to spinal motor neurons inhibit muscle atonia during active sleep (Chase and Morales 2005). Microdialysis experiments also show that glycine administered to motor neurons inhibits muscle activity and that this is reversed when strychnine is given (Morrison et al. 2002). Muscle activity is also controlled by GABA because studies during sleep show that antagonizing GABA receptors on motor neurons facilitate muscle activity (Morrison et al. 2003).

Excitatory neurotransmitters:

The principal neurotransmitters that neuromodulate motor neurons are serotonin and noradrenaline (Chau et al. 1998; Harvey et al. 2006; Rank et al. 2007) while dopamine has been suggested but not as well understood (Clemens and Hochman 2004; Garraway and Hochman 2001; Maitra et al. 1992) . These neurotransmitters are neuromodulatory because rather than eliciting excitatory postsynaptic potentials (EPSPs) or inhibitory postsynaptic potentials (IPSPs) (e.g. glutamate, GABA, and glycine), they generally enhance motor neuron responses to excitatory or inhibitory influences (Rye 2002). In this thesis, I will focus on dopamine since this neurotransmitter remains unclear in regulating muscle activity, however studies in narcoleptic
models show that by enhancing dopamine transmission, cataplexy is reduced (Kanbayashi et al. 2000; Shelton et al. 1995) while dopaminergic antagonism or agonism of D2 receptors decrease and increase cataplexy, respectively (Nishino et al. 1991; Reid et al. 1996).

**The Dopaminergic System:**

Dopamine in the brain, is localized to specific regions from A8 to A16 that are dispersed throughout the midbrain, diencephalon, and olfactory bulb (Bjorklund and Dunnett 2007; Dahlstroem and Fuxe 1964). From these nine regions that supply dopamine, the majority of dopaminergic neurons are found in the midbrain marked by the regions A8-A10. These regions encompass the substantia nigra as well as the VTA and supply most dopaminergic innervation within the CNS (Bjorklund and Dunnett 2007). The substantia nigra as well the VTA, project to the striatum, basal forebrain, cortex, and brainstem areas that participate in sleep (Jones 2005). The substantia nigra and VTA also receive reciprocal innervations from these same brainstem areas that include the raphe nucleus, locus coeruleus, LDT and PPT, tuberomammillary nucleus, lateral hypothalamus, preoptic area, and the basal forebrain (Monti and Monti 2007).

Another population of dopaminergic neurons that are suggested to be important in regulating sleep and wakefulness, are found in the vPAG (periaqueductal gray matter), an area of the A11 dopamine group (Lu et al. 2006). This group of neurons are active during wakefulness, and reduce firing during sleep however, their activity during cataplexy is unknown. The dopamine neurons in this region reciprocally interact with the major sleep areas and regulate patterns of sleep and wakefulness. These vPAG dopamine neurons, project to areas like the basal forebrain, locus coeruleus, dorsal raphe nucleus, lateral hypothalamus, LDT, and VLPO. It also receives afferent projections from the same regions it projects to (Lu et al. 2006). These neurons have been suggested to be the primary dopamine system that mediates arousal and sleep.
Importantly, the A11 dopamine neurons are unique in that they are the only known group of neurons to project to the spinal cord where motor neurons exist (Bjorklund and Dunnett 2007; Ridet et al. 1992; Rye 2002). Therefore, the dopaminergic modulation of muscle activity may be elicited through the A11 dopamine group directly. This thesis aims to determine how pharmacological manipulation of dopamine function affects motor control in narcoleptic mice.

Figure 1.6: This figure represents the distribution of dopamine cell groups in the rodent brain and their projections throughout the brain. The midbrain dopamine neurons A8-A10 (substantia nigra and VTA) constitutes the majority of dopamine in the CNS. They primarily project to the basal forebrain, striatum, and cortex and receive reciprocal innervations. The A11 dopamine cell group is the only group that projects to the spinal cord where motor neurons exist. Figure from Bjorklund and Dunnett 2007.
**Dopaminergic Receptors:**

Dopamine within the CNS as well as the spinal cord, are mediated by five types of receptors. These receptors are classified as: D1, D2, D3, D4, and D5 dopamine receptors and all belong to the superfamily of G-protein coupled receptors. These five receptors are further broken down into 2 distinct subtypes of receptors: D1-like and D2-like receptors.

D1 and D5 receptors belong to the D1-like family of dopamine receptors and are excitatory in nature. D2, D3, and D4 receptors belong to the D2-like family of dopamine receptors, which are inhibitory in nature (Girault and Greengard 2004). The distribution of these receptors within the brain vary remarkably. Firstly, out of the five types of receptors, the D1 and D2 receptors are the most abundant in the CNS, whereas D4 receptors are so scarce in the rat brain, that they are difficult to detect (Mansour and Watson 2000).

**D1-like family receptor distribution:**

D1 receptors are generally found in the neocortex, caudate-putamen, nucleus acumbens, amygdala, and the suprachiasmatic nucleus (Mansour and Watson 2000; Girault and Greengard 2004). D1 receptors are also found to a lesser degree in the cerebral cortex, thalamus, limbic system, hypothalamus, dorsal raphe nucleus and LC (Monti and Monti 2007).

D5 receptor distribution are located specifically in the hippocampus, hypothalamus, and thalamus (Mansour and Watson 2000). Both D1 and D5 receptors are postsynaptic receptors.

**D2-like family receptor distribution:**

D2 receptors are found in the caudate-putamen, nucleus acumbens, substantia nigra, VTA, and locus coeruleus (Mansour and Watson 2000; Girault and Greengard 2004). D2 dopamine receptors are important because they autoregulate dopamine release (Mansour and Watson 2000). D2 receptors are autoreceptors, that are located in the substantia nigra and VTA (Mansour and Watson 2000; Monti and Monti 2007).
D3 and D4 dopamine receptors are found at much lower levels compared to D2 receptors. D3 receptor distributions are found at moderately low levels in the cerebral cortex, nucleus accumbens, amygdala, hippocampus, and hypothalamus. D4 receptors distributions are found at extremely low levels in the frontal cortex, amygdala, hippocampus, and hypothalamus (Monti and Monti 2007).

At the level of the spinal cord, the dopamine receptors that exist are D1, D2, and D3 receptors (Zhao et al. 2007). However, it is suggested that D2 receptors have a more profound role in mediating motor activity since studies show that D2 agonists at the level of the spinal cord inhibit the monosynaptic stretch reflex in wild-type and D3 receptor knockout mice (Clemens and Hochman 2004) while D1 receptors are more involved in nociception (Zhao et al. 2007). Dopamine receptors in the spinal area typically receive most of their dopaminergic inputs from the A11 dopamine cell groups of the brain (Bjorklund and Dunnett 2007; Okura et al. 2004; Zhao et al. 2007) and this cell group interaction with D2 receptors, may mediate cataplexy at the level of the spinal cord.

V. The Hypocretin/Orexin System

Studies on encephalitis lethargica by von Economo in the early 20th century suggested a role for the hypothalamus in regulating both waking and sleep states. Lesions of the posterior hypothalamus and midbrain resulted in sleepiness while anterior hypothalamic inflammation resulted in insomnia (Zeitzer et al. 2006). Hence, the discovery of the neurotransmitter “hypocretin” located in the hypothalamus, supported von Economo’s theory that the hypothalamus is important for regulating sleep states since hypocretin innervates the brain areas involved in sleep and wakefulness (Zeitzer et al. 2006).

Hypocretin was simultaneously discovered in 1998 by two independent research groups. De Lecea et al. (1998) employed polymerase chain reaction subtraction techniques to extract...
mRNA in the hypothalamus when they discovered this novel pair of peptides and called them the *hypocretins* (hypocretin-1 and hypocretin-2) (de Lecea et al. 1998). The name hypocretin, was given to these peptides due to their distribution to the hypothalamic region as well as their similarity to the secretin family of peptides.

Sakurai et al. (1998) discovered this peptide by using reverse pharmacology techniques to search for endogenous ligands for G-protein-coupled receptors and discovered this peptide they called - *orexin* (orexin A and orexin B) (Sakurai et al. 1998). The naming of the orexin peptides (derived from the greek word *orexis* meaning appetite) were based on the appetite-stimulating effects when this peptide was administered to rodents in addition to the hypothalamus being implicated in feeding (Baumann and Bassetti 2005b; Willie et al. 2001). This thesis will refer to the term hypocretin because de Lecea et al. discovered and reported this peptide first.

**Hypocretin Neuropeptides:**

There are two neuropeptides that make up the hypocretin system: hypocretin-1 and 2 (Hcrt-1 and 2). These peptides are exclusively produced by a small group of neurons in the lateral and perifornical hypothalamus (Willie et al. 2001). These peptides are a product of a human prepro-hypocretin gene that is cleaved to produce mature hypocretin-1 (33 amino acid peptide) as well as hypocretin-2 (28 amino acid peptide) (Baumann and Bassetti 2005b). Hypocretin neurons have widespread projections throughout the CNS, including the cerebral cortex, olfactory bulb, hippocampus, amygdala, septum, thalamus, anterior and posterior hypothalamus, midbrain, brainstem, and spinal cord (Peyron et al. 1998; Willie et al. 2001).

**Hypocretin Receptors:**

The hypocretin peptides impart their effects through two G-protein-coupled receptors: hypocretin receptors 1 and 2 (HcrtR1 and HcrtR2). The receptors have differential affinities for each peptide. HcrtR1 has an specific affinity for only hypocretin-1, while HcrtR2 exerts equal affinities for both hypocretin-1 and 2 (Baumann and Bassetti 2005a; Willie et al. 2001). In
addition to the different affinities the receptors have for each peptide, receptor distribution throughout the CNS is quite diverse. Hypocretin-1 receptor mRNA, was found to be highly expressed in the prefrontal cortex, hippocampus, paraventricular thalamus, ventromedial hypothalamus, arcuate nucleus, dorsal raphe nucleus, and locus coeruleus (Willie et al. 2001). Hypocretin-2 receptor mRNA was found to be expressed in the cerebral cortex, septal nuclei, hippocampus, medial thalamic groups, dorsal and median raphe nuclei, tuberomammillary nucleus, dorsomedial hypothalamus, paraventricular hypothalamic nucleus, and ventral premammillary nucleus (Willie et al. 2001). The combination of both receptors, encompass a diverse distribution of hypocretin neurons throughout the CNS as illustrated by figure 1.7.

Figure 1.7: A sagittal section of a rat brain demonstrating the diverse projections of hypocretin neurons throughout the CNS illustrating the organization of the hypocretin system. Notice how hypocretin neurons are exclusively produced in the lateral hypothalamus. Hypocretin neurons have ascending projections to regions such as the cerebral cortex, hippocampus, amygdala (Amyg), septum, thalamus, anterior and posterior hypothalamus, midbrain and brainstem regions (ie. locus coeruleus (LC), pedunculopontine tegmental nucleus (PPN or PPT)), and the spinal cord. Figure from Willie et al. 2001.
The fact that hypocretin neurons widely project throughout the CNS to their respective receptors indicate hypocretin’s involvement in many other physiological functions apart from regulating sleep and wakefulness. These include neuroendocrine homeostasis, autonomic regulation and feeding (Willie et al. 2001).

**Dynamics of Hypocretin in Sleep, Wakefulness, and Motor Control:**

As it relates to sleep, wakefulness, and motor control, hypocretin release is typically highest during waking and REM sleep and lowest during NREM sleep (Kiyashchenko et al. 2002). The firing activity of hypocretin neurons across sleep is highest during active wakefulness when an individual is engaged in motor activities, reduced during quiet wakefulness when an individual is motorically quiet, and virtually silent during sleep (Lee et al. 2005). Hypocretin levels in canine CSF (cerebrospinal fluid) were greater during periods of sleep deprivation, and exercise (when the animal was motorically engaged) (Wu et al. 2002). In studies where hypocretin was administered to the cat midbrain, it enhanced locomotion and also facilitated postural muscle tone (Takakusaki et al. 2005).

Since hypocretin projects to dopamine neurons, it is possible that the loss of hypocretin interactions with dopaminergic areas predispose the sleep disorder *narcolepsy*. This thesis examines the role for dopamine on sleep and motor control by using a hypocretin knockout mouse model devoid of hypocretin.

**VI. The Neurobiology of Narcolepsy**

Narcolepsy is a chronic, disabling sleep disorder that affects approximately 0.03-0.16% of the population (Nishino et al. 2000). It is characterized by a series of symptoms. The more common symptoms are excessive daytime sleepiness (EDS), cataplexy (involuntary loss of skeletal muscle tone), disrupted nocturnal sleep, and REM intrusions into wakefulness
(SOREMP- sleep onset REM periods or sleep attacks). Other symptoms that are more variable include sleep paralysis (inability to move upon awakening) and hypnagogic hallucinations (dream events occurring at sleep onset) in individuals with narcolepsy (Siegel and Boehmer 2006; Siegel et al. 2001; Zeitzer et al. 2006). Narcolepsy and the symptoms associated with it, are linked to disruption of the hypocretin system.

**Reduced levels of hypocretin in narcolepsy:**

Individuals without narcolepsy, are normally endowed with approximately 70,000 hypocretin neurons in the lateral hypothalamus (Siegel and Boehmer 2006; Siegel et al. 2001). Studies have confirmed that in roughly 90% of patients that are diagnosed with narcolepsy, that these neurons are reduced to 10% of the normal levels (Mignot et al. 2002; Siegel and Boehmer 2006; Thannickal et al. 2000). A useful tool for clinicians to distinguish individuals with narcolepsy, have been a measure of hypocretin-1 (more physiologically stable than hypocretin-2) peptide levels in human cerebrospinal fluid (CSF) (Zeitzer et al. 2006). CSF levels are reduced by approximately 85-95% in narcoleptic patients (Gerashchenko et al. 2003; Kiyashchenko et al. 2002; Mignot et al. 2002).

**Figure 1.8:** An example of the distribution of hypocretin cells and axons in the perifornical and dorsomedial areas of the hypothalamus of normal and narcoleptic humans. Notice that in panels A,C,E, and G, represent the normal distribution of hypocretin cells in individuals without narcolepsy (photos taken at different magnifications). In panels B, D, F, and H, the number of hypocretin cells are vastly reduced approximately 85-90% of normal levels. There is a significant reduction in the level of hypocretin axonal staining in the narcoleptic individuals compared to normal individuals. Figure adopted from (Thannickal et al. 2000).


**Cause of narcolepsy:**

The symptoms of narcolepsy usually manifest in individuals within the second or third decade of life (Siegel and Boehmer 2006; Zeitzer et al. 2006). Narcolepsy is sporadic in nature, and there is a discordance of the disorder among identical twins (Siegel and Boehmer 2006; Thannickal et al. 2000; Zeitzer et al. 2006). This led researchers to believe that rather than narcolepsy being genetically predisposed within families, that it is linked to an autoimmune disease linked to the human leukocyte antigen (HLA-DQB1*0602). About 90-95% of all narcoleptic patients are positive for this antigen while only about 20-30% of normal patients exhibit this antigen (Siegel and Boehmer 2006; Zeitzer et al. 2006). The current view is that being positive for HLA DQB1*0602, causes an individual to be susceptible to developing narcolepsy. When this antigen is produced in response to other various exogenous infections such as pathogens or viruses, the DQB1*0602 antigen may recognize an epitope on hypocretin cells and orchestrate the destruction of these cells that causes narcolepsy (Siegel and Boehmer 2006; Zeitzer et al. 2006).

**Models of narcolepsy:**

Narcolepsy is a disabling disorder that has received much interest over the years. It has been useful for researchers to develop animals models of narcolepsy to study and gain valuable information to better understand the disease and to improve the quality of treatments offered.

**Human narcolepsy:**

The human form of narcolepsy as previously mentioned, is manifested by the loss of hypocretin neurons in the second or third decade of life. Hence, the system is disrupted because these neurons that are abolished, cannot produce the necessary hypocretin peptides to elicit their effects on the receptors (that are still intact). Therefore, the symptoms of narcolepsy are manifested due to the loss of the neurons.
Canine narcolepsy:

The canine model of narcolepsy was the first animal models used to study narcolepsy. Canine narcolepsy unlike the human form, is caused by mutations in the hypocretin receptor, specifically the HcrtR2 (Mignot et al. 1993a; Nishino et al. 2000). Therefore, it is the receptor component of the system that is disrupted. Even though the narcoleptic dog can produce both hypocretin ligands (hypocretin 1 and 2), they are unable to bind to the mutated receptor HcrtR2 (that has an affinity for both peptides) to elicit their effects. Therefore, the dog model although a useful tool for assessing narcolepsy and cataplexy, involves a physiological mechanism that is quite different from that of the human.

Rodent narcolepsy:

Recently, mouse/rat models of narcolepsy have been instrumental to understanding this disorder. There exist neuron ablated models of hypocretin, in narcoleptic rats and mice (Beuckmann et al. 2004; Hara et al. 2001). These animals are engineered so that hypocretin neurons die off with the expression of a toxic gene. This toxic gene (ataxin-3), is activated early in the life stage of the animal and kills the normal developing hypocretin neurons similar to how the hypocretin neurons die off in humans. However, the narcolepsy phenotype is accompanied by other undesirable phenotypes such as severe obesity despite the fact that the animals are hypophagic (Hara et al. 2001).

A more appropriate mouse model of narcolepsy (which is studied in this thesis) are hypocretin knockouts that are devoid of the hypocretin peptides. In these mice, the prepro-hypocretin gene that is responsible for translating the hypocretin 1 and 2 peptides, is removed (Chemelli et al. 1999). Therefore, the hypocretin ligand is not produced and the system is completely devoid of hypocretin; this is similar to human narcolepsy. Knockout mice can be argued as a better model compared to human narcoleptics for studying narcolepsy. This is because areas of the brain can be examined and manipulated to dissect their importance without
the confounding influences of hypocretin. In humans, a large proportion of hypocretin neurons
are abolished but the remaining neurons still produce hypocretin.

This thesis describes the effects of pharmacological manipulation of dopamine
neurotransmission in hypocretin knockout mice to understand the neurocircuity of cataplexy.
Since these mice are completely devoid of hypocretin, manipulating dopamine may elucidate the
mechanism that dopamine has in modulating sleep and cataplexy without the influences of
hypocretin.

**Excessive Daytime Sleepiness (EDS)**

Persistent sleepiness is a hallmark of narcolepsy. It is the initial complaint in undiagnosed
patients (Siegel and Boehmer 2006). Narcoleptic patients have difficulty maintaining
wakefulness during the day as well as exhibiting abnormal fragmented nocturnal sleep (Chemelli
et al. 1999; Mochizuki et al. 2004; Willie et al. 2001). Sleep in narcoleptic mice is similar to
sleep in narcoleptic humans (Willie et al. 2001). Although narcoleptics report excessive
sleepiness and have fragmented nocturnal sleep, studies demonstrate that narcoleptic individuals
do not exhibit any more or less sleep. Studies in narcoleptic mice also have fragmented sleep
despite having normal amounts of wakefulness and NREM sleep. However, REM sleep is
elevated in narcoleptics (Mochizuki et al. 2004).

Another interesting feature of narcoleptic sleep, is that they have frequent transitions
from one sleep state to another. However, a unique feature of narcoleptics is that they transition
directly from wakefulness into REM sleep (SOREMP –sleep onset REM periods or “sleep
attacks”). This is indeed a feature unique to narcoleptics and is exhibited frequently as
demonstrated by MSLT (Multiple Sleep Latency Tests).

These phenomena are demonstrated in studies using narcoleptic hypocretin knockout
mice (Chemelli et al. 1999; Willie et al. 2001). Figure 1.9 demonstrates sleep in narcoleptic mice
compared to wild-type mice, while also demonstrating more fragmented sleep exhibited in the
narcoleptic mice (hypnogram). In addition, comparing the hypnograms between narcoleptic mice and wild-type mice, demonstrates that in fact, narcoleptic mice exhibit direct transitions from wakefulness into REM sleep (SOREMP), whereas the wild-type mice do not.

1. Narcoleptic mice (KO) that exhibit nearly comparable overall levels of wakefulness and NREM sleep to wild-type mice (WT) while REM sleep is slightly greater. Sleep conducted over a 12:12 hr light: dark cycle. Figure adopted from (Mochizuki et al. 2004).

2. Comparison of sleep hypnograms between narcoleptic mice (orexin/hypocretin knockout) and wild-type mice. Lights off at 7p.m. The height of the horizontal line represents the vigilant state of the animal: R = REM sleep, S = NREM sleep, W = wakefulness. Notice how the narcoleptic mice exhibit highly fragmented sleep with more transitional states compared to the wild-type mice. Furthermore, the arrows in the narcoleptic mice hypnogram demarcate transitions from wakefulness directly to REM sleep or SOREMP (sleep onset REM periods), which is unique to narcoleptics and not to wild-type individuals. Figure adopted from (Chemelli et al. 1999).

Treatments for sleepiness:

Although narcoleptics have fragmented sleep as well as persistent sleepiness, there are many pharmacological approaches in treating these symptoms. The most common treatments for sleepiness are drugs that stimulate dopaminergic neurotransmission. Amphetamine-like stimulants have been instrumental in promoting wakefulness by counteracting the effects of
sleepiness. They do this by increasing endogenous dopamine levels and to a lesser extent by increasing noradrenaline (Billiard et al. 2006; Mignot and Nishino 2005; Zeitzer et al. 2006). Modafinil is a new drug that also increases dopamine levels and promotes waking (inhibiting reuptake of dopamine) however, unlike amphetamine it is not addictive (Billiard et al. 2006; Mignot and Nishino 2005; Willie et al. 2005; Zeitzer et al. 2006). It is also possible that synthetic hypocretin analogs may be used to treat sleepiness because studies in narcoleptic mice and dogs demonstrate that hypocretin peptides when centrally or peripherally administered, promote wakefulness (Mieda et al. 2004).

**Cataplexy**

Cataplexy is the other major symptom of narcolepsy. It can be severe (ie. complete loss of muscle tone) or moderate (ie. affecting voice, facial or limb muscles) (Houghton et al. 2004). Remarkably, during episodes of cataplectic attacks, respiratory as well as extraocular muscles are functional (Houghton et al. 2004).

Cataplexy is generally elicited by strong emotions. While cataplexy is often stimulated in the narcoleptic dog by vigorous play and by the presentation of food, it is stimulated in humans by laughter but also anger and fear (Houghton et al. 2004; Siegel and Boehmer 2006). This explains why it is often reported that when a person laughs uncontrollably, they experience a sensation of muscle weakness; however, it is hypothesized that hypocretin counteracts this emotionally-evoked inhibition and therefore, normal individuals are able to maintain posture while narcoleptics are not (Siegel and Boehmer 2006). Studies in the narcoleptic dog have demonstrated with single-unit recordings that neurons of the amygdala (part of limbic system regulating emotions) fire maximally just before and during periods of cataplexy (Gulyani et al. 2002) confirming that the emotionally stimulated cataplectic attacks are correlated with the limbic system.
Suggested mechanisms for muscle loss during cataplexy:

Cataplexy is best understood by examining the neural pathways that regulate muscle activity. Cataplexy may be mediated by the same mechanisms that cause muscle atonia in REM sleep. During REM sleep, there is a significant influence from cholinergic neurons from the LDT and PPT in the mesopontine area. Cholinergic neurons from the LDT and PPT project to the pons and medial medulla and activate medial medulla atonia neurons (GABA and glycine). These inhibitory neurons in turn project to spinal and brainstem motor neurons and release GABA and glycine that inhibit muscle tone (Houghton et al. 2004; Rye 2002).

During wakefulness, monoaminergic (noradrenergic) cell groups inhibit the medial medulla to sustain muscle tone (Houghton et al. 2004). Canine studies have demonstrated using unit recording that subsets of cells in the medial medulla fire only during cataplexy and during REM sleep (Siegel et al. 1991). Furthermore, studies using microinjections of hypocretin into medial medulla areas facilitate muscle activity (Mileykovskiy et al. 2002) suggesting that reduced muscle tone in cataplexy is likely a result of compromised “hypocretin-medial medullary” interactions.
The atonia in cataplexy is also caused by a loss of excitation on motor neurons from the LC (noradrenaline) (Houghton et al. 2004; Siegel and Boehmer 2006). Locus coeruleus neurons project directly to spinal motor neurons to facilitate postural muscle tone during wakefulness (Houghton et al. 2004; Siegel and Boehmer 2006). During transitions into sleep, LC neurons fall silent and remove the excitatory effects on spinal neurons contributing to atonia. Moreover, periods of cataplexy are a result of medial medulla neurons exerting inhibitory effects onto noradrenergic neurons of the LC (Houghton et al. 2004). Studies confirm that locus coeruleus activity in the narcoleptic dogs are virtually silent during periods of cataplexy (Wu et al. 1999). Hence, there is a reciprocal inhibition of muscle control during wakefulness (when LC neurons inhibit medial medulla cell groups) as well as during sleep and cataplexy (when medial medulla neurons inhibit LC cell groups) (Houghton et al. 2004; Siegel and Boehmer 2006).

Although dopamine modulate cataplexy, the mechanism is not understood. Across the sleep-wake cycle, it has been shown that dopaminergic neurons from the substantia nigra and VTA do not change their firing activity (Monti and Monti 2007). However, recent studies demonstrate that dopaminergic neurons in the vPAG fire maximally during waking and are reduced during sleep (Lu et al. 2006) (although there is no evidence that suggests the firing activity of these neurons during cataplexy).

Studies in narcoleptic canines show that manipulating dopamine, modulates cataplexy. Amphetamines that enhance endogenous dopamine have been shown to reduce cataplexy (Kanbayashi et al. 2000; Shelton et al. 1995). However, reductions in cataplexy are probably not related to inhibiting dopamine reuptake, since studies that used dopamine reuptake blockers have little effect on reducing cataplexy (Mignot et al. 1993b; Okura et al. 2004).

More recent studies have shown that the modulation of cataplexy via dopamine, is largely regulated through dopaminergic D2 receptors since dopamine D2 agonists exacerbate cataplexy while D2 antagonists reduce cataplexy (Nishino et al. 1991; Okura et al. 2004). Moreover,
studies administering dopamine D1 receptor agonists and antagonists to the narcoleptic canine, did not affect cataplexy suggesting a specific role for D2 receptors for inhibiting the muscle activity during cataplexy (Okura et al. 2004).

Manipulating dopamine in the narcoleptic dogs; through the enhancement of endogenous dopamine or through agonizing or antagonizing dopamine receptors, effectively modulates cataplexy however, this has never been demonstrated in the narcoleptic hypocretin knockout mice with an intact receptor system and lacking the hypocretin ligand.

Treatments for cataplexy:

Treatments for cataplexy are usually achieved by using tricyclic antidepressants. Antidepressants generally act by blocking the reuptake of monoamines such as noradrenaline, serotonin, and dopamine (Houghton et al. 2004; Mignot and Nishino 2005). This causes the increase of endogenous levels of these monoamines resulting in the reduction of cataplexy. Some common examples of anticataplectic compounds are Protryptiline, Imipramine, Clomipramine, and Venlafaxine that block monoamine reuptake (Mignot and Nishino 2005).

One of the more recent compounds used to treat cataplexy is γ- Hydroxybutyrate, more commonly known as GHB (Houghton et al. 2004; Mignot and Nishino 2005; Siegel and Boehmer 2006). The mechanism of how GHB treats cataplexy is poorly understood, but it is suggested that it may act to increase signaling through GABA-B receptors that inhibit brainstem atonia neurons (Houghton et al. 2004; Siegel and Boehmer 2006). Another suggestion is based on the fact that there are high concentrations of GABA receptors in the substantia nigra and VTA (dopaminergic areas). GHB may modulate dopamine neurons and reduce cataplexy through a dopamine pathway (Houghton et al. 2004).

Future treatments for cataplexy may involve the production of hypocretin synthetics, or hypocretin replacement therapy. Studies using ectopic expression of hypocretin in knockout mice that prevent cataplexy (Mieda et al. 2004; Siegel and Boehmer 2006).
With nearly a decade since the discovery of the hypocretin peptide, research can now focus on the interactions between hypocretin and monoaminergic neurons (e.g. dopamine), and how they regulate sleep and motor activity to better understand the mechanisms that regulate narcolepsy.

**Summary**: The goal of these experiments was to determine whether pharmacological manipulations of dopamine transmission in narcoleptic mice, affect either sleep or cataplexy.
Chapter Two:

MATERIALS AND METHODS
2. MATERIALS AND METHODS

I – Animal Colony and Genotyping Procedures

A. Animal Colony

Initially, the animal colony (murine mouse model) that was used in this experiment was derived from two breeding pairs of transgenic prepro-hypocretin knockout mice (heterozygous) (F2 C57Bl/6J-129SvEv mixed background littermates) that were generously provided by the Howard Hughes Medical Institute, Laboratory of Sleep Research (Investigator: Masashi Yanagisawa, MD/PhD).

Male and female heterozygous mice were then backcrossed with wild-type C57Bl/6 female and male mice ages 2-3 months (Charles River Laboratories), and housed in the Department of Cell and Systems Biology Animal Care Facility (University of Toronto). We wanted to outcross the animals with animals that were less closely related.

All F1 progeny were either of heterozygous or wild-type origins based on classical Mendelian genetics. The F1 generation progeny were then crossed with one another: heterozygous x heterozygous; heterozygous x wild-type; wild-type x wild-type, in order to produce a colony that would encompass animals that are 1 of 3 genotypes: (+/+) wild-type, (+/-) heterozygous, (-/-) knockouts.

B. Genotyping

Animals were bred under the guidelines specified by the Animal Care Facility (University of Toronto). Following 3 weeks of gestation, females gave birth to pups that remained with the female until 3-4 weeks when they were weaned and separated into littermates of the same sex. Genotyping was conducted over a 3 day period with each day achieving important steps towards identifying the animal.
DAY 1 (DNA SAMPLING) - Animals were genotyped via a DNA sample taken from a tail clipping. Each mouse was anesthetized under 5% isoflurane gas (1-chloro-2,2,2-trifluoroethyl ether) vaporized with medical grade oxygen (flow rate = 1 L/min), in a SurgiVet induction chamber (Surgivet, Waukesha, WI). Once the animal was unconscious, it was removed from the chamber and placed into a face mask that maintained the animal at 3% isoflurane until the tail was clipped. Using the hind limb withdrawal reflex to determine whether the animal was still unconscious, approximately 5 mm from the tip of the tail was clipped using medical grade scissors while forceps were used to retrieve the tip of the tail and transfer it to a 1.5ml test tube (311-08-051, Axygen, Union City, CA). After the clipping, the tail was treated with a hemostatic powder (Haver Lockhart Laboratories, Shawnee, KS) to prevent hemorrhaging. Each time the tail was clipped from a different animal, scissors as well as forceps, were rinsed in 100% anhydrous ethyl alcohol to prevent contamination from one sample to the other.

Once all tails were gathered into their respective labeled test tubes, they were treated with 300 ul of digestion buffer + 2 ul of Proteinase K (Sigma-Aldrich, St.Louis, MO). The digestion buffer was made from: 100 mM NaCl (41K8934, Sigma-Aldrich), 10 mM TrisCl (T2663, Sigma-Aldrich), 25 mM EDTA (E7889, Sigma-Aldrich), 0.5% SDS Lauryl Sulfate (L4390, Sigma-Aldrich), and double distilled water.

Each test tube was then wrapped in Parafilm laboratory film (Pechiney Plastic Packaging, Chicago, IL) and put onto a plastic test tube rack that was subsequently put into a shaking water bath (Precision Scientific) at 50 °C, 10 oscillations/ minute, and for at least 15 hours.

DAY 2 (DNA ISOLATION) – Each tube received 150 ul of Phenol:Chloroform:Isoamyl Alcohol (Invitrogen, Carlsbad, CA) and thoroughly mixed together for several minutes. Tubes were then centrifuged in an Eppendorf Mini Spin Plus (Eppendorf, Hamburg, Germany) at 12,000 rpm for 5 minutes. Post centrifugation, three layers were apparent in each tube. The top
layer (approximately 200-250 ul) was extracted via a 200 ul pipette (301-02-301, Axygen) and transferred to another 1.5 ml Axygen test tube. These new tubes received 35 ul of 7.5 M Ammonium Acetate (Sigma-Aldrich) + 600ul of 100% anhydrous ethyl alcohol and mixed together until DNA precipitated from the solution. Centrifugation was then again performed on the test tubes at 4000 rpm for 2 minutes to separate the DNA to the bottom of the tubes. The top liquid content was then discarded leaving behind the DNA precipitate. The tubes were then treated with 1000 ul of 70% anhydrous ethyl alcohol and mixed again followed by yet another centrifugation period at 4000 rpm for 2 minutes. Again, the supernatant was discarded leaving behind the DNA.

Test tubes were then left at room temperature for several hours with lids open to allow the evaporation of the remaining traces of alcohol (usually 2-3 hours). Finally, all test tubes were treated with 100 ul of Molecular Biology Reagent Water (W4502, Sigma-Aldrich) and left at room temperature until the following day.

**DAY 3 (PCR)** – The isolated DNA required dilution. 5 ul of the isolated DNA from each test tube was transferred to a 600 ul test tube (02-681-311, Fisherbrand, Pittsburgh, PA). These test tubes were then diluted by adding 100 ul of Molecular Biology Reagent Water (Sigma-Aldrich). The following step was to amplify the DNA via Polymerase Chain Reaction. A “Master Mix” solution was prepared composed of special custom primers. The ingredients of the master mix were prepared as follows per 3 reactions:
- 2 x PCR Master Mix (K0171, Fermentas, Burlington, ON)  25 ul
- 10 uM Primer Common 5’ (Invitrogen Custom Primer)  8 ul
  (5’- 3’) - TCA CCC CCT TGG GAT AGC CCT TCC
- 10 uM Primer WT 5’ (Invitrogen Custom Primer)  4 ul
  (5’- 3’) – GAC GAC GGC CTC AGA CTT CTT GGG
- 10 uM Primer KO 5’ (Invitrogen Custom Primer)  4 ul
  (5’ – 3’) – CCG CTA TCA GGA CAT AGC GTT GGC
- Molecular Biology Reagent Water (Sigma-Aldrich)  5.5 ul
- Platinum TAQ DNA Polymerase (10966-018, Invitrogen)  0.5 ul

From this prepared master mix stock, 14 ul would be added to 1 ul of diluted DNA from above, into a 0.2 ml thin wall, clear domed cap PCR tube (321-01-051, Axygen). These samples were then loaded into a PCR thermocycler (Eppendorf Mastercycler Gradient) to amplify the DNA. The thermocycler was programmed with the following temperatures and time settings:

- Cycle 1 – 94 °C @ 1 minute
- Cycle 2 – 94 °C @ 30 seconds
- Cycle 3 – 60 °C @ 30 seconds
- Cycle 4 – 72 °C @ 1 minute

The above temperatures and times were set to repeat 34 more times followed by:

- Cycle 5 – 72 °C @ 10 minutes
- Cycle 6 – 4°C hold indefinitely
**GEL ELECTROPHORESIS** - Gel electrophoresis was used to determine the banding patterns of the DNA samples in order to identify the genotypes of the animals.

Gels were prepared by adding 150 ml of Ultra Pure 10 x TAE buffer (15558-042, Invitrogen) diluted to 1 x TAE + 1.5 grams of Ultra Pure Agarose (15510-019, Invitrogen). The solution was then heated until the agarose powder dissolved. The solution was then cast in an Owl Easy Cast B2 electrophoresis system using the supplied well combs (Owl Separation Systems Inc, Portsmouth, NH).

Once the gel was casted (~ 30 minutes), it was put into its running orientation (- → +). Approximately 700 mL of 1 x TAE Buffer was placed into the electrophoresis system along with the gel. Eight ul of Gene Ruler 1 Kb ladder (SM0311, Fermentas) + 2 ul of 10 x BlueJuice (10816-015, Invitrogen) was added to the first well to serve as an indicator of protein banding size. Each remaining well was filled with a 8 ul sample of DNA + 2 ul of 10 x BlueJuice (10816-015, Invitrogen).

The Owl electrophoresis system was powered by a Thermo Electron power supply (EC250-90, Thermo Scientific, Waltham, MA) and operated at 120 volts, 500 mA, for a duration of 1-1.5 hours or until the DNA banding reached the “4” or “5” marking on the casting tray.

Once the gel electrophoresis finished running, the gel was removed from the tray and treated with 1-2 mL of ethidium bromide. The gel was left for 20-30 minutes to allow full saturation of the ethidium bromide solution. The gel was visually examined under U.V light to assess the banding patterning of the respective DNA samples using a FluorChem 8900 apparatus (Alpha Innotech Corporation, St. Leandro, CA).

Three unique banding patterns were observed:
<table>
<thead>
<tr>
<th>GENOTYPE</th>
<th>BANDING SIZE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Wild-type (+/)</td>
<td>400 bp</td>
</tr>
<tr>
<td>2. Heterozygous (+/-)</td>
<td>400 bp + 600 bp</td>
</tr>
<tr>
<td>3. Knockout (-/-)</td>
<td>600 bp</td>
</tr>
</tbody>
</table>

bp = Base Pairs

**Figure 2.1:** Gel electrophoresis revealing the mice genotype using a 1kb gene ruler/ladder. Wild-type animals banded at 400 base pairs, heterozygous animals banded at both 400 and 600 base pairs, knockout animals banded at 600 base pairs.
All of the experiments were carried out on male, C57Bl/6 mice, ages 2-4 months, weighing between 24 to 34 grams (mean = 27.4 ± 0.46 grams) and were derived from the colony. Eleven narcoleptic animals were used in this study where 5 animals were derived from heterozygous x heterozygous parents, 1 animal from a heterozygous x knockout parent, and 5 other animals from knockout x knockout parents. Nine wild-type animals were used in this study, that were littermates of the narcoleptic animals.

The colony was housed in the Department of Cell and Systems Biology Animal Care Facility (University of Toronto). All mice were housed in rectangular opaque boxes (32cm x 19 cm x 13 cm; Nalgene Labware, Rochester, NY, USA) and were fitted with standard laboratory corn cob bedding (Bed-o-cobs; The Andersons, Maumee, OH, USA). Mice were also supplied with the standard commercial rat/mouse chow (Lab Diet; PMI Nutrition International, St. Louis, MO, USA) and water ad libitum. The colony was maintained in a room at 20 ± °C, with a humidity index of 60%. The colony operated on a 12L/12D (light 12 hours: dark 12 hours) photoperiod cycle with lights on from 7 a.m. to 7 p.m.

Since behavioral experiments are sensitive to stress, the animals were moved from the Animal Care Facility to the laboratory 24 hours prior surgery, and remained there until the end of experiments. Laboratory environmental conditions were similar to the Animal Care Facility. Cages were changed twice a week (Monday and Thursday) using the same bedding materials, food, and water.
III – Surgical Techniques

A. Surgical Preparation

All preoperative and postoperative surgical procedures conformed to those suggested by the University of Toronto Division of Comparative Medicine’s policies on Survival Rodent Surgery. Aseptic surgical procedures were performed to avoid contamination; surgeries were conducted in an isolated room exclusive for rodent surgery. All surgical equipment followed proper sterilization by means of autoclaving and treatment with 70% anhydrous ethyl alcohol. All surgeons prepared for surgery by rigorous hand washing, surgical attire, gloves and face masks.

B. Surgical Procedure

Mice were anesthetically induced in a small clear plastic box (11.67 cm x 20.82 cm x 34.06 cm; Rubbermaid, Mississauga, ON, CA) using 5% isoflurane (1-chloro-2,2,2-trifluoroethyl ether), vaporized in medical grade oxygen at a flow rate of 1 L/min. Once the animal collapsed and there were no signs of consciousness, the mouse was taken out of the box and positioned into the stereotaxic apparatus (Model 900 Small Animal Stereotaxic Instrument; Kopf Instruments, Tujunga, CA, USA). Prior to the mouse being fitted into the stereotaxic apparatus, sterile cloths as well as a heating pad set to 37°C (TC-1000 Temperature Controller; CWE Inc., Ardmore, PA, USA) were placed to elevate the mouse into its respective surgical position. The mouse was held in the apparatus via special jaw bars (Model 921; KOPF Instrument), as well as a special mouse nose adapter (Model 907; KOPF Instruments) which maintained the mouse at 2-2.5 % isoflurane for the entire duration of the surgery. Using a cordless animal trimmer (Series 8900; Wahl Clippers Co, Sterling, IL, USA), the head and neck regions were shaved to expose bare skin for the surgical incision. To sterilize the skin region before the incision, Betadine (10% povidine-iodine topical solution USP) was applied because of its strong broad-spectrum microbicide
properties. An ophthalmic ointment – BNP (Bacitracin Neomycin Polymixin; Vetcom Inc., Upton, QC, CA), was also applied over the surface of the eyes to prevent desiccation.

To determine whether the animal was unconscious, the classical hind limb withdrawal reflex was used. Once the reflex was abolished, an incision was made from the surface of the skull running posteriorally from the frontal part of the head and terminating at the neck region to expose the skull surface as well as the nuchal musculature region. To keep the skull surface exposed from the surrounding skin, surgical hemostats were used to pull the skin away from the skull surface. Excess blood from the incision was removed using standard Q-tips and 0.9% saline was used to cleanse the area of any debris. Once the skull surface was dry (using standard Q-tips), hydrogen peroxide was applied to the skull surface which allowed one to discriminate the salient markings on the skull (ie. Bregma).

Four holes were drilled into the skull surface to allow chronic implantation of EEG (Electroencephalogram) electrodes. Using a stereotaxic drill with a cobalt bit (CHSD-73-10; Small Parts Inc., Miami Lakes, FL, USA) mounted onto a stereotaxic arm (944 Rat Alignment Tool; KOPF), bregma was referenced at zero. The 4 holes were then drilled via the following coordinates: 2 Anterior holes (mm from bregma: AP + 2.2; ML +/- 1.36; DV until the dura surface was reached); 2 Posterior holes (mm from bregma: AP – 3.0; ML +/- 1.36; DV until the dura surface was reached).

A specially designed head cap, dimensions 0.79 cm x 0.48 cm (Pinnacle Technology Inc., Lawrence, KS, USA) was fitted to the skull surface via the skull screws (0.12mm, Pinnacle Technology Inc.) that also served as the recording electrodes. The screws were manually inserted by hand with a small flat headed screwdriver until the head cap was flush with the skull surface. To ensure proper electrical conductivity, silver epoxy (#8331; Mg Chemicals, Burlington, ON, CA) was applied to the gap between the screws and the head cap using a 30 gauge syringe tip (#305106; BD, Franklin Lakes, NJ, USA). Once the head cap was securely fastened to the skull,
a few drops of Instant Crazy Glue (#601062; Elmers Products, Scarborough, ON, CA) was applied between the head cap and the skull surface in order to further adhere the head cap to the skull surface.

Following implantation of the EEG screws/electrodes, two EMG (Electromyogram) wires were inserted into the neck muscle. At this point, a combination of 3 mL of 5% dextrose solution and 2 mL of Buprenorphine (0.04 mg/kg), was subcutaneously injected to rehydrate the mouse and minimize pain.

To permanently fix the head cap to the skull, dental cement (#56061, 3M Ketac Cem Aplicap; 3M, St. Paul, MN, USA), was prepared using a 3M ESPE Rotomix (3M) and administered around the perimeter of the head cap making sure that there was adhesion to the skull surface. Once the dental cement dried, the opened wound was then sutured. Using a needle driver and 6-0 P-10 cutting Sofsilk sutures (SS-1639; Syneture, Norwalk, CT, USA), 3-4 stitches were used to close the loose skin around the head cap making sure to allow ample slack. Polysporin (Vitamin E Enriched Complete) was applied around the sutured skin to prevent infection and speed recovery. Mice were then released from the stereotaxic apparatus while the nose piece was still affixed to deliver pure oxygen for 5 minutes.

**C. Post Surgery**

Immediately after surgery, mice were placed into a clean cage (32cm x 19 cm x 13 cm; Nalgene Labware), lined with paper towels, with half of the cage sitting on a heating pad. Mice would often arise from anesthesia very quickly (~ 5-10 minutes) and would remain in this cage until they resumed their typical ambulatory, locomotor functions (ie. walking, eating, drinking, grooming). Following this period, they were transferred to their regular cages with their standard corn cob bedding.

Animals were monitored over the next couple days to insure that there were no signs of infections or overt changes in their typical behaviour. They received their regular diet as well as
water *ad libitum*. Mice were allowed a minimum of 10 days to fully recover from surgery, before they were used for experimentation. Figure 2.2 depicts a schematic illustration of a fully equipped mouse ready for experimentation.

*Figure 2.2:* A diagrammatic representation of a mouse instrumented with a custom head cap used to record sleep and wakefulness in a freely behaving mouse. Electroencephalogram (EEG) screws were chronically implanted into the skull and allowed for the recording of sleep states. Electroencephalogram (EEG) allowed for electrophysiological brain recordings while electromyogram (EMG) wires were inserted into the neck muscle and allowed for the recording of musculature activity to characterize each sleep state: Wake, NREM sleep, REM sleep. (Diagram created by Gavin Tse, 2005)
IV – Data Acquisition Methods

A. Sleep Recording Procedures

The purpose of our study was to examine sleep and muscle activity simultaneously and to do so, the study followed a series of procedures. On the initial day of experimentation, mice were placed into a sound-attenuated, ventilated and brightly lit chamber. Mice were induced in a clear plastic box (11.67 cm x 20.82 cm x 34.06 cm; Rubbermaid) under 5% isoflurane, vaporized in medical grade oxygen until the animal ceased movement. While the mouse was unconscious, the head cap was connected to a custom preamplifier (1.87 grams, 100 V/V, Pinnacle Technologies Inc.) and tethered to a low torque rotating cable and placed into a clear Plexiglas cylinder (Diameter = 17.8 cm, Height = 20.3 cm; Department of Cell and Systems Biology Workshop). The conditions in the plastic cylinder were similar to the mouse’s home cage in that the cylinder was similar in size and contained the same bedding, lab chow, and water. The preamplifier that was connected to the low torque rotating cable was subsequently connected to an integrated conditioning acquisition system amplifier (Model 4100, Pinnacle Technologies Inc.). This amplifier was then connected to a Dell computer (Optiplex GX620; Dell Computers, North York, ON, CA) via a powered USB hub (DY-USB2; Dynex, Richfield, MN, USA) that contained the software (Version 1.6.2, Pinnacle Technologies Inc.) necessary to operate the hardware.

EEG and EMG signals were amplified and filtered (Model 4100 DASC Amplifier, Pinnacle Technologies Inc.). EEG signals were filtered between 0.5 Hz to 35 Hz while EMG signals were filtered between 10 Hz to 80 Hz. Signals were then sampled and displayed using the Pinnacle Technologies software (Version 1.6.2, Pinnacle Technologies Inc.) at a 400 Hz sampling rate. Figure 2.3 outlines the experimental setup.
Figure 2.3: Experimental recording apparatuses: (a) Sleep recording chamber: the mouse is placed in a Plexiglas cylinder equipped with fresh corn cob bedding, food, and water, within a sound-proof box, while the head piece is attached to a tether (b) Mouse head piece hooked up to the custom preamplifier (c) Low torque tether and data acquisition system that amplifies and filters EEG and EMG signals (d) Computer System with Pinnacle Technologies Software for data acquisition of all sleep and muscle activity
B. Videography

Videography was used in the study to visualize motoric behaviour and confirm sleep-wake states. Video imaging was captured via an infrared video camera (DCR-TRV260, Sony Digital 8 Handy Cam, Sony Electronics Inc.,) mounted on a tripod (T37, Optex, Markham, ON, CA) within the recording chamber. Video acquisition was acquired with a movie maker software program (Windows Movie Maker, Microsoft Corporation, Redmond, WA, USA), and video clips were automatically created at the culmination of the video recording periods for analysis. In order to synchronize the video imaging with the EEG/EMG recording at the exact time, a stop timer (U of T medstore) was placed in the recording chamber such that the video camera visually recorded it along side the Plexiglas chamber that held the mouse. The EEG/EMG acquisition software (version 1.6.2, Pinnacle Technologies Inc.), contained a built in stop timer that allowed the user to initiate a “count-up” timer sequence at the start of a button. Hence, at the onset of the recording session, the in-chamber stop timer, and the EEG/EMG acquisition timer (version 1.6.2, Pinnacle Technologies Inc.) were synchronized. In this context, one could verify the timing of a behavioural observation in the video, with EEG/EMG sleep-wake behaviour. This allowed the observer to depict what was happening from an electroencephalogram/electromyogram perspective (ie. cataplexy; waking EEG and low muscle EMG).

V – Experimental Protocol and Pharmacological Interventions

The studies performed in this thesis were conducted under freely behaving conditions in mice in a controlled environment. Mice were tethered to the recording apparatus, and were put into the recording chamber between 10-11 a.m. to allow the animals to acclimatize to their environmental setting before experimentation. We recorded sleep from 7 p.m. to 10 p.m. in the dark with an infrared camera. This was performed so that behavioural observations could be
viewed while depicting the EEG and EMG activity. These experiments were performed under baseline (unhandled animal), injection of 0.9% saline (handled animal), as well as candidate drug interventions (see below). Baseline and saline treatments were always conducted first followed by the various candidate drugs which were randomly administered. Each animal was allowed 2 days in between drug treatments so that previous drugs did not affect behaviour.

All drugs used in the study were given intraperitoneally with a 1.0 mL syringe (#309602, BD) and a 30 gauge needle (#305106; BD). This required preparation of drug concentrations in a consistent 0.3 mL volume administered to all animals. All drugs were reconstituted in double distilled water at room temperature, and were prepared fresh 30 minutes before the injection. Drugs that had low solubility, were sonicated (#08849-00, Ultrasonic Cleaner, Cole Palmer Instrument Company, Vernon Hills, IL, USA) to aid the dissolving process. Certain drugs that were stable in their reconstituted form, were prepared and then stored in the dark at -20°C until the experiment. Injections took less than 10 seconds and were done approximately 5 minutes before 7 p.m. Candidate drugs considered for this study were amphetamine and quinpirole.

**Amphetamine:** Amphetamine increases the release of dopamine and to a lesser extent, noradrenaline and serotonin (Billiard et al. 2006). These stimulating effects promote wakefulness in addition to being effective in treating symptoms of narcolepsy (Kitahama and Valatx 1979; Shelton et al. 1995). We used dextroamphetamine (d-amphetamine, (+)-α-Methylphenethylamine sulfate salt, MW= 368.49, Sigma-Aldrich Inc.) and examined the effect it had on sleep and behavioural arrests in wild-type and knockout animals. We gave a dose of 2 mg/kg because previous data demonstrate it increases waking (Kitahama and Valatx 1979).
**Quinpirole:** Quinpirole HCl (D2/D3 dopamine receptor agonist, (-)-Quinpirole monohydrochloride, MW= 255.79, Sigma-Aldrich Inc.) was used because it agonizes dopaminergic D2 receptors. D2 receptor agonism has been demonstrated to promote wakefulness as well as locomotion (Isaac and Berridge 2003). Importantly, it has also been shown to increase behavioural arrests such as cataplexy in narcoleptic dogs (Mignot et al. 1993; Reid et al. 1996). Hence, we aimed to determine the effects of quinpirole on sleep and behavioural arrests (e.g. cataplexy) by using a 0.5 mg/kg concentration on both wild-type and narcoleptic mice.

**VI – Data Analysis for Sleep and Behavioural Arrests**

**SLEEP MEASUREMENTS**

**EEG and EMG Scoring of Sleep and Wakefulness**

EEG and EMG activity were quantified using a Pinnacle Technologies script, which integrated EEG and EMG values into 10-second epochs. Scoring the data required assessing the sleep states of the animal over 3 continuously hours from 7p.m. to 10 p.m. Three hour periods were used in our study since the half life exhibited by each drug, were no more than this time period. Amphetamine’s half life is approximately 2 hours and quinpirole’s half life is approximately 1.8 hours in rodents (Haefely et al. 1976; Whitaker and Lindstrom 1987). Scoring was achieved by a software program that took the recorded sleep data over the 3 hours, and integrated it into a scoring file (Sirenia SCOR E, Pinnacle Technologies Inc.). Since the sleep recorded data was quantified into 10-second epochs, 3 hours of continuous recording produced 1080 epochs that was later visually scored by the user. For the purpose of this study, each 10 second epoch was characterized as 1 of 3 sleep states: waking, NREM sleep, and REM sleep; and later identified as 1 of 4 behavioural arrest states when compared with videography (see
The criteria for scoring sleep is described in the following table 2.1. Figure 2.4 visually illustrates the 3 sleep states, as well as the muscle activity that occurs during each state.

**Table 2.1:** Names and descriptions of sleep states used to score all data under behaving conditions. Data was analyzed in 10-second epochs.

<table>
<thead>
<tr>
<th>Sleep-Wake States</th>
<th>Description</th>
<th>EEG Waves</th>
<th>EMG Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Waking*</td>
<td></td>
<td>Low voltage, high frequency waveforms</td>
<td>High and variable activity, corresponding to periods of locomotion such as walking, grooming, eating, etc.</td>
</tr>
<tr>
<td>2. NREM*</td>
<td></td>
<td>High voltage, low frequency waveforms</td>
<td>Hypotonia</td>
</tr>
<tr>
<td>3. REM*</td>
<td></td>
<td>Low voltage, high frequency waveforms, regular waveform patterns and would closely resemble waking activity.</td>
<td>Atonia</td>
</tr>
</tbody>
</table>

* Each 10-second epoch was scored as a sleep state if 60% of that epoch was the respective sleep state. If there was 50% of one sleep state and 50% of another sleep state that resided within an epoch, then the epoch would be designated a “Transitional” state. If a sleep state was unclear, it was scored as an “Artifact” and excluded from the sleep analysis.

**Figure 2.4:** This figure illustrates the EEG of 3 sleep states that were analyzed in this study: waking, NREM, and REM sleep. The muscle activity is also depicted in this figure with waking activity typically exhibiting high muscle activity in the EMG, NREM sleep exhibiting decreased muscle activity (hypotonia) and REM sleep exhibiting virtually absent muscle activity (atonia).
**Analysis of Sleep**

Once 3 hours of sleep activity were scored according to the guidelines above, the scoring program allowed viewing of the relative percentage of time spent in waking, NREM, and REM sleep over 3 hours. The percentage of time spent in waking, NREM, and REM sleep summed to 100% at any given time (e.g. if over 3 hours, waking = 60%, NREM sleep = 35%, and REM sleep = 5%, then waking = 108 minutes, NREM sleep = 63 minutes, and REM sleep = 9 minutes). For the purpose of this study, we looked at the overall percentages of waking, NREM, and REM sleep over 3 complete hours, while also comparing the percentages of waking, NREM, and REM sleep at 30 minute intervals over the 3 hours. The percentages of waking, NREM, and REM sleep were compared across baseline, saline, and drug interventions in narcoleptic and wild-type mice.

**Statistical Analysis of Sleep**

Total percent time (minutes) of waking, NREM, and REM sleep were compared over the total 3 hours between narcoleptic and wild-type mice using unpaired t-tests (SigmaStat 2.03; Systat Software Inc.) with P< 0.05 differences declared significant. Data are presented as mean ± SEM unless noted differently. Total percent time (minutes) of waking, NREM, and REM sleep that compared saline with drug interventions within narcoleptic or wild-type mouse groups, used paired t-tests (SigmaStat 2.03; Systat Software Inc.) with P< 0.05 differences declared significant.

Percent time (using minutes) of waking, NREM, and REM sleep that were compared in 30 minute intervals over 3 hours, between narcoleptic and wild-type mice, were compared using a 2-Way ANOVA Repeated Measures (RM) tests. Results were further analyzed using a post-hoc Student-Newman Keuls test and differences were considered significant if the null hypothesis was rejected at P< 0.05. Analyses were performed using SigmaStat (Version 2.03; Systat Software Inc.). The percent time of waking, NREM, and REM sleep (compared in 30
minute intervals between saline and drug interventions over 3 hours) between narcoleptic or wild-type mouse groups, were compared using a 2-Way ANOVA Repeated Measures (RM) test that was further analyzed using a post-hoc Student-Newman Keuls test and differences were considered significant if the null hypothesis was rejected at $P < 0.05$.

**BEHAVIOURAL ARREST MEASUREMENTS**

**Video Scoring of Behavioural Arrests**

Video scoring was necessary in order to quantify changes in behaviour across sleep and wakefulness. Video recordings were analyzed the subsequent day to assess whether animals experienced episodic periods of behavioural arrest or cataplexy (cessation of muscle activity). The number of episodes as well as the time durations of those respective episodes, were recorded and compared to the electroencephalogram (EEG) and electromyogram (EMG) to verify the accuracy of the video observations. Video clips were automatically created at the culmination of the video recording by the Windows Movie Maker program. Each video clip was evaluated twice to ensure that behavioral arrests were correctly identified.

A subset of criteria was used to evaluate a behavioural arrest in an animal and is outlined in the following table:

Table 2.2: Criteria for defining episodes of behavioural arrests during the recording period.

<table>
<thead>
<tr>
<th>Defining Behavioral Arrests</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Immediate Termination of Movement *</td>
<td>Animals would roam around the recording chamber, drinking, or eating and would be interrupted by a rapid termination of movement.</td>
</tr>
<tr>
<td>2. Loss of Posture **</td>
<td>After the termination of movement, postural muscle tone decreased causing the animals to collapse to one side. Often, the animal would appear to be elongated in a relaxed state with its head stretched out.</td>
</tr>
<tr>
<td>3. Full Resumption of Movement</td>
<td>Animals would transition from an absence of movement, to fully resuming their typical locomotor behaviour such as walking, and grooming.</td>
</tr>
</tbody>
</table>

* At certain times during the onset of a behavioral arrested episode and termination of movement, the tail went limp or sporadically whipped around before the animal collapsed. ** During a period of behavioral arrest, animals at times would display accelerated/rapid breathing patterns. Furthermore, animals would sometimes exhibit rocking motions during an episode as if they were resisting a behavioural arrest.
Verifying EEG and EMG with Behavioural Arrests

Once the EEG/EMG data as well as the video data was scored, they were compared with one another to assess what was occurring in the EEG/EMG parallel to each behavioural arrest. Four distinct types of behavioural arrests were characterized upon comparing them with the EEG and EMG. The following table explains these 4 types of behavioural arrests:

Table 2.3: Four types of behavioural arrests observed while comparing videography to EEG/EMG recordings.

<table>
<thead>
<tr>
<th>Observations</th>
<th>Descriptions</th>
<th>EEG Transitions</th>
<th>EMG Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Cataplexy (see figure 3.10) p77</td>
<td>Continuous waking activity</td>
<td></td>
<td>Atonia</td>
</tr>
<tr>
<td>2. Wake-REM (see figure 3.11) p77</td>
<td>Waking activity $\rightarrow$ REM sleep activity</td>
<td></td>
<td>Atonia</td>
</tr>
<tr>
<td>3. Gradual Arrest (see figure 3.12) p78 (GA NREM) *</td>
<td>Waking activity $\rightarrow$ NREM sleep $\rightarrow$ Waking activity</td>
<td></td>
<td>Hypotonia</td>
</tr>
<tr>
<td>4. Gradual Arrest (see figure 3.13) p78 (GA NREM-REM) *</td>
<td>Waking activity $\rightarrow$ NREM sleep $\rightarrow$ REM sleep $\rightarrow$ Waking activity</td>
<td></td>
<td>Hypotonia/Atonia</td>
</tr>
</tbody>
</table>

* Gradual arrests appeared visually similar to the criteria used in defining behavioral arrests from Table 2.2 and gradual arrests did not resemble normal sleep positioning and posture i.e) Huddling/curling up of the animals prior to sleep.

Analysis of Behavioural Arrests

The 4 types of behavioural arrests in our study were quantified by 3 different measurements:

- mean number of behavioural arrests
- mean duration (seconds) of the behavioural arrests
- mean total time spent in behavioural arrest (seconds) (number of episodes x duration)

All behavioural arrests in this study were examined over a 3 hour period. Furthermore, when we observed the different measurements of each behavioural arrest, we took each behavioural arrest and normalized it to the time the animal spent awake. The reason this was performed was the fact that no animal could exhibit any of the 4 types of behavioural arrests while sleeping (NREM or
REM sleep). There must have required a transition from wakefulness into each behavioural arrest state. It is clear from our first set of studies on sleep, that over the course of 3 hours, the animals did not spend all of their time in wakefulness. They also exhibited time spent in NREM and REM sleep. Hence, we normalized our measurements by the time spent awake (in units of minutes), since our sleep study quantified the time spent in waking, NREM and REM sleep in minutes.

Behavioural arrests were only examined in narcoleptic animals since wild-type animals never exhibited any forms of behavioural arrests. Although we studied 11 narcoleptic animals, certain animals did not exhibit any behavioural arrests (during the saline treatment) in the 3 hour observation period.

We excluded animals that did not exhibit behavioural arrests during saline treatment; because we made the “a priori” hypothesis that amphetamines would reduce cataplexy (Shelton et al. 1995) and behavioural arrests. Therefore, 6 narcoleptic animals out of the 11 were examined with amphetamine during cataplexy, 8 narcoleptic animals out of the 11 were examined with amphetamine during W-REM transitions, 9 narcoleptic animals out of the 11 were examined with amphetamine during GA NREM transitions, and 7 narcoleptic animals out of the 11 were examined with amphetamine during GA NREM-REM transitions.

We used 7 narcoleptic animals that were treated with quinpirole and although some of these animals never exhibited any of the behavioural arrests with the saline treatment, we still included them because we made the “a priori” hypothesis that quinpirole would increase or exacerbate cataplexy consistent with other studies (Nishino et al. 1991). It was also speculated that quinpirole may increase the other types of behavioural arrests because those arrests are transitions into sleepier states, and D2 agonists (like quinpirole) increase drowsiness (Ferrari and Giuliani 1993).
Statistical Analysis of Behavioural Arrests

Mean number of episodes, duration, and total time spent in each type of behavioural arrests were compared over 3 hours within narcoleptic mice using paired t-tests comparing baseline and saline treatments, and also between saline and drug interventions with P< 0.05 differences declared significant (SigmaStat 2.03; Systat Software Inc.).
Chapter Three:

RESULTS
3. RESULTS

STUDY 1: Determining the role for dopamine on excessive daytime sleepiness (EDS)

Because narcoleptics are excessively sleepy, we wanted to determine how a potent stimulant such as amphetamine, would affect sleep in narcoleptic mice. These studies were designed to compare how d-amphetamine affects the amounts of sleep and wakefulness in narcoleptic mice and their wild-type counterparts. We also wanted to compare how a dopamine D2 agonist such as quinpirole, would affect sleep in narcoleptic mice since D2 receptors are implicated in autoregulation and can decrease the endogenous levels of dopamine. Hence, we also aimed to determine how quinpirole affected the amounts of sleep and wakefulness in narcoleptic and wild-type mice. Since mice are nocturnal creatures, studying them at night is equivalent to studying their daytime activities because they are awake.

A. Comparing the different levels of sleep and wakefulness between narcoleptic (hypocretin knockout) and wild-type mice.

Since narcoleptics have disrupted wake patterns (specifically increased REM sleep) (Mochizuki et al. 2004), we wanted to compare the baseline differences in sleep and wakefulness between narcoleptic and wild-type mice. By comparing the percentages of waking, NREM, and REM sleep (over the total 3 hours of sleep recording) between narcoleptic (n=11) and wild-type mice (n=9), we found that there was a difference in sleep and wakefulness between these two genotype groups. With respect to percent wakefulness, we found that there was a 21 percent increase in waking in narcoleptic mice compared to the wild-type mice (from $57 \pm 4\%$ waking in wild-type mice vs. $69 \pm 2\%$ waking in narcoleptic mice). With respect to NREM sleep, there was a 36 percent decrease in NREM sleep in the narcoleptic mice compared to the wild-type mice (from $39 \pm 3\%$ NREM sleep in wild-type mice to $25 \pm 3\%$ NREM sleep in narcoleptic mice).
Lastly, with respect to REM sleep, there was a 48 percent increase in REM sleep in the narcoleptic mice compared to the wild-type mice (from 4 ± 1% REM sleep in wild-type mice to 6 ± 1% REM sleep in narcoleptic mice). The respective increases/decreases of sleep and wakefulness, are represented by figure 3.1 that illustrate the baseline temporal percentages of waking, NREM, and REM sleep between narcoleptic and wild-type mice. With respect to the percent of waking, narcoleptics exhibited a higher percentage of wakefulness compared to wild-type mice (2-Way RM ANOVA, P= 0.008). Furthermore, because narcoleptic mice exhibited a higher percentage of wakefulness, they had a lower percentage of NREM sleep compared to wild-type mice (2-Way ANOVA, P= 0.003). Lastly, narcoleptics had more REM sleep than did wild-type mice (2-Way ANOVA, P= 0.047).
Figure 3.1: Amounts of sleep and wakefulness in narcoleptic, KO (n=11) and wild-type mice, WT (n=9). The figure represents the percentages of waking, NREM, and REM sleep recorded in 30 minute intervals, of both KO and WT mice from 7 P.M to 10 P.M. There was a significant difference between KO and WT mice in wakefulness, NREM, and REM sleep, P< 0.05.
B. Comparing the effects of saline injections on sleep and wakefulness

We determined if handling mice during injections affected sleep and wakefulness. Rodent sleep is affected by stress (Cheeta et al. 1997) such as handling; and since our protocol required using drug manipulations by handling and injecting them, we tested the handling effect by comparing baseline (non-handling) to saline injections.

**Narcoleptic Mice:** By handling the mice and injecting them with saline, wakefulness decreased by 10 percent (from 69 ± 2% in baseline to 62 ± 4% with saline), NREM sleep increased by 31 percent (from 25 ± 3% in baseline to 33 ± 3% with saline), and REM sleep decreased by 12 percent (from 6 ± 1% in baseline to 5 ± 1% with saline); however, the respective increases/decreases of sleep and wakefulness are shown in figure 3.2 and demonstrates that there were no significant differences between baseline and saline with respects to waking (n=11, 2-Way RM ANOVA, P= 0.112), NREM sleep (n=11, 2-Way RM ANOVA, P= 0.070), and REM sleep (n=11, 2-Way RM ANOVA, P= 0.388).

**Wild-type Mice:** In wild-type mice, wakefulness decreased by 5 percent (from 57 ± 4% in baseline to 55 ± 3% with saline), NREM sleep increased by 11 percent (from 39 ± 3% in baseline to 43% ± 3 with saline), and REM sleep decreased by 42 percent (from 4 ± 1% in baseline to 2% with saline). The respective increases/decreases of sleep and wakefulness are illustrated in figure 3.3 and show that there were no significant difference between baseline and saline in waking (n=9, 2-Way RM ANOVA, P= 0.650), and NREM sleep (n=9, 2-Way RM ANOVA, P= 0.436); however, there was a significant difference between baseline and saline in REM sleep (n=9, 2-Way RM ANOVA, P= 0.026).

Although there were no significant differences between baseline and saline treatments in narcoleptic mice, there was a difference in REM sleep between these two treatments in the wild-
type mice. Hence our study compared drug manipulations to saline, since both drug and saline treatments entail the handling of animals during injection.
Figure 3.2: This figure represents the comparison between the baseline (unhandled) and saline (handled) effects on sleep and wakefulness in narcoleptic mice (n=11). Recorded percentages of waking, NREM, and REM sleep were plotted in 30 minute intervals. There were no significant differences in wakefulness, NREM and REM sleep, between the baseline and saline treatments in narcoleptic mice, P> 0.05.
Figure 3.3: This figure represents the comparison between the baseline (unhandled) and saline (handled) effects on sleep and wakefulness in wild-type mice (n=9). Recorded percentages of waking, NREM, and REM sleep were plotted in 30 minute intervals. There were no significant differences in wakefulness and NREM sleep, \( P > 0.05 \), however there was a significant difference in REM sleep, between the baseline and saline treatments in wild-type mice, \( P < 0.05 \).
C. Determining the effects of d-amphetamine on sleep and wakefulness in narcoleptic and wild-type mice

Amphetamine induces hyperactivity (Kitahama and Valatx 1979) and increase wakefulness while reducing sleepiness (Mignot and Nishino 2005; Zeitzer et al. 2006). Pharmacokinetics of d-amphetamine demonstrate in rodents that the half life of this drug is about 3 hours (the time course we observe our animals) (Haefely et al. 1976; Lokiec et al. 1978). We wanted to examine the effects of d-amphetamine (2mg/kg) (Kitahama and Valatx 1979) in narcoleptic and wild-type mice to determine whether it affected sleep and wakefulness. We also wanted to establish whether differences in responses to d-amphetamine, occurred between wild-type and narcoleptic mice.

d-amphetamine in Narcoleptic Mice: Narcoleptic mice treated with amphetamine compared to saline increased waking by 28 percent (from 62 ± 4% with saline to 80 ± 2% with amphetamine), decreased NREM sleep by 43 percent (from 33 ± 3% with saline to 19 ± 2% with amphetamine), and decreased REM sleep by 71 percent (from 5 ± 1% with saline to 1 % with amphetamine). The increases/decreases of sleep and wakefulness are illustrated in figure 3.4 and show that there was a significant difference between saline and amphetamine such that wakefulness was increased by amphetamine over the first 2 hours and then returned to basal levels (n=11, 2-Way RM ANOVA, P< 0.001). NREM sleep was suppressed by amphetamine for 2 hours and then returned to basal levels (n=11, 2-Way RM ANOVA, P< 0.001). REM sleep was also suppressed by amphetamine for the first 2 hours and then returned to basal levels (n=11, 2-Way RM ANOVA, P= 0.001).

d-amphetamine in Wild-type Mice: In wild-type mice treated with amphetamine, wakefulness increased by 66 percent (from 55 ± 3% with saline to 91 ± 3% with amphetamine), NREM sleep
decreased by 78 percent (from 43 ± 3% with saline to 9 ± 3% with amphetamine), and REM sleep decreased by 99 percent (from 2% with saline to virtually 0% with amphetamine). Figure 3.5 demonstrates that there was a significant difference between saline and amphetamine, such that wakefulness was increased by amphetamine over the entire 3 hours of sleep recording (n=9, 2-Way RM ANOVA, P< 0.001). NREM sleep was reduced by amphetamine for the entire 3 hours (n=9, 2-Way RM ANOVA, P< 0.001). REM sleep was virtually abolished by amphetamine over the 3 hours (n=9, 2-Way RM ANOVA, P< 0.001).
Figure 3.4: This figure compares the effects of d-amphetamine and vehicle saline on sleep and wakefulness in narcoleptic mice (n=11). Recorded percentages of waking, NREM, and REM sleep were plotted in 30 minute intervals. There were significant differences in wakefulness, NREM and REM sleep, between amphetamine and saline in narcoleptic mice, P< 0.05.
Figure 3.5: This figure compares the effects of d-amphetamine and vehicle saline on sleep and wakefulness in wild-type mice (n=9). Recorded percentages of waking, NREM, and REM sleep were plotted in 30 minute intervals. There were significant differences in wakefulness, NREM and REM sleep, between amphetamine and saline in wild-type mice, P<0.05.
Comparing the time course differences of sleep and wakefulness between narcoleptic and wild-type mice in the presence of d-amphetamine:

Figure 3.6 compares how amphetamine differentially affects sleep and wakefulness in narcoleptic (n=11) versus wild-type (n=9) mice. The results show that the time course effects of amphetamine lasted longer in the wild-type animals. Amphetamine produced a greater and longer lasting increase of wakefulness in wild-type mice compared to the narcoleptic mice (2-Way ANOVA, P= 0.007), despite the fact that narcoleptics exhibited higher basal levels of wakefulness compared to wild-type mice (illustrated in figure 3.1). Amphetamine produced a longer suppression of NREM sleep in the wild-type compared to the narcoleptic mice (2-Way ANOVA, P= 0.014). REM sleep was also suppressed for a longer period by amphetamine in wild-type compared to narcoleptic mice (2-Way ANOVA, P< 0.001). REM sleep was suppressed for the entire 3 hour recording period in wild-type mice; however, in narcoleptics, REM sleep started to return to basal levels after 2 hours.
Figure 3.6: Comparing the time course differences of sleep and wakefulness between narcoleptic (KO) (n=11) and wild-type mice (WT) (n=9) in the presence of d-amphetamine. The figure represents the percentages of waking, NREM, and REM sleep recorded in 30 minute intervals, of both KO and WT mice in the presence of amphetamine. There were significant differences in the responses to amphetamine, between KO and WT mice in wakefulness, NREM, and REM sleep, P< 0.05.
D. Determining the effects of quinpirole on sleep and wakefulness in narcoleptic and wild-type mice

Quinpirole (a selective D2/D3 receptor agonist) administered to rats and chicks have been shown to increase sleep (Ferrari and Giuliani 1993; Nishino et al. 1991). We aimed to determine if quinpirole (0.5 mg/kg) given intraperitoneally affected sleep and wakefulness in the narcoleptic and wild-type mice.

**Quinpirole in Narcoleptic Mice:** Figure 3.7 shows that there were no significant differences between saline and quinpirole in wakefulness (n=7, 2-Way RM ANOVA, P= 0.977), NREM sleep (n=7, 2-Way RM ANOVA, P= 0.686), and REM sleep (n=7, 2-Way RM ANOVA, P= 0.793).

**Quinpirole in Wild-type Mice:** Figure 3.8 shows that there were no significant differences between saline and quinpirole in wakefulness (n=6, 2-Way RM ANOVA, P= 0.612), NREM sleep (n=6, 2-Way RM ANOVA, P= 0.686), and REM sleep (n=6, 2-Way RM ANOVA, P= 0.793).
Figure 3.7: This figure compares the effects of quinpirole to vehicle saline, on sleep and wakefulness in narcoleptic mice (n=7). Recorded percentages of waking, NREM, and REM sleep were plotted in 30 minute intervals. There were no significant differences in wakefulness, NREM and REM sleep, between quinpirole and saline in narcoleptic mice, P> 0.05.
Figure 3.8: This figure compares the effects of quinpirole to vehicle saline, on sleep and wakefulness in wild-type mice (n=6). Recorded percentages of waking, NREM, and REM sleep were plotted in 30 minute intervals. There were no significant differences in wakefulness, NREM and REM sleep, between quinpirole and saline in wild-type mice, P> 0.05.
Comparing the effects of quinpirole on sleep and wakefulness between narcoleptic and wild-type mice:

Figure 3.9 shows that there were no significant differences between wild-type and narcoleptic mice in wakefulness treated with quinpirole (2-Way ANOVA, P= 0.862), NREM sleep (2-Way ANOVA, P= 0.849), and REM sleep (2-Way ANOVA, P= 0.589).
Figure 3.9: The effects of quinpirole on the % changes of sleep and wakefulness relative to saline, between narcoleptic mice, KO (n=7) and wild-type mice, WT (n=6). Saline treatments for both KO and WT groups are normalized to axis “0”, and quinpirole treatments are represented as a % change relative to “0”. Percent changes of waking, NREM, and REM sleep relative to saline, were plotted in 30 minute intervals. There were no significant differences of the responses to quinpirole, between KO and WT mice, P> 0.05.
The second set of studies was aimed to determine the role for dopamine in regulating cataplexy. We did this by pharmacologically manipulating dopamine in narcoleptic mice using amphetamine or quinpirole. Although wild-type mice were also treated with the same drugs as the narcoleptic mice, they never exhibited behavioural arrests. This is consistent with the observation that wild-type mice did not exhibit cataplexy (Chemelli et al. 1999). Hence the focus of this section is exclusively on narcoleptic mice.

**Characterizing the types of behavioural arrests:**

Chapter 2 described the criteria that we employed to define behavioural arrests. We identified four types of arrests that we categorized based on examining the EEG and EMG of each animal. These behavioural arrests are consistent with another study that examined narcoleptic mice (Willie et al. 2003). These four types of arrests were characterized into two categories—abrupt and gradual arrests:

**ABRUPT ARRESTS**

1. Cataplexy
2. Waking $\rightarrow$ REM sleep

**GRADUAL ARRESTS**

3. Waking $\rightarrow$ NREM sleep $\rightarrow$ waking
4. Waking $\rightarrow$ NREM sleep $\rightarrow$ REM sleep $\rightarrow$ waking

Abrupt arrests as characterized by Willie et al. (2003) were very sudden transitions into states of postural collapse; whereas, gradual arrests were more gradual transitions into states of postural collapse.
For simplicity, the remainder of this paper will address the types of arrests as: cataplexy, W-REM, GA NREM, and GA NREM-REM. The following figures describe the EEG during each of the four types of behavioural arrests and the transitioning of each behavioural state. Note that the figures are merely a representation of transitions between states, and that the behavioural arrests depicted in real life, are actually longer in duration.

**CATAPLEXY**

![Figure 3.10: This figure is an example of the EEG and EMG of narcoleptic mice during periods of cataplexy. During periods of waking EEG activity, the electromyogram (EMG) is accompanied by high muscle activity. During periods of cataplexy, waking activity is still maintained while muscle activity is greatly reduced.](image)

**W-REM**

![Figure 3.11: This figure is an example of the EEG and EMG of narcoleptic mice during periods of W-REM transitions. During transition periods from waking to REM sleep, the EMG is accompanied by a loss of muscle activity followed by a resumption of muscle activity when the EEG returns to waking.](image)
Figure 3.12: This figure is an example of the EEG and EMG of narcoleptic mice during periods of GA NREM transitions. During transition periods from waking to NREM sleep, the EMG is accompanied by a loss of muscle activity followed by a resumption of muscle activity when the EEG returns to waking. This type of gradual arrest transition to NREM sleep, are short periods that are typically shorter than NREM sleep periods during normal sleep.

Figure 3.13: This figure is an example of the EEG and EMG of narcoleptic mice during periods of GA NREM-REM transitions. During transition periods from waking to NREM sleep to REM sleep, the EMG is accompanied by a loss of muscle activity followed by a resumption of muscle activity when the EEG returns to waking. This type of gradual arrest transition to NREM sleep and then to REM sleep, are short periods that are typically shorter than NREM to REM sleep periods during normal sleep.
A. Determining whether the stress of handling an animal affects the frequency or duration of behavioural arrests

We wanted to demonstrate whether there was a difference in the frequency or duration of behavioural arrests between treating the mice with saline (handled) compared to baseline (unhandled). We examined the four types of behavioural arrests and quantified the mean number of episodes, mean duration, and total time spent in behavioural arrests over 3 hours.

ABRUPT ARRESTS (Baseline versus Saline)

Figure 3.14 shows how saline treatment affected cataplexy. Although there was a 70 percent increase in the mean number of cataplexy episodes (n=11, Paired t-test, P= 0.156), a 239 percent increase in the mean cataplexy duration (n=11, Paired t-test, P= 0.091), and a 641 percent increase in the total time spent in cataplexy (episodes x duration) (n=11, Paired t-test, P= 0.088); there were no significance differences in these three observations. This figure also illustrates the temporal variation of the number of episodes, duration, and total time of cataplexy across the 3 hour recording period in 30 minute increments. This demonstrates that at different periods of time, there are large variations in the data.

Figure 3.15 illustrates how saline affected W-REM transitions. There was a 36 percent decrease in the number of episodes (n=11, Paired t-test, P= 0.353), a 33 percent increase in the duration (n=11, Paired t-test, P= 0.564), and a 23 percent decrease in the total time spent in W-REM transitions (n=11, Paired t-test, P= 0.503); however, there were no significance in these three observations.

GRADUAL ARRESTS (Baseline versus Saline)

Figure 3.16 illustrates how saline affected GA NREM transitions. There was no change in the number of episodes (n=11, Paired t-test, P= 0.943), a 244 percent increase in the duration (n=11, Paired t-test, P= 0.002), and a 126 percent increase in the total time spent in GA NREM transitions (n=11, Paired t-test, P= 0.013). There were no significant differences in the number of
episodes of GA NREM transitions; however, there were significant differences in the duration and total time spent in GA NREM transitions.

Figure 3.17 illustrates how saline affected GA NREM-REM transitions. There was a 28 percent decrease in the number of episodes (n=11, Paired t-test, P= 0.275), no change in the duration (n=11, Paired t-test, P= 0.982), and a 22 percent decrease in the total time spent in GA NREM-REM transitions (n=11, Paired t-test, P= 0.438); however, there were no significant differences in these three observations.

We demonstrated that there was a difference between the baseline and saline treatments but only in the gradual arrest NREM transition (GA NREM). Therefore, we compared our drug manipulations in our study with saline and not baseline.
Figure 3.14: This figure compares the effects of vehicle saline (handling) to baseline (unhandled) on the levels of cataplexy over 3 hours in narcoleptic mice. The left panels represent the grand mean (mean x mean) number of episodes, duration, and total time spent in cataplexy (episodes x duration). There were no significant differences between the baseline and saline treatments on cataplexy (n=11, Paired t-test, P> 0.05). The right panels represent the mean trends of cataplexy at each half hour increments over 3 hours. These panels represent the number of episodes, duration, and total time spent in cataplexy and the variation that exists at different time points.
Figure 3.15: This figure compares the effects of vehicle saline (handling) to baseline (unhandled) on the levels of W-REM transitions over 3 hours in narcoleptic mice. The left panels represent the grand mean (mean x mean) number of episodes, duration, and total time spent in W-REM transitions (episodes x duration). There were no significant differences between the baseline and saline treatments on W-REM transitions (n=11, Paired t-test, P>0.05). The right panels represent the mean trends of W-REM transitions at each half hour increments over 3 hours. These panels represent the number of episodes, duration, and total time spent in W-REM transitions and the variation that exists at different time points.
Figure 3.16: This figure compares the effects of vehicle saline (handling) to baseline (unhandled) on the levels of GA NREM transitions over 3 hours in narcoleptic mice. The left panels represent the grand mean (mean x mean) number of episodes, duration, and total time spent in GA NREM transitions (episodes x duration). There was no significant difference in the mean number of episodes of GA NREM transitions between the baseline and saline treatments (n=11, Paired t-test, P>0.05) however, there were significant differences in the mean duration and total time spent in GA NREM transitions (n=11, Paired t-test, P<0.05). The right panels represent the mean trends of GA NREM transitions at each half hour increments over 3 hours. These panels represent the number of episodes, duration, and total time spent in GA NREM transitions and the variation that exists at different time points.
Figure 3.17: This figure compares the effects of vehicle saline (handling) to baseline (unhandled) on the levels of GA NREM-REM transitions over 3 hours in narcoleptic mice. The left panels represent the grand mean (mean x mean) number of episodes, duration, and total time spent in GA NREM-REM transitions (episodes x duration). There were no significant differences between the baseline and saline treatments on GA NREM-REM transitions (n=11, Paired t-test, P> 0.05). The right panels represent the mean trends of GA NREM-REM transitions at each half hour increments over 3 hours. These panels represent the number of episodes, duration, and total time spent in GA NREM-REM transitions and the variation that exists at different time points.
B. Determining the effects of amphetamine on behavioural arrests

Although amphetamines are classically used to treat the primary symptom of narcolepsy which is excessive daytime sleepiness (EDS), it has also been demonstrated in the canine model to reduce cataplexy (Shelton et al. 1995). We wanted to test the effects of amphetamine to determine whether amphetamine reduced cataplexy and EDS. Apart from observing the effects of amphetamine on cataplexy, the other 3 types of behavioural arrests in our study (W-REM, GA NREM, GA NREM-REM) are transition states into states of sleepiness. We were also interested in how amphetamine affected these three types of behavioural arrests.

**Cataplexy:** Figure 3.18 demonstrates that amphetamine reduced the number of episodes of cataplexy by 52 percent compared to saline but this was not statistically significant (n=6, Paired t-test, P=0.105). Amphetamine significantly reduced the duration of cataplexy by 88 percent compared to saline (n=6, Paired t-test, P=0.027), and it also reduced the total time spent in cataplexy by 90% compared to saline but was not statistically significant (n=6, Paired t-test, P=0.068).

**W-REM transitions:** In figure 3.19, amphetamine significantly reduced the number of W-REM episodes by 92 percent (n=8, Paired t-test, P=0.016), reduced the duration of W-REM transitions by 79 percent (n=8, Paired t-test, P=0.024), and reduced the total time spent in W-REM transitions by 91 percent (n=8, Paired t-test, P=0.022) compared to saline.
**GA NREM TRANSITIONS**: In figure 3.20, amphetamine significantly reduced the number of GA NREM episodes by 63 percent (n=9, Paired t-test, P= 0.011), reduced the duration of GA NREM transitions by 73 percent (n=9, Paired t-test, P=0.011), and reduced the total time spent in GA NREM transitions by 72 percent (n=9, Paired t-test, P= 0.003) compared to saline.

**GA NREM-REM TRANSITIONS**: In figure 3.21, amphetamine significantly reduced the number of GA NREM-REM episodes by 87 percent (n=7, Paired t-test, P= 0.004), reduced the duration of GA NREM-REM transitions by 65 percent (n=7, Paired t-test, P=0.017), and reduced the total time spent in GA NREM-REM transitions by 80 percent (n=7, Paired t-test, P= 0.003) compared to saline.
Figure 3.18: This figure illustrates the effects of d-amphetamine compared to vehicle saline on cataplexy in narcoleptic mice. This figure represents the mean number of episodes, mean duration, and total time spent in cataplexy over 3 hours. There were no significant differences in the mean number of episodes and total time spent in cataplexy with amphetamine compared to saline (n=6, Paired t-test, P> 0.05) however, there was a significant difference in the mean duration of cataplexy with amphetamine (n=6, Paired t-test, P< 0.05).
Figure 3.19: This figure illustrates the effects of d-amphetamine compared to vehicle saline on W-REM transitions in narcoleptic mice. This figure represents the mean number of episodes, mean duration, and total time spent in W-REM transitions over 3 hours. There were significant differences in the mean number of episodes, mean duration and total time spent in W-REM transitions with amphetamine compared to saline (n=8, Paired t-test, P>0.05).
Figure 3.20: This figure illustrates the effects of d-amphetamine compared to vehicle saline on GA NREM transitions in narcoleptic mice. This figure represents the mean number of episodes, mean duration, and total time spent in GA NREM transitions over 3 hours. There were significant differences in the mean number of episodes, mean duration and total time spent in GA NREM transitions with amphetamine compared to saline (n=9, Paired t-test, P> 0.05).
Figure 3.21: This figure illustrates the effects of d-amphetamine compared to vehicle saline on GA NREM-REM transitions in narcoleptic mice. This figure represents the mean number of episodes, mean duration, and total time spent in GA NREM-REM transitions over 3 hours. There were significant differences in the mean number of episodes, mean duration and total time spent in GA NREM-REM transitions with amphetamine compared to saline (n=7, Paired t-test, P> 0.05).
C. Determining the effects of quinpirole on behavioural arrests

Local perfusion studies using quinpirole (D2/D3 agonist) into the VTA and substantia nigra of the dog, greatly exacerbates cataplexy (Honda et al. 1999). Furthermore, peripheral administration of quinpirole (intravenously) also has a potent affect on precipitating cataplexy in the narcoleptic dog (Mignot et al. 1993; Nishino et al. 1991). Our study administered quinpirole to our narcoleptic mice and examined its effects on the different types of behavioural arrests.

**CATAPLEXY:** Figure 3.22 demonstrates that quinpirole increased the number of cataplexy episodes by 248 percent compared to saline but this was not statistically significant (n=7, Paired t-test, P= 0.125). Quinpirole significantly increased the duration of cataplexy by 923 percent compared to saline (n=7, Paired t-test, P=0.013), and it also increased the total time spent in cataplexy by 616 percent compared to saline (n=7, Paired t-test, P= 0.028).

**W-REM TRANSITIONS:** In figure 3.23, there was a trend for quinpirole to reduce the number of W-REM episodes by 40 percent (n=7, Paired t-test, P= 0.308), reduce the duration of W-REM transitions by 54 percent (n=7, Paired t-test, P=0.235), and also reduce the total time spent in W-REM transitions by 18 percent (n=7, Paired t-test, P= 0.756) compared to saline but none of these observations reached statistical significance.

**GA NREM TRANSITIONS:** In figure 3.24, there was a trend for quinpirole to reduce the number of GA NREM episodes by 32 percent (n=7, Paired t-test, P= 0.363), reduce the duration of GA NREM transitions by 53 percent (n=7, Paired t-test, P=0.125), and also reduce the total time spent in GA NREM transitions by 50 percent (n=7, Paired t-test, P= 0.163) compared to saline but none of these observations reached statistical significance.
GA NREM-REM TRANSITIONS: In figure 3.25, there was a trend for quinpirole to reduce the number of GA NREM-REM episodes by 42 percent (n=7, Paired t-test, P= 0.101), reduce the duration of GA NREM-REM transitions by 32 percent (n=7, Paired t-test, P=0.367), and also reduce the total time spent in GA NREM-REM transitions by 44 percent (n=7, Paired t-test, P= 0.080) compared to saline but none of these observations reached statistical significance.
Figure 3.22: This figure illustrates the effects of quinpirole compared to vehicle saline on cataplexy in narcoleptic mice. This figure represents the mean number of episodes, mean duration, and total time spent in cataplexy over 3 hours. There was no significant difference in the mean number of episodes of cataplexy (n=7, Paired t-test, P> 0.05), however there were significant differences in the mean duration and total time spent in cataplexy with quinpirole compared to saline (n=7, Paired t-test, P< 0.05).
Figure 3.23: This figure illustrates the effects of quinpirole compared to vehicle saline on W-REM transitions in narcoleptic mice. This figure represents the mean number of episodes, mean duration, and total time spent in W-REM transitions over 3 hours. There were no significant differences in the mean number of episodes, mean duration, and total time spent in W-REM transitions with quinpirole compared to saline (n=7, Paired t-test, P> 0.05)
Figure 3.24: This figure illustrates the effects of quinpirole compared to vehicle saline on GA NREM transitions in narcoleptic mice. This figure represents the mean number of episodes, mean duration, and total time spent in GA NREM transitions over 3 hours. There were no significant differences in the mean number of episodes, mean duration, and total time spent in GA NREM transitions with quinpirole compared to saline (n=7, Paired t-test, P> 0.05)
Figure 3.25: This figure illustrates the effects of quinpirole compared to vehicle saline on GA NREM-REM transitions in narcoleptic mice. This figure represents the mean number of episodes, mean duration, and total time spent in GA NREM-REM transitions over 3 hours. There were no significant differences in the mean number of episodes, mean duration, and total time spent in GA NREM-REM transitions with quinpirole compared to saline (n=7, Paired t-test, P > 0.05)
Chapter Four:

DISCUSSION
AND
FUTURE DIRECTIONS
4. DISCUSSION & FUTURE DIRECTIONS

The main goal of this thesis was to explore how manipulations of the dopaminergic system regulated sleep and wakefulness, as well as behavioural arrests, in wild-type and narcoleptic mice. Our findings demonstrated that narcoleptic mice had a greater percentage of wakefulness and less NREM sleep than wild-type mice. Narcoleptic mice however, had more REM sleep than wild-type mice consistent with previous studies. We found that amphetamines increased the amount of wakefulness in both wild-type and narcoleptic mice while reducing the amounts of NREM and REM sleep. More importantly, narcoleptic mice did not respond as well as wild-type mice to the effects of amphetamine. Quinpirole did not affect the amount of wakefulness, NREM, or REM sleep in either wild-type or narcoleptic mice.

1. Differences in wakefulness, NREM, and REM sleep between wild-type and narcoleptic mice:

Mochizuki et al. (2004), examined the sleep-wake patterns of narcoleptic mice over a 24 hour period (12 hr light: 12 hr dark), and compared them with wild-type animals. They observed that both genotypes exhibited similar percentages of waking and NREM sleep; however, REM sleep was slightly greater in the narcoleptic animals during the dark phase when mice were active. Our study found (fig 3.1) that the narcoleptic mice were awake more over 3 hours from 7-10 p.m., and exhibited less NREM sleep; however, consistent with Mochizuki et al. (2004), narcoleptic mice had more REM sleep than wild-type mice.

A possible explanation for the increased waking in narcoleptic mice may have resulted from the 3 hour window that we used in observing these animals. Narcoleptic mice have more fragmented sleep patterns compared to wild-type mice. Consider the scenario where wild-type mice sleep while narcoleptic animals are frequently awake as a result of their fragmented sleep pattern. If narcoleptic mice are constantly awake they will continue to be while transitioning into
their active dark phase (7 p.m.). Wild-type mice may just start to wake up at the onset of the active dark phase (7 p.m.) and this may be represented in them exhibiting less wakefulness in the time that our study observed sleep (from 7 p.m. to 10 p.m.). However, this trend may not have been evident over the entire dark phase (12 hours) in which Mochizuki et al. (2004) examined the mice for.

II. Dopaminergic modulation of the sleep-wake cycle in wild-type and narcoleptic mice

Effects of amphetamine during sleep and wakefulness:

Classically, amphetamines have been used to improve wakefulness by manipulating three areas that regulate endogenous levels of dopamine. Amphetamine improves dopamine transmission, inhibits DAT (dopamine active transporter), and inhibits monoamine oxidase (enzyme that inactivates dopamine) (Kanbayashi et al. 2000). While amphetamines affect dopaminergic pathways, they also act on noradrenergic pathways. Studies have shown that the effects of dopamine may be more pronounced in inducing wakefulness than noradrenaline, since dopamine-specific reuptake blockers were more effective at promoting wakefulness compared to noradrenergic reuptake blockers in both wild-type and narcoleptic canines (Lu et al. 2006; Nishino et al. 1998; Nishino and Mignot 1997). Also, studies in the cat have demonstrated that lesioning of the locus coeruleus (disrupting the majority of noradrenaline in the brain), was still able to maintain wakefulness due to the wake-promoting effects of amphetamine (Jones et al. 1977) suggesting a profound dopaminergic role in cortical arousal. Furthermore, Lu et al. (2006), showed that dopaminergic neurons were active during wakefulness in the vPAG (ventral periaqueductal gray) due to Fos expression however, no fos expression was seen in the locus coeruleus (noradrenaline site) (Lu et al. 2006).

Our study demonstrated that the use of the d-amphetamine isoform, which is more potent than the other amphetamine derivative (e.g. l-amphetamine) in stimulating EEG cortical arousal
and wakefulness (Kanbayashi et al. 2000), significantly increased the amount of wakefulness, while also reducing amounts of NREM and REM sleep in both wild-type and narcoleptic mice. This stimulation in arousal and hyperactivity is consistent with other studies in mice using amphetamines (Kitahama and Valatx 1979) as well as studies in canines (Kanbayashi et al. 2000; Shelton et al. 1995; Wisor et al. 2001).

The more salient component of our results, are comparing the effects of amphetamine between wild-type and narcoleptic animals. Figure 3.6 demonstrates that although both wild-type and narcoleptic mice showed increased wakefulness with amphetamine, the narcoleptic mice did not exhibit the robust changes to amphetamine that their wild-type cohorts did. The wake promoting effects of amphetamine were greater and longer lasting in the wild-type mice (despite the fact that our study showed that narcoleptic mice exhibited more wakefulness than wild-type mice on average - fig 3.1).

It is possible that the more potent wake-promoting effects of amphetamine in wild-type mice compared to the narcoleptic animals may result from an intact hypocretin system, which generally excites monoaminergic cell bodies (Siegel and Boehmer 2006). Amphetamine acts not only on dopamine but also on noradrenaline and noradrenaline is also implicated in maintaining wakefulness. Siegel and Boehmer (2006) suggest that hypocretin stimulates arousal through monoaminergic pathways via noradrenergic and dopaminergic innervation. Since narcoleptic mice have reduced abilities to excite noradrenaline and dopamine release due to a lack in the hypocretin ligand, wild-type mice may exhibit longer lasting wake-promoting effects of amphetamine since their non-disrupted hypocretin system may increase the drive for noradrenergic and dopaminergic release and subsequent increase in wakefulness and reduced NREM sleep.
**Effects of quinpirole during sleep and wakefulness:**

Previous studies show that quinpirole increases sleepiness while dopamine antagonists conversely increase arousal in narcoleptic dogs (Nishino et al. 1991). Furthermore, studies in rats and chicks demonstrated that administration of selective D2 agonists (i.e. B-HT 920) also tended to induce sleepiness (Ferrari and Giuliani 1993). As such, we hypothesized that treatments with quinpirole in our narcoleptic mice would increase sleep and reduce wakefulness. Our results showed no significant changes in sleep or wakefulness in either narcoleptic or wild-type animals.

An explanation for the absence of change in sleep or wakefulness with quinpirole may be due to simultaneous agonism of both pre and postsynaptic dopamine D2 receptors. As previously mentioned, D2 agonists perfused to the VTA (rich in dopamine D2 autoreceptors) increased a state of sleepiness (Reid et al. 1996). Also, studies using D2 agonists that have a preferential selectivity for D2 autoreceptors, produced a drowsy state in rats and chicks (Ferrari and Giuliani 1993). Conversely, studies that used intracerebroventricular (i.c.v) applications of D2 agonists produced waking effects and increased locomotion, suggesting a role for D2 postsynaptic receptor agonism as opposed to D2 presynaptic autoreceptor agonism (Isaac and Berridge 2003). Since our route of administering quinpirole was given intraperitoneally, it is possible that the drug acted at both pre and postsynaptic receptor sites on dopamine cell bodies, and that this may simultaneously cancel the effects of sleep and wakefulness in our mice. We know the dose of quinpirole was effective because it increased cataplexy.

**III. Dopaminergic modulation of the different types of behavioural arrests in hypocretin knockout mice**

In addition to observing the effects of dopaminergic manipulations on sleep and wakefulness, we wanted to explore the effects of dopamine on the different types of behavioural arrests in narcoleptic mice. We initially demonstrated that stressing the animals during our experiments produced a trend for increasing cataplexy as well as increasing GA NREM
transitions. Treatments with amphetamine reduced cataplexy as well as all other forms of “sleep attacks” (W-REM, GA NREM, and GA NREM-REM transitions). Conversely, we found that quinpirole generally increased cataplexy; however, there was a trend for reducing the three other types of sleep attacks.

One of the most unique methods of our study in narcoleptic mice is the fact that we observed spontaneous occurring cataplexy. The symptom of cataplexy is elicited due to strong emotional stimuli (Siegel and Boehmer 2006). For example, cataplexy in human narcoleptics is predictably elicited with strong emotions such as laughter (Siegel and Boehmer 2006). Cataplexy in the narcoleptic canine was induced by play and food. Quantifying cataplexy in the dog involves the FECT (Food-Elicited Cataplexy Test) in which observers use 12 pieces of food presented in a circular arrangement, and count the number of cataplexy episodes as well as the duration of each episode taken for each canine to complete the test (eat all 12 pieces of food) (Kanbayashi et al. 2000; Shelton et al. 1995). Narcoleptic mice that are housed together (allowed to interact and play) exhibit more cataplectic attacks than mice housed individually (Chemelli et al. 1999). Our study did not stimulate cataplexy with emotional stimuli and thus the degree of behavioural arrests that the narcoleptic mice exhibited were spontaneous and unpredictable. However, our study aimed to demonstrate whether emotional stress of handling the animal affected cataplexy and the other three forms of sleep attacks.

The effects of stress on behavioural arrests:

During our study, we tested the effects of handling an animal (injection with vehicle saline) versus baseline (ie. unhandled). This was performed to determine whether stress had an effect on behavioural arrests. Although our study found a significant difference between handled versus unhandled animals during the sleep attack (GA NREM transition – fig 3.16), we were more interested in cataplexy, since it is induced by strong emotions and stress is an emotional stimulus. We observed a trend for increased cataplexy with the number of episodes (ie. P=
0.156), duration (ie. P= 0.091), and total time spent in cataplexy (ie. P= 0.088) (fig 3.14); however, this did not reach significance. It is possible that a larger sample size of mice may generate the statistical significance for increasing cataplexy when a stress (emotional stimuli) is subjected to a narcoleptic individual. Our predictions are based on the fact that during our study, we noticed that there was high variability between animals in the number of cataplexy events each animal exhibited. This is also consistent with the study by Chemelli et al. (1999) using these narcoleptic mice. Therefore, a larger sample size may dampen the high variability found when using a small sample size of highly variable animals. This is also reinforced by the power value in our experiment (0.175).

**Effects of amphetamine on cataplexy:**

Although amphetamines are useful in the treatment of daytime sleepiness, they also suppress cataplexy in the narcoleptic canine (Kanbayashi et al. 2000; Shelton et al. 1995). Our study also demonstrated a similar effect with amphetamine on cataplexy in narcoleptic mice. Although amphetamine did not reduce the number of episodes of cataplexy, we found that it reduced the duration as well as the total time spent in cataplexy (fig 3.18). However, our study measured spontaneous cataplexy, while the canine model measured food-elicited cataplexy. The number of cataplexy episodes may not have reached significance based on the fact that we looked at cataplexy as it appeared naturally. However, the fact that the duration of cataplexy, as well as the total time spent in cataplexy, was reduced with amphetamine suggests that amphetamine reduces cataplexy through a mechanism similar to the canine model of narcolepsy. It appears that the reduction in cataplexy is modulated through the enhancement of similar monoaminergic transmission pathways in both the mice and dog despite the fact that the models result from different changes in hypocretin – mice deficient in the hypocretin ligand while the dog possesses a deficient receptor for the hypocretin ligand.
Effects of amphetamine on sleep attacks:

In addition to examining cataplexy, the other forms of behavioural arrests or “sleep attacks” (W-REM, GA NREM, and GA NREM-REM) can be considered markers for sleepiness. Willie et al. (2003), in one of their initial studies, characterized behavioural arrest states using both hypocretin knockout mice as well as hypocretin receptor-2 deficient mice (similar to narcoleptic canines). They reported similar findings as our study did and distinctly characterized two types of behavioural arrests: abrupt and gradual in both genotypes of mice.

Our study demonstrated that treating our hypocretin knockout mice with amphetamine significantly reduced the amount of W-REM sleep attacks, as well as both types of gradual arrests. Interestingly, Willie et al. (2003) found that treating their mice with two different drug types clomipramine (anticataplectic agent) and caffeine (psychostimulant to treat sleepiness), produced distinct reductions in each type of behavioural arrest. Clomipramine reduced the frequency of abrupt arrests such as cataplexy and W-REM transitions with no augmentation of gradual arrests. On the other hand, caffeine reduced the frequency of gradual arrests without changing the frequency of abrupt arrests. When both drugs were administered together, both types of arrests (abrupt and gradual) were reduced suggesting the mechanisms of both drugs are independent.

Our study demonstrated that amphetamine reduced both abrupt and gradual arrests; however, when we look closer at Willie et al. (2003), we see that their drugs functioned by manipulating different neurotransmitters. Clomipramine is a tricyclic antidepressant used to treat cataplexy and does so via a monoaminergic uptake blocker with heavy emphasis on reducing noradrenaline reuptake (Mignot and Nishino 2005). Caffeine is a psychostimulant that treats sleepiness via the blockade of adenosine receptors that elevates dopamine transmission in the CNS (Cauli and Morelli 2005).
Amphetamines have been shown to reduce cataplexy as well as sleepiness as previously mentioned. Amphetamine reduced nearly all types of behavioural arrests. Enhancement of dopaminergic transmission appears to be important in mediating these four types of behavioural arrests.

**Effects of quinpirole on cataplexy:**

Studies in the narcoleptic canine have demonstrated that cataplexy is modulated through dopaminergic D2 receptors. D2 agonists aggravate cataplexy while dopaminergic antagonists reduce it (Honda et al. 1999; Mignot et al. 1993; Nishino et al. 1991). Quinpirole (D2/D3 agonist) has been shown in narcoleptic dogs to increase cataplexy when administered centrally to dopaminergic cell bodies in both the ventral tegmental area (VTA) (Okura et al. 2004; Reid et al. 1996) and the substantia nigra (Honda et al. 1999). Peripheral administration of quinpirole also induced cataplexy in the narcoleptic dog (Mignot et al. 1993; Nishino et al. 1991).

Our experiments demonstrated that treatments with quinpirole also increased cataplexy. Quinpirole produced a trend of increasing the number of episodes; however, this was not statistically significant (i.e. P=0.105). Much in the same way that amphetamine did not reach significance in reducing the number of cataplexy episodes, this could have possibly been due to spontaneously occurring cataplexy in the mice as opposed to emotionally elicited cataplexy in the dog. However, the duration as well as the total time spent in cataplexy was largely increased with quinpirole and is consistent with the canine studies (Okura et al. 2004).

Apart from many studies in the narcoleptic canine that focused on the VTA and substantia nigra as the major constituents of dopamine in the CNS, it was shown that when dopaminergic D2 agonist and antagonist were administered directly to the periventricular gray (PVG) area that constitutes the A11 dopamine cell group (projecting directly to spinal motor neurons), that cataplexy increased and decreased respectively (Okura et al. 2004). As such, it is
possible that dopaminergic D2 receptors modulate cataplexy via dopamine projections that
directly modulate motor neuron activity.

**Effects of quinpirole on sleep attacks:**

Unlike cataplexy, quinpirole had no effect on sleep attacks; a result consistent with the
observation that quinpirole had no effect on sleep itself. It was previously suggested that
quinpirole did not affect sleep due to the simultaneous agonism of pre and postsynaptic
receptors. This same mechanism may be related to why sleep attacks were unchanged since sleep
attacks are essentially representations of sleep. Therefore, although D2 receptor agonism
increased cataplexy, D2 receptor dynamics do not seem to be involved in sleep attacks. This
phenomenon is reinforced by studies where D2 receptor antagonism reduces cataplexy, but fails
to change sleep (Okura et al. 2000).

**Future Studies:**

There are a number of topics to consider for future studies. Firstly, dopaminergic
modulation of muscle activity at the level of spinal motor neurons should be investigated. Since
the A11 dopamine cell group projects directly to the spinal cord and that dopaminergic D2
agonists and antagonists increase and decrease cataplexy at the level of the A11 dopamine cell
group (Okura et al. 2004), it is suggested that D2 receptors modulate muscle control at the level
of the spinal cord. Lu et al. (2006) measured the activity of these A11 dopamine neurons during
sleep and wakefulness, but their activity during cataplexy is not yet known.

What should also be considered for future studies is examining the innervations between
dopamine cell groups such as the VTA and substantia nigra with the locus coeruleus (major site
for noradrenaline in the CNS). Studies have shown that modulation of dopamine cell groups such
as the VTA and substantia nigra either excite or inhibit locus coeruleus activity (Honda et al.
1999; Okura et al. 2004). This suggests that augmenting dopamine neuron activity, secondarily
modulates locus coeruleus noradrenergic neurons. Since noradrenaline is critically implicated in
sleep and motor control (LC projects directly to the spinal cord), it may be important to study the connections between dopamine and noradrenergic cell groups and how they both may interact to regulate muscle tone during cataplexy.

**Conclusions:**

In summary, the results from our experiments developed a number of conclusions: (1) The emotional stress of handling a narcoleptic animal can increase cataplexy during behavioural experiments (2) Amphetamine increased wakefulness and decreased NREM and REM sleep in both wild-type and narcoleptic mice with more potent effects in wild-type littermates. This may be a result of a fully functional hypocretin system in wild-type mice that excites dopaminergic and noradrenergic cell groups that narcoleptic mice are devoid of. (3) Quinpirole did not affect sleep or wakefulness in either wild-type or narcoleptic mice, suggesting that both pre and postsynaptic D2 receptors are being simultaneously agonized to dampen the effects of any changes in sleep or wakefulness (4) Amphetamine reduced cataplexy and sleep attacks suggesting that these 4 behavioural arrests are regulated by increasing dopamine transmission. (5) Quinpirole increased cataplexy in narcoleptic mice similar to the narcoleptic dog which suggests a similar dopamine D2 receptor mechanism of eliciting muscle atonia despite their different forms of narcolepsy (dog with mutated receptor and mice with a deficiency in the ligand). (6) Quinpirole did not affect sleep attacks consistent with quinpirole failing to affect sleep.
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