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Catecholamines and Arousal

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Catecholamines and Arousal

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An abstract of
A dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
in Graduate Division of Biological and Biomedical Science
Molecular and Systems Pharmacology
2010

Abstract

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By Heather A. Mitchell

The catecholamines norepinephrine (NE) and dopamine (DA) are involved in the initiation and maintenance of arousal states. Arousal is primarily associated with sleep and wake, but also includes behaviors such as locomotor activity and aggression. Sleep is a crucial process that is found in all animals studied to date, from humans to flies, and is regulated through a complex network of neuromodulatory systems. Despite such detailed regulation, sleep and arousal disorders are common, and are treated with a wide variety of pharmacotherapies, many of which require catecholaminergic mechanisms for their efficacy. A common symptom of depression is a disturbance in arousal levels, and this can often be exacerbated by antidepressant medications. In order explore the role of NE in antidepressant-induced changes in arousal, we administered several antidepressants that inhibit the NE transporter (NET) and increase extracellular NE levels, and measured locomotor behavior. When administered either acutely or chronically, NET blocking antidepressants decreased locomotor behavior, unless combined with DA transporter (DAT) blockade (e.g. the dual NET/DAT inhibitor antidepressant bupropion). To further investigate the importance of catecholamines in arousal pharmacology, we examined modafinil, a commonly-prescribed treatment for narcolepsy and excessive sleepiness. While the molecular mechanism for modafinil is unknown, previous research has suggested that both NE and DA are involved in its wake-promoting actions. We used mice lacking NE, in combination with antagonists for both dopaminergic and adrenergic receptors, to show that complex NE/DA interactions likely underlie modafinil efficacy. Finally, we characterized behavior in a mouse model for Lesch-Nyhan Disease (LND), a devastating condition with symptoms of self-injurious behavior and losses of DA in the brains of human patients. We found that this mouse model had "hyperarousal-like" alterations similar to those found in human patients, as measured by increased aggression and a unique form of amphetamine-induced stereotypy. These studies used diverse models to explore the importance of catecholamines in basal and pharmacologically-induced arousal states.

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Acknowledgements

Over the last six years in graduate school I have received help and guidance from a number of people. First and foremost, I would like to acknowledge my mentor, Dr. David Weinshenker. I could not imagine a better PI than David; he has always been enthusiastic about both science and mentoring his students, and has given me great encouragement throughout my tenure in his lab. Not only have I learned to be a better researcher from David, but under his guidance I have become a stronger writer and presenter as well.

I would also like to thank my thesis committee for their insight and many helpful suggestions over the years: Dr. Joseph Cubells, Dr. Randy Hall, Dr. Michael Iuvone, and Dr. David Rye. Many collaborators have also been valuable resources, particularly Dr. Glenda Keating in Dr. Rye's lab for assistance with the rat EEG experiments, and Dr. James Thomas, Dr. Alaine Keebaugh and Jamie Davis for allowing me to participate in the Lesch-Nyhan Disease project. The Molecular and Systems Pharmacology graduate program and the Department of Human Genetics both have been extremely helpful, and created a wonderful environment for learning and collaborations.

The members of the Weinshenker lab, both past and present, have made the last six years not only an educational experience, but a fun one, and I've thoroughly enjoyed spending my days with them. Past members include: Karen Rommelfanger, Jesse Schank, and Kroshona Tabb, and current members are: Debra Cooper, Alisha Epps, Meriem Gaval, Canaan Jerome, Darlene Mitrano, Yvonne Ogbonmwan, and Jason Schroeder.

No research can occur without funding, and I would like to acknowledge mine from the Molecular and Systems Pharmacology Training Grant T32 GM 008602 and Cephalon Pharmaceuticals. I have also been supported by an ARCS Foundation Fellowship.

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Abbreviations

5-HT	serotonin
AD	Alzheimer's disease
ADHD	attention deficit/hyperactivity disorder
AR	adrenergic receptor
BDNF	brain-derived neurotrophic factor
CRIN	Clinical Research In Neurodegeneration
DA	dopamine
DAT	dopamine transporter
DBH	dopamine β hydroxylase
DOPS	L-3,4-dihydroxyphenylserine
DRN	dorsal raphe nucleus
DSP-4	N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine
EEG	electroencephalogram
EPI	epinephrine
GPCR	G-protein coupled receptor
HPRT	hypoxanthine-guanine phosphoribosyltransferase
KO	knockout
LC	locus coeruleus
LH	lateral hypothalamus
LND	Lesch-Nyhan disease
MAOI	monoamine oxidase inhibitor
MPOA	medial preoptic area
MSA	medial septal area
NE	norepinephrine
NET	norepinephrine transporter
PD	Parkinson's disease
PFC	prefrontal cortex
PRTFDC1	phosphoribosyl transferase domain containing protein
REM	rapid eye movement
RLS	restless legs syndrome
SCN	suprachiasmatic nucleus
SERT	serotonin transporter
SLA	spontaneous locomotor activity
SNP	single nucleotide polymorphism
SSRI	selective serotonin reuptake inhibitor
TCA	tricyclic antidepressant
TMN	tuberomammillary nucleus
VLPO	ventrolateral preoptic area
vPAG	ventral periaqueductal grey
WGA	whole genome amplification
WT	wildtype

CHAPTER 1:

CATECHOLAMINERGIC PHARMACOLOGY AND AROUSAL

Adapted from: Mitchell HA, Weinshenker D. Good Night and Good Luck: Norepinephrine in Sleep Pharmacology. Biochem Pharmacol. 2010 Mar 15;79(6):801-9.

1.1 Abstract

Sleep and arousal states are crucial biological processes that are regulated through complex interactions between multiple brain regions and neuromodulators. As disorders of these systems can have deleterious impacts on health and quality of life, a wide variety of pharmacotherapies have been developed to treat conditions of heightened arousal and excessive sleepiness. The neurotransmitters norepinephrine (NE) and dopamine (DA) impact the efficacy of many wake- and sleep-promoting medications. Wake-promoting drugs such as amphetamine and modafinil increase extracellular levels of NE and DA, enhancing transmission along the wake-promoting pathway. Cocaine exerts its stimulant effects through a combination of dopaminergic and noradrenergic systems. While caffeine, the most popular psychoactive stimulant, inhibits adenosine receptors and does not directly act on NE or DA, there is considerable interaction between the adenosine system and DA. Many antidepressants that affect the signaling of NE are also used for treatment of insomnia. Therefore, both NE and DA are shown to be crucial players in both arousal and sleep pharmacology.

1.2 Sleep and arousal states

Sleep is one of the most universal biological processes in existence. It is highly conserved, and creatures from Drosophila melanogaster and Caenorhabditis elegans to humans experience at least some form of it (Cirelli and Tononi, 2008). Depriving an organism of sleep altogether can be extremely detrimental, and may even lead to death (Rechtschaffen et al., 1989). Sleep is therefore considered necessary for life, but why this is so remains unclear. Sleep is subdivided into rapid eye movement sleep (REM), which is characterized by high-frequency electroencephalogram (EEG) recordings and muscle atonia (Remy et al., 2005), and non-REM (slow-wave) sleep, characterized by low frequency EEG recordings and body rest (Stenberg, 2007). Arousal, which could be considered the converse of sleep, is a state of wakefulness, responsiveness to sensory stimulation or excitability, or readiness to make behavioral responses. Therefore, arousal can manifest in a variety of ways, including attention, cognitive performance and activity levels.

1.3 Disorders of the arousal system

Although sleep and arousal are tightly controlled processes orchestrated by multiple regulatory systems, disorders do occur, some as a result of disruptions in sleep circuitry, some secondary to other conditions, and others as a result of modern lifestyles. Sleep complaints are in fact the second-leading cause for seeking medical attention, after pain (Colten and Altevogt, 2006). Sleep disorders can be categorized broadly into conditions of excessive wakefulness (e.g., insomnia) and excessive sleepiness (narcolepsy, shift-work disorder, jet lag). Insomnia is defined as difficulty falling asleep and maintaining adequate sleep, and is the most commonly reported sleep problem in the United States. In addition to the associated lack of nighttime sleep, there are a host of daytime consequences, as well, such as tiredness, stress, and attentional deficits (Colten

and Altevogt, 2006). Conversely, narcolepsy and hypersomnias involve excessive daytime sleepiness and sleep attacks or unintended napping. This can have a profound impact on an individual's ability to function and limits even basic tasks like driving a car (Colten and Altevogt, 2006). In addition to the effects these conditions have on sleep itself, problems such as cardiovascular disease and diabetes are often worsened by insufficient sleep (Knutson et al., 2007). Furthermore, patients with conditions from depression to Parkinson's and Alzheimer's diseases often experience co-morbid sleep disturbances (Adolfsson et al., 1979; Comella, 2006; Sanchez et al., 2007).

Moreover, conditions of heightened arousal can also be detrimental. Attention-deficit/hyperactivity disorder (ADHD), schizophrenia, and aggression could all be considered extreme versions of arousal, and many drugs of abuse also induce a hyperaroused state. ADHD affects about 5% of children worldwide, and manifests with behavioral symptoms of inattention, hyperactivity, and impulsivity (Genro et al., 2010). Interestingly, this condition is frequently comorbid with sleep disturbances (Tsai and Huang, 2010). Difficulties with falling asleep and maintaining sleep are also common in schizophrenia, a condition that is characterized in part by delusions and social withdrawal (Cohrs, 2008; Simpson et al., 2010). Thus, medications to treat arousal disorders are a necessity in our society, and understanding the pathways and neurotransmitter systems that regulate sleep and arousal is crucial.

1.4 Regulation of arousal states

Sleep is a global process, controlled by regions throughout the brain and by multiple neurotransmitters and neuropeptides. The first studies that attempted to localize regions of the brain responsible for sleep maintenance were conducted in the early twentieth century by von Economo, who noticed correlations between sleeping sickness and lesions in certain brain areas (Reid et al., 2001). He reported that patients who had encephalitis lethargica and slept for 20

hours a day had lesions at the base of the midbrain, and he hypothesized that this site might be the origin for an ascending arousal pathway. Subsequent studies revealed an arousal system with two main branches (reviewed in (Saper et al., 2005)). The first branch begins in the cholinergic neurons of the pedunculopontine and laterodorsal tegmental nuclei and projects up, relaying through the thalamus, and into the cortex; the second begins in monoaminergic neurons, the noradrenergic locus coeruleus (LC), dopaminergic neurons in the ventral periaqueductal grey (vPAG), the serotonergic dorsal raphe nucleus (DRN), and also the histaminergic tuberomammillary nucleus (TMN), and progresses to the cerebral cortex. These neurons also receive inputs from orexin neurons in the perifornical area of the lateral hypothalamus (Carter et al., 2009). The orexins are especially important for controlling transitions between sleep and wake and between stages of sleep, and as a result, disruptions in this system cause narcolepsy (Lin et al., 1999). These two branches also interact with the circadian pacemaker of the brain, the suprachiasmatic nucleus (SCN), mainly via relay through the dorsal medial hypothalamus (Aston-Jones et al., 2001; Pace-Schott and Hobson, 2002). This provides the intersection of the homeostatic regulation of sleep, based on accumulated sleep drive or tiredness, and circadian regulation of sleep, based on a 24-hour cycle set by the SCN via integration of light inputs from the retina.

While the cholinergic and monoaminergic systems act to promote wakefulness in conjunction with the orexins, there are other neuronal groups that act to promote sleep. The primary population of sleep-promoting neurons is located in the preoptic area, specifically the ventrolateral preoptic area (VLPO). These neurons express c-Fos protein during sleep, a marker of neuronal activation, and have elevated discharge rates specifically during sleep, and lesions to this area prevent sleep (Lu et al., 2000; Sherin et al., 1996; Szymusiak et al., 1998). In addition, these VLPO neurons project to, and share mutual inhibition with, the neuronal groups involved in the ascending arousal pathways (Chou et al., 2002; Gallopin et al., 2000). The majority of these cells are GABAergic, and some also contain enkephalin or galanin (Gallopin et al., 2000; Gaus et

al., 2002). The interaction of the VLPO neurons with the ascending arousal systems provides what has been described by Saper and colleagues as a “flip-flop switch” that regulates and controls transitions between sleep and wake (Saper et al., 2005).

1.5 Norepinephrine and arousal

As described above, NE is one of the main neurotransmitters involved in arousal. LC neurons fire in a wake-dependent manner; they are highly active during wake, slow-firing during non-REM sleep, and almost completely quiescent during REM sleep (Aston-Jones and Bloom, 1981). The A1 and A2 brainstem noradrenergic nuclei also provide input to regions of the hypothalamus known to be involved in sleep regulation (Berridge, 2006). Pharmacological suppression of LC activity leads to sedation and drives forebrain EEG recordings into sleep-like patterns. We know that dopamine β-hydroxylase knockout (*Dbh -/-*) mice, which lack norepinephrine, have altered sleep and arousal patterns. They show decreased latency to sleep after stress, require stronger stimuli to wake them after sleep deprivation, and have increased overall sleep, albeit with less REM, in a 24-hour period (Hunsley and Palmiter, 2003; Hunsley and Palmiter, 2004; Ouyang et al., 2004). In addition, these mice exhibit decreases in locomotor behavior when placed in a novel environment, indicating deficits in arousal, though they are hypersensitive to the stimulant effects of amphetamine and cocaine (Schank et al., 2006; Weinshenker et al., 2002a).

Pharmacological studies have revealed robust wake-promoting effects of α1- and β-adrenergic receptor (AR) agonists when administered to the medial septal area (MSA) and the medial preoptic area (MPOA), both wake-promoting regions, reviewed in detail by Berridge (Berridge, 2006; Berridge, 2008). Conversely, blockade of ARs results in sedation. These effects appear to be mediated mostly by antagonism of α1ARs, though there are synergistic effects when combined with βAR antagonists. While one group has found that microinjection of the α1AR

antagonist prazosin into the MPOA induces sleep rather than wake, the magnitude of changes in wake time were relatively small and depended on ambient temperature (Kumar et al., 2007; Vetrivelan et al., 2005). Thus, it seems likely that NE primarily acts through α1ARs in the MPOA to increase wake, although stimulation of α1ARs in this brain region may be sedative under some conditions.

1.6 Dopamine and arousal

The other catecholamine, DA, is also important for arousal. While DA was not originally thought to be involved in regulation of sleep/wake, recent evidence indicates that it has a role. Unlike noradrenergic neurons, the firing rate of dopaminergic neurons does not significantly change over the course of the sleep-wake cycle, although DA release increases during REM sleep (Maloney et al., 2002; Miller et al., 1983). Dzirasa further explored the role of DA in sleep through studies in mice with either increased extracellular DA (dopamine transporter knockout (DAT KO)) or depletion of DA (DAT KO mice treated with a tyrosine hydroxylase inhibitor that prevents DA production) (Dzirasa et al., 2006). It was found that hyperdopaminergic DAT KO mice exhibit a wake state with EEG properties similar to REM, while DA-depleted mice display a wake state similar to slow wave sleep, with complete suppression of REM. Furthermore, there is a population of dopaminergic neurons in the vPAG that is wake-active and has reciprocal projections with other neuronal groups in the ascending arousal system (Lu et al., 2006).

The impact of DA on sleep appears to be primarily mediated through D2-family DA receptors. Dzirasa noted that haloperidol, a D2 antagonist, attenuated the impact of genetic increases in extracellular DA on atypical EEG activity, and that D2 agonists can restore REM sleep in DA-depleted mice (Dzirasa et al., 2006). In humans, sleep deprivation increased binding of DA to D2 receptors, as measured by decreased competitive binding of the radioligand raclopride, and these changes correlated with feelings of fatigue and impaired performance on a

visual attention task (Volkow et al., 2008). Mice lacking D2 receptors display decreased wakefulness, and increased sleep fragmentation (Qu et al., 2010).

The following sections will discuss various medications that affect the arousal system, and how the catecholamines impact their mechanisms of action.

1.7 Stimulants

Stimulants are among the most commonly used pharmacotherapies in our society. Patients suffering from conditions of excessive daytime sleepiness, such as narcolepsy and shift-work sleep disorder, are often prescribed modafinil or amphetamine, which are also used off-label and recreationally. Other frequently abused stimulants include methylphenidate and cocaine. Caffeine, ubiquitous in coffee, soda, and energy drinks, is the most popular psychoactive substance in the world. While all these compounds work through diverse mechanisms, NE and/or DA appear to contribute, either directly or indirectly, to their wake-promoting activities.

Amphetamines

Amphetamines were developed in 1927 and first used to treat narcolepsy in 1935 (Banerjee et al., 2004). The amphetamines include several compounds, each with its own wake-promoting efficacy and abuse liability. Those typically used to treat excessive sleepiness are dexamphetamine (Dexedrine) and methylphenidate (Ritalin), though methylphenidate is most commonly used for ADHD. Amphetamines are highly successful at promoting wakefulness, but they possess several unpleasant and even dangerous side effects that limit their usefulness. Amphetamines induce sympathomimetic effects, such as increased heart rate and blood pressure, and are also known for their addictive properties. Despite these caveats, amphetamines have remained a firstline treatment for conditions of excessive sleepiness, such as narcolepsy and phase-shift disorder.

Amphetamine has been shown to increase wakefulness in rats when administered either acutely or chronically (Andersen et al., 2009). When given acutely, amphetamine increased sleep latency, and decreased the amount of sleep and sleep efficiency compared with saline, as measured by EEG. Furthermore, it eliminated REM sleep for the duration of the three-hour test session. When administered chronically (every day for seven days), amphetamine decreased sleep efficiency on the first day, but efficiency improved gradually over subsequent days. Similarly, whereas amphetamine drastically reduced slow-wave sleep on the first day of testing, this decrease was attenuated over subsequent days, indicating the development of a tolerance to its wake-promoting effects. Sleep latency was increased by amphetamine on each day of the test. In addition to decreasing sleep, amphetamine also increases locomotor behavior and arousal, which manifests as stereotypy at higher doses (Roffman and Raskin, 1997). Like amphetamine, methylphenidate also increases locomotor behavior in rats, with evidence of stereotypy at very high doses, and many studies indicate that these effects increase over time (reviewed in (Askenasy et al., 2007)).

In humans, both dexamphetamine and methylphenidate increase sleep latency and improve self-reported sleepiness (Banerjee et al., 2004). Dexamphetamine reduces total sleep, both REM and slow-wave, as measured by EEG, and decreases sleep efficiency. It is effective at sustaining wake in military pilots, in addition to its more traditional use as a narcolepsy treatment. However, administration of amphetamines typically results in rebound hypersomnolence when the individual is allowed to sleep, and because of the associated drop off in sleep efficiency, individuals usually feel residual sleepiness. Furthermore, users can develop a tolerance to both methylphenidate and low-dose dexamphetamine (Ross et al., 2002; Strakowski et al., 2001). There is evidence that amphetamine increases aggression in humans; although the exact mechanism is still unknown, it may be due to impaired impulse control combined with paranoia (Dawe et al., 2009). As a result of its euphoric and stimulant effects, amphetamine is frequently

abused, complicating its use for arousal disorders. Similarly, methylphenidate is taken illicitly, particularly by high school and college students (Bogle and Smith, 2009).

Amphetamine and methylphenidate act on the monoamine systems to facilitate release and block reuptake of serotonin (5-HT), DA, and NE. As described, all three monoamines are known to play a role in the regulation of arousal state. The DRN, the LC, and DA neurons in the vPAG are primary components of the ascending arousal system. Elevated extracellular levels of these neurotransmitters promote wakefulness through excitatory actions on the ascending arousal pathway, while simultaneously preventing sleep by inhibiting the sleep-promoting neurons of the VLPO.

NE is a particularly important component because, aside from wake-promoting effects of its own, it also acts to facilitate the firing of DA neurons, thereby increasing their arousing effects (Weinshenker and Schroeder, 2007). Furthermore, the LC has reciprocal connections with 5-HT neurons in the DRN (Kim et al., 2004). The importance of NE is underscored by several findings. For example, the wake-promoting effects of low-dose amphetamine observed in wildtype mice is abolished in *Dbh -/-* mice that specifically lack NE (Hunsley and Palmiter, 2004). Direct infusion of low-dose amphetamine into sleep-related adrenergic projection fields containing both α1 and β ARs increases wakefulness and arousal, as measured by EEG (Berridge, 2006). Microdialysis studies have shown a correlation between NE release in the prefrontal cortex (PFC), which receives direct innervation from the LC, and the time spent awake after administration of low-dose amphetamine. Central infusion of βAR antagonists in anesthetized animals can completely block the effects of low-dose amphetamine on wake, although no effect is seen in unanesthetized rats. This potential discrepancy could be evaluated further in normally sleeping animals, and the addition of α1AR antagonists may yield more insight. Combined, these results indicate that facilitation of NE release is critical for the wake-promoting actions of amphetamine.

DA is also crucial for the impact of the amphetamines on arousal. In addition to being an important component in the ascending arousal system, DA is well known to be responsible for

modulating locomotor activity, which is frequently used as an index of arousal in behavioral paradigms. Furthermore, DA influences types of heightened arousal such as abuse of these stimulants and aggressive behaviors (de Almeida et al., 2005). ADHD, considered a type of hyperarousal, is thought to be the result of impaired DA signaling, though the exact nature of this dysfunction has not yet been elucidated, and other mechanisms may be involved (Oades et al., 2005; Tripp and Wickens, 2009). Though methylphenidate affects both the noradrenergic system and the dopaminergic system, it is suggested that its ability to ameliorate the symptoms of ADHD are due to its dopaminergic modulation (Tripp and Wickens, 2009). Effects of amphetamine can be attenuated by DA receptor antagonists, administered either acutely or chronically (Dunn and Killcross, 2006). In addition, infusion of the D1/D2 receptor antagonist into the prefrontal cortex of rats completely blocked amphetamine-induced locomotor behavior (Bast et al., 2002).

Cocaine

Cocaine was initially produced from the coca plant of South America, and in that form has been used as a stimulant for over 1000 years (reviewed in (Johanson and Fischman, 1989)). In the last 100 years, the active ingredient was isolated, synthesized, and while it was originally used as a supplement and a treatment for a variety of ailments, it is currently most often used illicitly as a recreational drug.

The acute effects of cocaine on sleep are similar to those of amphetamine and include longer sleep latency, decreased total sleep time, and decreased REM (Boutrel and Koob, 2004; Schierenbeck et al., 2008). Insomnia is a frequent adverse effect of cocaine. In addition, much like amphetamine, cocaine also increases locomotor activity in animal models. Abuse of cocaine in humans leads to sleep disturbances, most frequently fragmented sleep and alterations in REM (Valladares and Irwin, 2007). Even during abstinence from cocaine, some of these disturbances continue. However, modafinil is able to normalize sleep architecture and decrease daytime sleepiness in cocaine-dependent subjects (Morgan et al., 2010). Modafinil has also been shown to

be effective at reducing cocaine intake in cocaine-dependent individuals, and this may indicate that improving sleep quality is an important factor in that reduction (Dackis et al., 2005; Hart et al., 2008).

Cocaine acts by inhibiting the monoamine transporters, and thereby increasing extracellular concentrations of DA, NE, and 5-HT. All of these neurotransmitters are important for arousal, and their primary nuclei are parts of the ascending arousal pathway. The locomotor effects of cocaine seem primarily due to its inhibition of DAT. Cocaine-induced locomotor behavior is normal in NE transporter (NET) and SERT KO mice, but DAT KO mice are nonresponsive to acute cocaine (Hall et al., 2009). DAT KO mice also exhibit decreased self-administration of cocaine, indicating that DA is also responsible for at least some of the rewarding effects of this drug (Thomsen et al., 2009). However, while DAT blockers such as JHW007 can occlude the locomotor effects of cocaine (Desai et al., 2005), cocaine-induced locomotion is also significantly decreased in mice lacking the α1bAR (Drouin et al., 2001). Furthermore, when pretreated with an α1AR antagonist, mice displayed decreased development and expression of cocaine sensitization (Jimenez-Rivera et al., 2006). In-depth studies of cocaine on sleep utilizing EEG monitoring have not been conducted.

Modafinil

Modafinil (Provigil) was first developed in the early 1990s and was approved for the treatment of narcolepsy in the United States in 1998. Since then, it has become the most prescribed medication in the United States for treatment of excessive daytime sleepiness; it is also used off-label for conditions as diverse as ADHD and cocaine dependence. One reason for the success of modafinil is its lack of negative side effects that are common to other medications used to treat excessive sleepiness. For example, unlike amphetamines, modafinil does not induce sympathomimetic effects, does not cause sleep rebound, and has low abuse liability (Ballon and Feifel, 2006; Edgar and Seidel, 1997; Minzenberg and Carter, 2007).

Initial animal studies revealed that modafinil increased wake in a dose-dependent manner, with no signs of sleep rebound, as measured by EEG recordings (Edgar and Seidel, 1997; Lin et al., 1992). Amphetamine, in comparison, also dose-dependently increased wake, but with a subsequent sleep rebound period and considerable side effects like hyperactivity (Lin et al., 1992). When compared with methamphetamine in rats, modafinil increased wake to a similar extent, but with longer bouts of sustained wakefulness and less marked locomotor effects (Edgar and Seidel, 1997). In humans, modafinil is approved to treat excessive daytime sleepiness associated with narcolepsy, shift-work sleep disorder, and obstructive sleep apnea (reviewed by (Ballon and Feifel, 2006)). In several trials for narcolepsy, modafinil decreased daytime sleepiness without affecting nighttime sleepiness, and when tested for shift-work sleep disorder, it increased sleep latency during nighttime shifts, enabling patients to stay awake when necessary. Modafinil is also well tolerated by patients who experience sleepiness secondary to other conditions or illnesses. When compared with amphetamine, volunteers given modafinil showed less need for recovery sleep after sleep deprivation and fewer sleep disturbances with no REM sleep deficit (Buguet et al., 1995). Further, modafinil has been shown to boost performance and alertness to a similar level as caffeine (Wesensten et al., 2002).

Despite the high prevalence of its use, however, the exact molecular mechanism underlying modafinil's efficacy has yet to be elucidated. An early screen of possible targets revealed only low-affinity binding to the DAT. Subsequent studies confirmed this, and also found potential binding to the NET (Madras et al., 2006; Mignot et al., 1994; Zolkowska et al., 2009). Moreover, DAT knockout, α1AR antagonists or knockout, and DA receptor antagonists blunt modafinil-induced arousal, highlighting the importance of catecholamine signaling (Stone et al., 2002; Wisor et al., 2001).

As described above, both NE and DA are important components of the ascending arousal pathway, and both are important for modafinil efficacy. Modafinil activates the LC in animal models and in humans (Fiocchi et al., 2009; Hou et al., 2005; Minzenberg et al., 2008). NE

neurons in both the LC and in the A1/A2 groups form reciprocal connections with the sleep-promoting neurons of the VLPO and decrease VLPO neuron firing via presynaptic α2ARs (Chou et al., 2002; Matsuo et al., 2003). Wake-active DA neurons in the vPAG also project to the VLPO (Jones et al., 1985; Lu et al., 2006). Importantly, these vPAG neurons express and are activated by α1ARs (Jones et al., 1985; Pieribone et al., 1994). These data have led us to propose a model in which modafinil blocks both DAT and NET, thereby increasing extracellular DA and NE, which then act downstream. We believe that the NE could promote wake in several different ways in parallel: (1) by providing excitatory input on wake-promoting DA neurons via α1ARs, (2) by activating wake-promoting neurons in the hypothalamus via α1ARs and βARs, and (3) by providing inhibitory input onto sleep-promoting neurons in the hypothalamus (potentially via α2ARs) (Fig. 3.9). The higher levels of NE may also be stimulating histamine neurons, as modafinil administration increases histamine release (Ishizuka et al., 2003). However, this is most likely a downstream effect, because modafinil does not appear to bind histamine receptors directly (Prast et al., 1991). The focus of Chapter 3 of this document is exploration of the catecholaminergic regulation of modafinil-induced arousal.

Caffeine

Caffeine is the most popular psychoactive substance in the world; eighty percent of the population reports regular use of caffeine. It is present in coffee, tea, chocolate, and sodas, as well as many over-the-counter remedies.

Animal studies have shown that caffeine dose-dependently increases wakefulness at the expense of slow-wave sleep, REM sleep, and total sleep time (Yanik et al., 1987). Much of caffeine's effects on sleep and arousal in humans have been self-reported, but there have also been some laboratory-controlled studies (reviewed in (Roehrs and Roth, 2008)). At doses equivalent to a single cup of coffee, caffeine has been found to increase latency to sleep and

decrease total sleep time without affecting REM sleep. In a model of chronic caffeine administration, total sleep time was decreased over the course of the study, but some tolerance developed over time. In EEG studies, caffeine decreased slow-wave activity, consistent with "shallower" sleep. When compared with methylphenidate, caffeine had a greater effect on increasing sleep latency and reducing total sleep.

Caffeine is an antagonist of adenosine receptors, blocking the actions of adenosine, a purine that is synthesized throughout the brain. Levels of adenosine accumulate during wakefulness and decrease during sleep, especially in specific brain regions, such as the basal forebrain (Landolt, 2008). Adenosine's effects on sleep regulation are diffuse and involve multiple neurotransmitter systems. It acts to inhibit wakefulness by decreasing the frequency of action potentials of orexin neurons, and also inhibits histamine release via excitation of GABA neurons in the TMN (Hong et al., 2005; Liu and Gao, 2007). In parallel, it increases sleep by exciting a subset of neurons in the VLPO (Gallopin et al., 2005). The effects of adenosine are mediated by four distinct G protein-coupled receptors (GPCRs) (A1, A2A, A2B, and A3). Those receptors with the most convincing evidence for sleep involvement are the A1 and A2A receptors (Landolt, 2008). Caffeine is a selective antagonist of the A2A receptors, as studies in knockout (KO) mice have revealed that caffeine effectively increases wakefulness in A1 receptor KO mice, but has no effect on A2A receptor KO mice (Huang et al., 2005). By inhibiting adenosine binding, caffeine blocks its sleep-promoting effects in the VLPO, and prevents its inhibition of wake-promoting effects on the histamine and orexin systems.

Caffeine is one of the rare stimulant-type drugs that does not appear to act through the noradrenergic system, and there is scant evidence of even an indirect role for NE. Indeed, *Dbh -/-* mice lacking NE respond normally to caffeine (Hunsley and Palmiter, 2004). Rather, caffeine prevents the actions of adenosine on other elements of the sleep regulatory system, such as histamine, orexin, and the GABAergic neurons of the VLPO. However, interactions between D2 and A2A receptors may underlie the locomotor-promoting effects of caffeine (reviewed in (Cauli

and Morelli, 2005)). This effect is most likely due to the reciprocal inhibition between D2 receptors and A2A receptors, where activation of A2A receptors leads to reduction in D2 receptor signaling, and vice versa. Indeed, some of the behavioral phenotypes seen in DA deficient mice are thought to be at least partially a result of increased adenosine activation. Administration of caffeine to DA deficient mice restores feeding behavior to the same level of a DA precursor, and also increases locomotor behavior in a dose-dependent manner (Kim et al., 2003).

1.8 Antidepressants

Although their first-line use is for the treatment of affective disorders, antidepressants are also used off-label to treat sleep disorders. Depressed patients often suffer from sleep disturbances, and antidepressant therapy can ameliorate these disturbances; however, it is unclear whether this is a direct effect of the antidepressant on sleep, or whether the insomnia fades as mood improves (Erman, 2005). On the other hand, antidepressants often have unwanted side effects on sleep and wakefulness, ranging from drowsiness to insomnia. Antidepressants are categorized by mechanism, and include tricyclic antidepressants (TCAs), monoamine oxidase inhibitors (MAOIs), selective serotonin reuptake inhibitors (SSRIs), and others, which typically act through combinations of transporter and receptor blockade. These variations in mechanism lead to a range of effects on sleep (as reviewed extensively by (Mayers and Baldwin, 2005)).

The effects of antidepressants on sleep are diverse, even within a class of medications. Sedation and drowsiness are common side effects of the TCAs (e.g., desipramine (Norpramin), imipramine (Tofranil), amitriptyline (Elavil)). Amitriptyline increases drowsiness and shortens sleep latency compared with placebo, whereas imipramine actually increases sleep latency and decreases total sleep time. MAOIs and SSRIs (e.g., fluoxetine (Prozac), sertraline (Zoloft), citalopram (Celexa)) can cause insomnia and decreased sleep efficiency, as does bupropion (Wellbutrin). Compared with other antidepressants, fluoxetine is associated with significantly

more fractured and less efficient sleep. Notably, all these classes of antidepressants suppress REM sleep to some degree.

Trazodone (Desyrel) is an antidepressant that is also commonly prescribed for insomnia (Mendelson, 2005). In a study examining its effects on primary insomnia, trazodone improved self-reports of sleep latency, sleep duration, and decreased number of wakings. In another study, trazodone improved self-reports of sleep, but when measured by EEG, had no impact on sleep duration or sleep latency, with only a modest reduction in number of wakings. However, even trazodone has been shown to suppress REM sleep. Besides being observed in the clinic, animal models also show suppression of REM sleep following antidepressant administration (Sanchez et al., 2007).

Most antidepressants act through monoamine systems, though their specific mechanisms are variable, which may account for the diverse effects on sleep. Some TCAs are specific for NET blockade (e.g., desipramine), while others block both NET and SERT (e.g., imipramine). TCAs also have interactions with histamine, serotonin, and adrenergic receptors. SSRIs are specific for the SERT (Mayers and Baldwin, 2005), whereas MAOIs interfere with monoamine metabolism. Bupropion, the antidepressant most commonly prescribed for smoking cessation, is a dual NET/DAT inhibitor. Trazodone, the most commonly prescribed antidepressant for insomnia, is a weak inhibitor of serotonin synaptosome reuptake and is also an antagonist at 5-HT1A, 5HT1C, 5-HT2, and α-adrenergic receptors (Mendelson, 2005). The contribution of NE to these effects on sleep is not clear. Among the tricyclics, the NET-selective desipramine is the least effective at promoting sleep (Mayers and Baldwin, 2005). However, the dual NET/SERT inhibitor imipramine, which is also less effective at promoting sleep than other TCAs, decreases c-Fos in the LC (de Medeiros et al., 2005). In rats, antidepressants that act by inhibiting the NET, either alone or in combination with SERT, showed the highest amount of sleep disruption (Sanchez et al., 2007). These seemingly paradoxical effects could be because treatments that increase extracellular NE, such as NET blockers, increase availability not only at excitatory

α1ARs and βARs, which would be expected to increase wake, but also at α2ARs, which act as inhibitory autoreceptors to decrease LC activity and NE release. While antidepressants do not typically involve DA (with the exception of bupropion), a meta analysis indicates that dopaminergic treatments may help improve quality of life in depressed patients (IsHak et al., 2009), and dopaminergics are being increasingly investigated as novel treatments for depression (Kulkarni and Dhir, 2009).

Interestingly, short-term sleep deprivation has antidepressant effects. While the mechanism of this is not yet clear, research indicates that it may be linked to NE from the LC (reviewed in (Payne et al., 2002)). Sleep deprivation increases the expression of a variety of genes, including brain-derived neurotrophic factor (BDNF) and its receptor TrkB, both of which are hypothesized to play a role in the antidepressant effects of sleep deprivation. However, if the LC is lesioned, the upregulation of these genes following sleep deprivation does not occur. Moreover, sleep deprivation increases LC firing and levels of extracellular NE. Behavioral studies should be conducted in the future to explore the impact of LC lesions on sleep deprivation as an antidepressant therapy.

1.9 Summary

Sleep disorders of both insomnia and excessive sleepiness present major public health problems, making medications that can successfully treat these disorders without side effects very valuable. Sleep is regulated by multiple neurotransmitter systems, and pharmacotherapies act on many aspects of its regulation to treat various disorders. Wake-promoting medications frequently act by increasing transmission along an ascending arousal pathway, whereas sleep-promoting medications typically inhibit this pathway through neurons in the VLPO, the main sleep-promoting nucleus in the brain.

A common thread in the mechanism of action of many sleep pharmacotherapies is NE. A pictorial representation integrating and summarizing the anatomical connections between noradrenergic neurons and the wake- and sleep-promoting systems, as well as potential sites of action of wake- and sleep-promoting drugs, is depicted in Fig. 1.1. For example, amphetamine directly induces release and blocks reuptake of NE and DA, increasing extracellular concentrations and activating the ascending arousal pathway. Although the mechanism of modafinil has not been determined conclusively yet, its actions are blocked by α1AR antagonists, and there is some evidence that it may act as a dual NET/DAT inhibitor. In contrast, caffeine is one wake-promoting agent that does not appear to have a significant adrenergic component, though DA may increase its efficacy through interactions with the adenosine system. Finally, most antidepressants, including those used as insomnia treatments, affect the adrenergic system.

The diversity of the mechanisms of these drugs demonstrates the breadth of transmitter systems involved in sleep regulation. One obstacle to developing effective treatments for sleep disorders is that all these neurotransmitters are crucial for numerous biological processes in addition to sleep, and enhancing or inhibiting their activity often leads to a number of unpleasant or even dangerous side effects. However, as we learn more about how to differentiate the effects on arousal from other systemic effects, we may be able to develop increasingly specific medications that target the arousal system more directly. Rodent models are extremely useful tools for elucidating the role of specific molecules in both normal and pathological physiology. The work described in this dissertation utilized both rats and knockout mice to explore the function of catecholamines in arousal pharmacology.

While antidepressants are known to have a variety of effects on sleep, some of these due to NE, their effects on locomotor behavior have not been systematically analyzed. We explored various categories of antidepressants (tricyclics, SSRIs, and other transporter blockers) and their impact on locomotor behavior. All pharmacotherapies were administered to mice both acutely and chronically at physiologically relevant concentrations, and the impact of each drug on

locomotor behavior was measured. In addition, mice lacking the NET were also used for this study, to further tease apart the role of NE and NET in how antidepressants impact locomotor behavior.

One of the great unanswered questions in sleep pharmacology is the mechanism by which modafinil exerts its wake-promoting effects. Learning this will not only provide valuable information about arousal pathways, but will also lead toward development of the next generation of arousal-producing pharmacotherapies. In order to solve this question, *Dbh -/-* mice were used in behavioral paradigms of sleep latency and locomotor activity. Because *Dbh -/-* mice lack NE but have overactive DA signaling, we hypothesized that if modafinil acts primarily by a noradrenergic mechanism, the mice would be nonresponsive, whereas if modafinil acts primarily through dopaminergic mechanisms the mice would be hypersensitive. Subsequent studies with catecholaminergic receptor antagonists were conducted to further analyze the role that DA and NE have in modafinil's effects on the arousal system. Based on the findings of these experiments, a hypothetical model was developed proposing that modafinil acts as a low-affinity antagonist for NET and DAT, thereby increasing extracellular NE and DA, which act downstream to increase wake. Aspects of this model were then explored through local infusion studies in mice and rats.

Use of knockout and transgenic mouse models of disease states have been of tremendous value in elucidating the importance of catecholamines in arousal. This work also details the behavioral characterization of a novel mouse model for Lesch-Nyhan Disease (LND). LND is a genetic condition caused by dysfunction of the hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene, leading to a variety of metabolic and behavioral symptoms and DA loss in the striatum. While patients with LND do not display sleep disturbances, their levels of arousal, as demonstrated by locomotor behavior and increased aggression, are altered. Although the genetic cause of LND is known, the cause of the debilitating behavioral symptoms is still a mystery, in part because HPRT-deficient mice do not display LND-like neurological phenotypes. We tested the novel theory that the behavioral issues of LND are due to an interaction between HPRT and

phosphoribosyl transferase domain containing protein (PRTFDC1), a gene in the HPRT family that is absent in the mouse lineage. It has been hypothesized that both loss of HPRT and expression of PRTFDC1 are necessary for the expression of the characteristic LND behavior (Keebaugh et al., 2007). In order to examine this hypothesis, HPRT knockout mice were crossed with mice transgenic for human PRTFDC1, and tested in behavioral paradigms for a hyperaroused state, including locomotor behavior, amphetamine-induced stereotypy, and aggression.

The experiments included in this dissertation all utilize behavioral paradigms in rodent models to explore the importance of catecholamines in arousal. Behavioral studies are particularly useful in that they provide the opportunity to observe the direct impact of a pharmacological treatment or removal of a gene/protein on the actions of an animal, and thereby learn more about how that treatment or protein might impact human disease states. Each of the studies included herein offer new information about how catecholamines impact arousal, which then can be used to investigate further treatment options for neurological conditions.

Acknowledgements

David Weinshenker was supported in part by NIH/NIDA (DA017963). Heather Mitchell has been supported by T32 GM008602.

Figure 1.1 Arousal pathways and sites of action of arousal-promoting pharmacotherapies

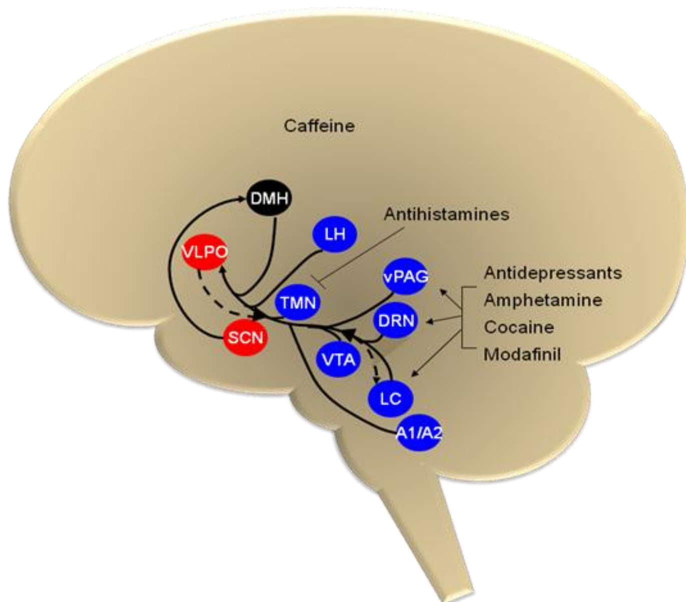


Figure 1.1 Arousal pathways and sites of action of arousal-promoting pharmacotherapies

The ascending arousal pathway is pictured here with sites of actions of the aforementioned medications. Wake-promoting regions are shown in blue, sleep-promoting regions are shown in red, and relays are shown in black. The noradrenergic LC and A1/A2, along with the dopaminergic VTA and vPAG, the serotonergic DRN, and the histaminergic TMN share reciprocal inhibition with the sleep-promoting neurons of the GABAergic VLPO. The orexinergic neuros of the LH further increase wake by enhancing the actions of these aminergic neurons. The circadian clock influences these pathways via relays through the DMH. Antidepressants increase extracellular monoamines through a variety of mechanisms, which then affect both the arousal pathway, and the reverse inhibitory pathway through autoreceptors. Antihistamines suppress wake by preventing the actions of histamine on the arousal pathway. Amphetamine blocks monoamine transporters to increase activity along the wake-promoting pathways, and while the mechanism of modafinil remains unclear, it is hypothesized to act in a similar manner. Finally, caffeine acts throughout the brain, by suppressing the inhibitory effects of adenosine.

CHAPTER 2:

THE EFFECTS OF NOREPINEPHRINE TRANSPORTER INACTIVATION ON LOCOMOTOR ACTIVITY IN MICE

Adapted from:

Mitchell HA, Ahern TH, Liles LC, Javors MA, Weinshenker D. The effects of norepinephrine transporter inactivation on locomotor activity in mice. Biol Psychiatry. 2006 Nov 15;60(10):1046-52.

2.1 Abstract

Acute administration of different classes of antidepressants can enhance or reduce spontaneous locomotor activity (SLA) in a novel environment, but the effects of chronic antidepressant treatment on SLA in novel and familiar environments are less well characterized. Because norepinephrine (NE) is an important regulator of SLA, we speculated that NE transporter (NET) blockade contributes to the effects of some antidepressants on SLA. In order to investigate this, antidepressant drugs (reboxetine, desipramine, imipramine, venlafaxine, bupropion) were administered acutely (i.p. injection) or chronically (via osmotic minipump) to control and NET knockout (NET KO) mice, and SLA in novel or familiar environments was recorded. It was found that acute treament with most NET blocking antidepressants decreased SLA in a novel environment, while chronic treatment decreased SLA in both novel and familiar environments. The exception was the NET/dopamine transporter (DAT) blocker bupropion, which tended to increase SLA. Co-administration of reboxetine and the DAT blocker GBR12909 also increased SLA. NET KO mice had low basal SLA that was increased by bupropion, while reboxetine had no effect in NET KO mice. These results indicate that acute or chronic NET inactivation decreases SLA in novel and familiar environments unless coupled with DAT blockade.

2.2 Introduction

The monoamines dopamine (DA), serotonin (5-HT), NE, and epinephrine (EPI) are important modulators of both psychomotor activity and mood (Brown and Gershon, 1993; Fishman et al., 1983; Ressler and Nemeroff, 1999; Stone et al., 2003b). Alterations of the monoamine systems likely contribute to depression (Brocco et al., 2002; Brunello et al., 2002; Frazer, 2000; Stone et al., 2003b), which interestingly is often marked by abnormal, usually retarded, psychomotor activity (Caligiuri and Ellwanger, 2000; Sachdev and Aniss, 1994; Sobin and Sackeim, 1997). In addition, some forms of depression in humans and animal models show psychomotor hyperactivity or agitation, thought to be increased responsiveness to stressful stimuli (Marks et al., 1971; Sobin and Sackeim, 1997).

The standard pharmacological treatments for depression block one or more monoamine transporter, increasing extracellular monoamine availability. Although these drugs alleviate such symptoms as anhedonia and despair in both humans and animals, one of their most common, and perhaps debilitating, side-effects is sedation and decreased psychomotor activity (Brocco et al., 2002; Tucker and File, 1986). Currently there is no generally accepted explanation for the phenomenon, and surprisingly few studies have characterized the effects of antidepressants on motor activity. Furthermore, previous animal work has three main limitations: first, many studies have used antidepressants with targets other than monoamine transporters (e.g., desipramine; (Rommelspacher et al., 1989; Vogel et al., 1986)); second, none have used a paradigm that approaches therapeutic conditions (i.e., chronic therapeutic serum drug levels); and third, none have assessed long-term (e.g. 24 hour) locomotor activity during chronic treatment.

Our study focused on NE and NET inhibitor antidepressants and was designed to address all three limitations. Using the mouse model, we systematically tested the effects of chronic antidepressant administration on short-term SLA in a novel environment and long-term (24 hour) SLA in a familiar environment. To address drug specificity, we used the selective NET inhibitor

reboxetine, which does not interact with other transporters or receptors (Wong et al., 2000), and NET KO mice, which have a specific deletion of the gene encoding NET. To mimic human antidepressant treatment, we administered reboxetine via osmotic minipump for 18-20 days at a dose that produced therapeutic serum levels of drug. Finally, we systematically tested the chronic effects of 4 other antidepressant NET inhibitor classes on SLA at therapeutically relevant serum levels: desipramine (tricyclic NET inhibitor), imipramine (tricyclic NET and serotonin transporter [SERT] inhibitor), venlafaxine (selective NET and SERT inhibitor) and bupropion (selective NET and DAT inhibitor).

2.3 Methods

Animals and housing

For the first round of experiments, dopamine β-hydroxylase heterozygote (*Dbh +/-*) control mice, maintained on a C57BL6/J and 129SvEv background, were bred and used from our knockout (*Dbh -/-*) colony. *Dbh -/-* males were crossed to *Dbh +/-* females, with half the progeny being *Dbh +/-* and the other half being *Dbh -/-*. Originally, we wished to include the analysis of the *Dbh -/-* mice that completely lack NE, but they did not tolerate the minipump surgeries well and showed signs of general malaise, so they were not included. *Dbh +/-* mice have normal NE levels and are indistinguishable from wildtype littermates for all previously tested phenotypes (Szot et al., 1999; Thomas et al., 1998; Thomas et al., 1995; Thomas and Palmiter, 1997b), including SLA in a novel (Fig. 2.1) and familiar (Thomas and Palmiter, 1997a) environment. For the second set of experiments, NET KO and wildtype (WT) control mice, maintained on a pure C57BL6/J background, were generated from NET +/- heterozygote breeders obtained from Mark Caron (Duke University). Adult male and female mice, 3 to 7 months old at testing, were used, and all experimental subjects had age-matched controls. No sex or age differences were observed

and results were combined. The colony room was maintained at 22°C with lights on from 0700 to 1900 throughout the experiment. Food and water were available ad libitum, with animals maintained according to the *NIH Guide for Care and Use of Laboratory Animals*. All experiments were approved by the Emory Institutional Care and Use Committee.

Compounds

Drugs used in this study were: reboxetine (Pfizer, Groton, CT), desipramine (Sigma-Aldrich, St. Louis, MO), imipramine (Sigma-Aldrich), bupropion (Sigma-Aldrich), GBR12909 (Sigma-Aldrich) and venlafaxine (Wyeth, Monmouth Junction, MJ).

Antidepressant administration

Drugs were administered acutely via i.p. injection or chronically via Alzet® osmotic minipumps (Model #2004, 0.25 µl/hr, 28 d; Durect, Cupertino, CA). For chronic administration, antidepressants were dissolved in either 0.9% sterile saline (reboxetine, 20 mg/kg/d; imipramine, 120 mg/kg/d; venlafaxine, 20 mg/kg/d; bupropion, 40 mg/kg/d) or an aqueous solution containing 50% ethanol and 0.9% sterile saline (desipramine, 20 mg/kg/d), and loaded into pumps. Doses were chosen to achieve serum levels that fell within or very close to human therapeutic range (Ahern et al., 2006). Minipumps containing 0.9% sterile saline or a 50% ethanol/0.9% sterile saline aqueous solution were used as vehicle controls. All pumps were placed in a sterile 37°C saline bath for 1 day before implantation. Mice were anesthetized with isoflurane and minipumps were implanted in the intraperitoneal cavity. Buprenorphine (2.5 mg/kg, s.c.) was given immediately following surgery for pain management. For acute administration, all antidepressants were dissolved in vehicle (0.9% sterile saline) except for GBR12909 (1.5% DMSO, 0.9% sterile saline) and injected i.p. as a bolus (10 ml/kg) using the same doses as for the chronic paradigm. The exceptions were (1) imipramine, which caused profound ataxia and sedation when 120 mg/kg

was injected acutely, so 20 mg/kg was used instead, and (2) GBR12909 (20 mg/kg), which is not used clinically as an antidepressant and was not tested in the chronic experiment.

Locomotor activity testing

For acute experiments in a novel environment, drugs were injected i.p. 30 minutes prior to placement in unfamiliar transparent plexiglass cages (40 x 20 x 20 cm) situated in racks with 7 infrared photobeams spaced 5 cm apart, each end beam 5 cm from the cage wall (San Diego Instruments Inc., LaJolla, CA). Activity chambers were connected via an interface to a computer, and ambulations (consecutive beam breaks) were recorded. Testing began between 0900-1000 and continued for 2 hours. For chronic experiments in a novel environment, mice were tested, as described above, 18-20 days after minipump implantation. For chronic experiments in a familiar environment, mice were housed with food and water in the activity chambers for 48 hours, and activity was recorded during the second 24 hours.

Statistics

Group differences were analyzed by ANOVA followed by Bonferroni post-hoc tests using Graphpad Prism for Macintosh.

2.4 Results

Acute administration

Selective NET blocker antidepressants and tricyclics typically reduce SLA in a novel environment when administered acutely (e.g. (Brocco et al., 2002; Tucker and File, 1986)), while dual selective SERT/NET and DAT/NET inhibitors increase SLA. To confirm these findings in our paradigm, we administered reboxetine, desipramine, imipramine, venlafaxine, and bupropion to *Dbh +/-* mice via i.p. injection and measured SLA in a novel environment 30 minutes later.

Similar to previous reports, we found that most selective NET inhibitor and tricyclic antidepressants that we tested decreased SLA in a novel environment, especially during the first 30-60 min. For reboxetine (Fig. 2.2a) and desipramine (Fig. 2.2b), there was a significant effect of time (reboxetine: $F_{(3,56)} = 54.02$, $P < 0.0001$; desipramine: $F_{(3,56)} = 28.31$, $P < 0.0001$), treatment (reboxetine: $F_{(1,56)} = 29.6$, $P < 0.0001$; desipramine: $F_{(1,56)} = 8.26$, $P < 0.01$), and a time x treatment interaction (reboxetine: $F_{(3,56)} = 3.47$, $P < 0.05$; desipramine: $F_{(3,56)} = 3.67$, $P < 0.05$). For imipramine (Fig. 2.2c), there was a significant effect of time ($F_{(3,56)} = 23.17$, $P < 0.0001$) and a time x treatment interaction ($F_{(3,56)} = 2.95$, $P < 0.05$). In contrast, venlafaxine (Fig. 2.2d) had no effect on SLA (significant effect only of time: $F_{(3,56)} = 36.17$, $P < 0.0001$), while bupropion (Fig. 2.2e) potently enhanced SLA at all time points (time: $F_{(3,56)} = 2.95$, $P < 0.05$; treatment: $F_{(1,56)} = 109.4$, $P < 0.0001$). Bupropion is the only NET/DAT blocker antidepressant, suggesting that the increase in SLA by simultaneous DAT blockade overrode the ability of NET blockade to reduce SLA. Indeed, when the selective DAT blocker GBR12909 was co-administered with reboxetine (Fig. 2.2f), SLA was enhanced (treatment: $F_{(1,56)} = 73.27$, $P < 0.0001$), confirming this hypothesis.

Chronic administration

To determine how chronic treatment with various classes of NET inhibitor antidepressants influences exploratory activity, we administered reboxetine, desipramine, imipramine, venlafaxine, and bupropion to *Dbh +/-* mice for 18-20 days via osmotic minipump and measured SLA in a novel environment (Fig. 2.3). Similar to the acute administration experiment, most antidepressants decreased SLA. For reboxetine (Fig. 2.3a), there was a significant effect of time ($F_{(3,88)} = 21.16$, $P < 0.0001$), treatment ($F_{(1,88)} = 54.13$, $P < 0.0001$), and a time x treatment interaction ($F_{(3,88)} = 4.18$, $P < 0.01$). For desipramine (Fig. 2.3b), imipramine (Fig. 2.3c), and venlafaxine (Fig. 2.3d), there was a significant effect of time (desipramine: $F_{(3,84)} = 23.07$, $P < 0.0001$; imipramine: $F_{(3,84)} = 21.76$, $P < 0.0001$; venlafaxine: $F_{(3,88)} = 29.65$, $P < 0.0001$) and treatment (desipramine: $F_{(1,84)} = 23.59$, $P < 0.0001$; imipramine: $F_{(1,84)} = 11.39$, $P <$

0.01; venlafaxine: $F_{(1,88)} = 19.99$, $P < 0.0001$). In contrast, bupropion had no effect on SLA (Fig. 2.3e). None of the treatments caused obvious sedation in the mice. We also assessed the effects of chronic reboxetine treatment on long-term SLA in a familiar environment. Chronic reboxetine significantly reduced SLA over 24 hours (time: $F_{(48,686)} = 5.19$, $P < 0.0001$; treatment: $F_{(1,686)} = 5.25$, $P < 0.05$), and this effect was specific to the dark phase (time: $F_{(5,84)} = 3.69$, $P < 0.01$; treatment: $F_{(1,84)} = 5.75$, $P < 0.05$) (Fig. 2.4). The reboxetine-treated group appeared to be slightly more active during the light phase, but the difference was not significant.

Antidepressants in NET KO mice

To confirm that a reduction of SLA could be mediated solely by NET blockade, we examined the SLA of NET KO mice. We found that NET KO mice had low SLA in a novel environment, as previously reported (Fig. 2.5a; (Xu et al., 2000)). There was a significant effect of time ($F_{(3,52)} = 23.66$, $P < 0.0001$), genotype ($F_{1,52} = 31.02$, $P < 0.0001$), and a genotype x time interaction ($F_{3,52} = 6.59$, $P < 0.001$). Moreover, both acute (Fig. 2.5a) and chronic (Fig. 2.5b) treatment of WT mice with reboxetine reduced SLA in a novel environment (acute: time: $F_{(3,52)} = 29.95$, $P < 0.0001$; treatment: $F_{(1,52)} = 24.29$, $P < 0.0001$; chronic: time: $F_{(3,68)} = 38.61$, $P < 0.0001$; treatment: $F_{(1,68)} = 32.84$, $P < 0.0001$), while reboxetine had no effect in NET KO mice, demonstrating that the effects of reboxetine on SLA in WT mice were mediated exclusively by NET blockade. To determine whether the locomotor activating effects of bupropion were dependent on the NET, we tested the effects of bupropion on SLA in WT and NET KO mice (Fig. 2.6*)*. When administered acutely, bupropion increased SLA in both WT (time: $F_{3,52} = 15.77$, $P < 0.0001$; treatment: $F_{1,52} = 5.6$, $P = 0.0001$) and NET KO mice (time: $F_{3,56} = 22.14$, $P < 0.0001$; treatment: $F_{1,56} = 72.37$, $P < 0.0001$; time x treatment interaction: $F_{3,56} = 8.05$, $P < 0.001$) demonstrating that the NET is not required for bupropion to increase SLA in a novel environment (Fig. 2.6a). Chronic bupropion treatment also increased SLA in a novel environment in both WT (time: $F_{3,52} = 13.72$, $P < 0.0001$; treatment: $F_{1,52} = 22.56$, $P < 0.0001$) (Fig. 2.6b) and NET KO

mice (time: $F_{3,56} = 12.11$, $P < 0.0001$; treatment: $F_{1,56} = 6.11$, $P < 0.05$) (Fig. 2.6c). Finally, chronic bupropion increased 24 hour activity in WT mice (treatment: $F_{1,156} = 10.72$, $P < 0.01$) (Fig. 2.6d). Similar to the results with reboxetine (Fig. 2.4), the drug-induced change in activity was restricted to the dark phase. Chronic bupropion had a tendency to slightly increase 24 hour activity in NET KO mice, especially early in the dark phase, (Fig. 2.6d), but the result was not quite significant (treatment: $F_{1,168} = 3.07$, $P = 0.08$). Dark phase activity was markedly reduced in NET KO mice compared to WT (genotype: $F_{1,156} = 92.35$, $P < 0.0001$; genotype x time interaction: $F_{11,156} = 7.21$, $P < 0.0001$) (Fig. 2.6d).

2.5 Discussion

While previous studies have shown that acute or quasi-chronic (multiple i.p. injection) administration of NET inhibitors decreases motor activity in rodents (Tucker and File, 1986; Vogel et al., 1986), the experiments presented here constitute the first study that has systematically evaluated the chronic effects of various classes of NET inhibitor antidepressants on SLA at therapeutically relevant serum drug concentrations. In addition, the effect of chronic antidepressant administration on long-term locomotor activity in a familiar environment had never been examined. Because compounds used in some studies such as desipramine and imipramine act at sites other than monoamine transporters such as ion channels and acetylcholine, histamine, and adrenergic receptors (Frazer, 2000), the effects could not be directly attributed to NET blockade. Furthermore, lacking a measure of circulating serum levels of drug (Vogel et al., 1986), the therapeutic relevance was unclear (Tucker and File, 1986). Brocco and colleagues thoroughly investigated the effects of acute antidepressant administration, and found that drugs that block either SERT alone or SERT and NET increased SLA, while selective NET inhibitors either decreased SLA or had no effect (Brocco et al., 2002). Our acute results are in agreement for the selective NET inhibitor reboxetine, but not for the dual selective NET/SERT inhibitor

venlafaxine (increase in SLA in the Brocco study, no effect in our study). The reason for this difference is unclear. However, when given chronically, all tricyclics, reboxetine, and venlafaxine decreased SLA, highlighting the importance of examining both acute and chronic drug administration. Furthermore, chronic reboxetine administration decreased 24 hour SLA in a familiar environment, an effect that was restricted to the dark phase. NET KO mice had low SLA in a novel environment as previously reported (Xu et al., 2000), and we further demonstrated low dark phase activity in a familiar environment. Importantly, reboxetine had no further effect on SLA in the absence of NET. Taken together, these results demonstrate that both acute and chronic NET blockade inhibits SLA at times when mice are typically most active (exposure to novel environment, dark phase of the circadian cycle).

NE and EPI typically enhance SLA, while blockade of adrenergic signaling (particularly α_1 adrenergic receptors) reduces SLA (Harro et al., 1995; Stone et al., 1999; Weinshenker et al., 2002b). How then can we reconcile the increase in extracellular NE with the decrease in SLA after NET blockade? One possibility is that NET blockade increases signaling via inhibitory $\alpha 2$ autoreceptors, thus decreasing noradrenergic neuron firing and evoked NE release. In addition, chronic NET inactivation results in many compensatory changes in the noradrenergic system, including a down-regulation of tyrosine hydroxylase (the rate-limiting enzyme in NE synthesis; (Nestler et al., 1990)), burst firing of the locus coeruleus (LC, the major brain noradrenergic nucleus; (Grant and Weiss, 2001)), the NET (Benmansour et al., 1999; Weinshenker et al., 2002b), and adrenergic receptors (Bergstrom and Kellar, 1979; Invernizzi and Garattini, 2004; Xu et al., 2000). Thus, despite elevated basal extracellular NE levels, NET blockade may result in an overall decrease in NE signaling under some conditions.

Interestingly, the only NET inhibitor we examined that did not reduce SLA was bupropion, which also blocks DAT. Bupropion increased SLA in a novel environment when given acutely in both control strains tested. When given chronically, bupropion also increased

SLA in both a novel environment and during the dark phase in a familiar environment in WT C57BL6/J mice, but had no effect on activity in *Dbh* +/- mice (mixed C57BL6/J and 129SvEv). This difference is not surprising, as strain differences in dopaminergic sensitivity and signaling are well documented (He and Shippenberg, 2000; Kuzmin and Johansson, 2000; Womer et al., 1994). The locomotor stimulating effects of acute and chronic bupropion in a novel environment persisted in NET KO mice, indicating that the NET is not required for this effect. Because DA is a potent enhancer of locomotor activity and bupropion has amphetamine-like stimulant properties (Tucker and File, 1986), we hypothesized that simultaneous DAT blockade may override the attenuation of SLA by NET blockade. In support of this idea, co-administration of reboxetine and the selective DAT blocker GBR12909 potently increased SLA.

Our results have potential clinical implications. Alterations (up or down) in psychomotor activity are a common hallmark of a depressive state (Sobin and Sackeim, 1997). Because NET inhibitors given chronically decrease SLA in mice, these compounds might be useful for treating "agitated" depressive individuals. Interestingly, olfactory bulbectomized rats, a commonly used rodent model of depression, display hyperactive exploratory behavior that is suppressed by chronic administration of NET inhibitor antidepressants (Harkin et al., 1999). In contrast, patients with depressed activity levels may wish to avoid NET inhibitors or use bupropion, which can increase psychomotor activity under some conditions.

Acknowledgements

We thank Marc Caron for providing NET KO breeding pairs, Pfizer for providing reboxetine, Wyeth for providing venlafaxine, and David Archer for use of anesthesia equipment. This work was supported in part by the Epilepsy Foundation and the National Alliance for Research on Schizophrenia and Depression. Heather Mitchell has also been supported by NIH grant T32 GM 008602.

Figure 2.1 Comparison of spontaneous locomotor activity in *Dbh* +/+ and *Dbh* +/- mice in a novel environment

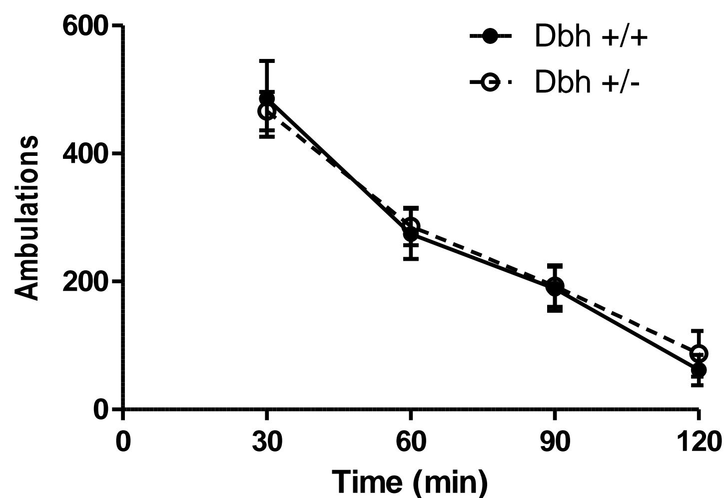


Figure 2.1 Comparison of spontaneous locomotor activity in *Dbh* +/+ and *Dbh* +/- mice in a novel environment. Mice were placed in activity chambers, and SLA was recorded for 2 hours. Shown are mean ± SEM ambulations (consecutive beam breaks).

Figure 2.2 Effects of acute antidepressant drug treatment on spontaneous locomotor activity in a novel environment

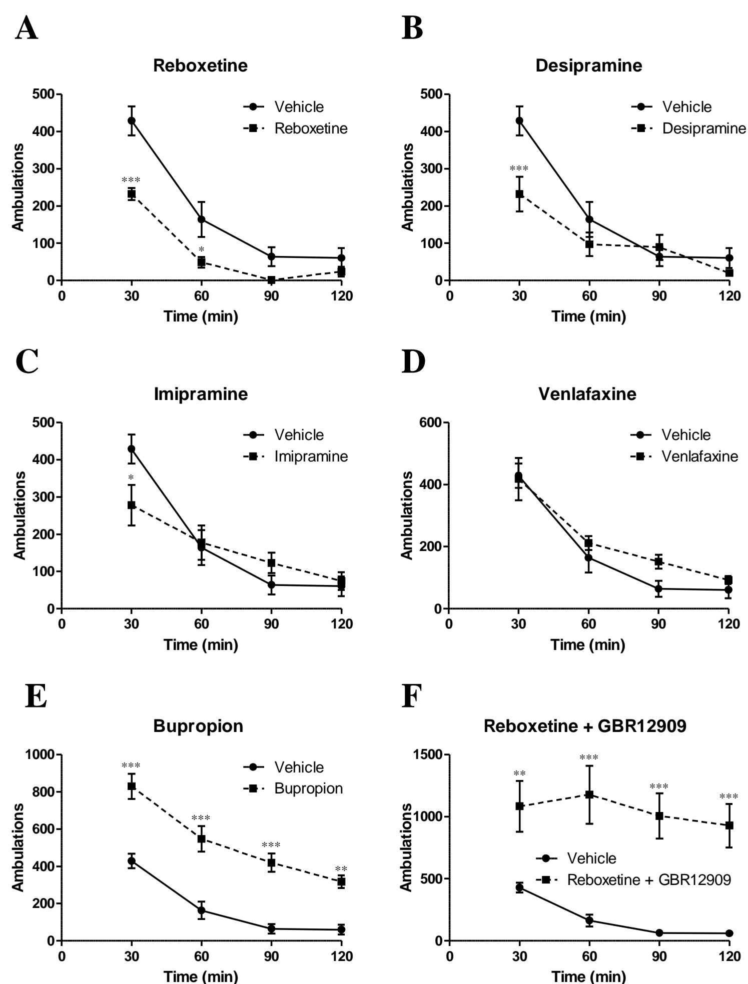


Figure 2.2 Effects of acute antidepressant drug treatment on spontaneous locomotor activity in a novel environment. Shown are mean ± SEM ambulations (consecutive beam breaks) of *Dbh +/-* mice administered (**A**) reboxetine (20 mg/kg/d), (**B**) desipramine (20 mg/kg/d), (**C**) imipramine (20 mg/kg/d), (**D**) venlafaxine (20 mg/kg/d), (**E**) bupropion (40 mg/kg/d), or (**F**) reboxetine + GBR12909 compared to vehicle (0.9% saline) via i.p. injection 30 min prior to test (n = 8 per group; *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ compared to vehicle).

Figure 2.3 Effects of chronic antidepressant drug treatment on spontaneous locomotor activity in a novel environment

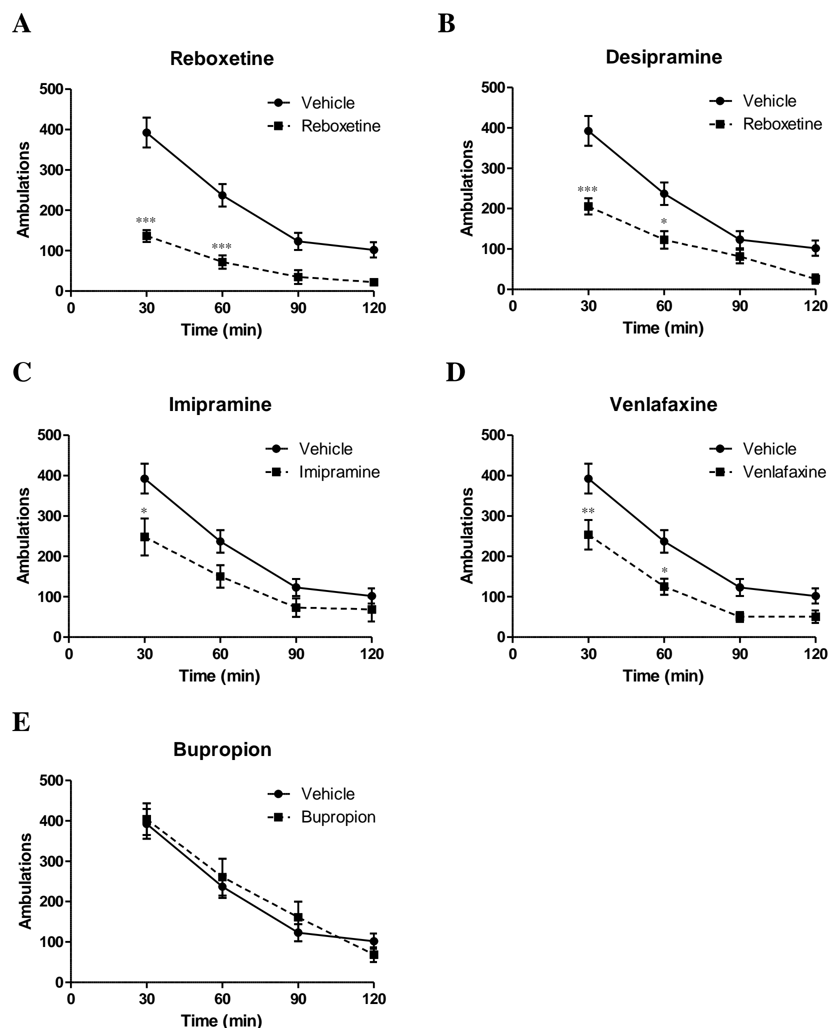


Figure 2.3 Effects of chronic antidepressant drug treatment on spontaneous locomotor activity in a novel environment. Shown are mean ± SEM ambulations (consecutive beam breaks) of *Dbh +/-* mice administered vehicle (0.9% saline or 50% EtOH in 0.9% saline), (**A**) reboxetine (20 mg/kg/d), (**B**) desipramine (20 mg/kg/d), (**C**) imipramine (120 mg/kg/d), (**D**) venlafaxine (20 mg/kg/d), or (**E**) bupropion (40 mg/kg/d) via osmotic minipump for 18-20 days (n = 7 – 15 per group); *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ compared to vehicle.

Figure 2.4 Effects of chronic reboxetine administration on 24 hour spontaneous locomotor activity

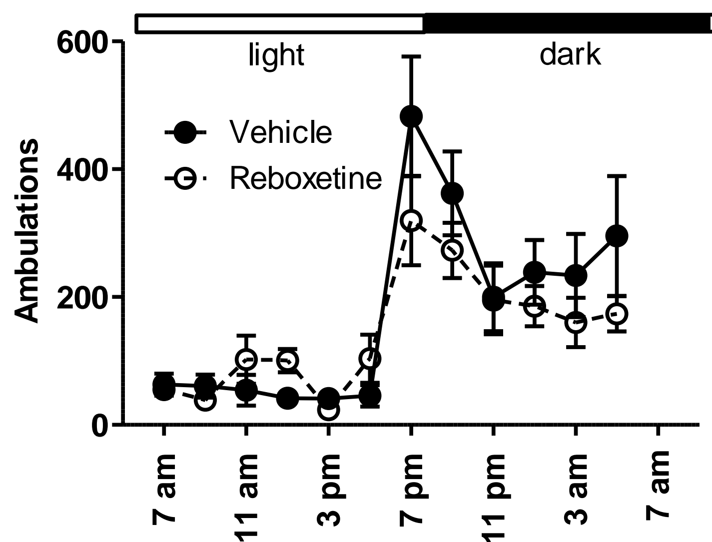


Figure 2.4 Effects of chronic reboxetine administration on 24 hour spontaneous locomotor activity. Shown is mean ± SEM ambulations (consecutive beam breaks) of *Dbh +/-* mice administered vehicle (0.9% saline) or reboxetine (20 mg/kg/d) via osmotic minipump for 18-20 days (n = 8 per group).

Figure 2.5 Effects of reboxetine administration and NET genotype on spontaneous locomotor activity in a novel environment

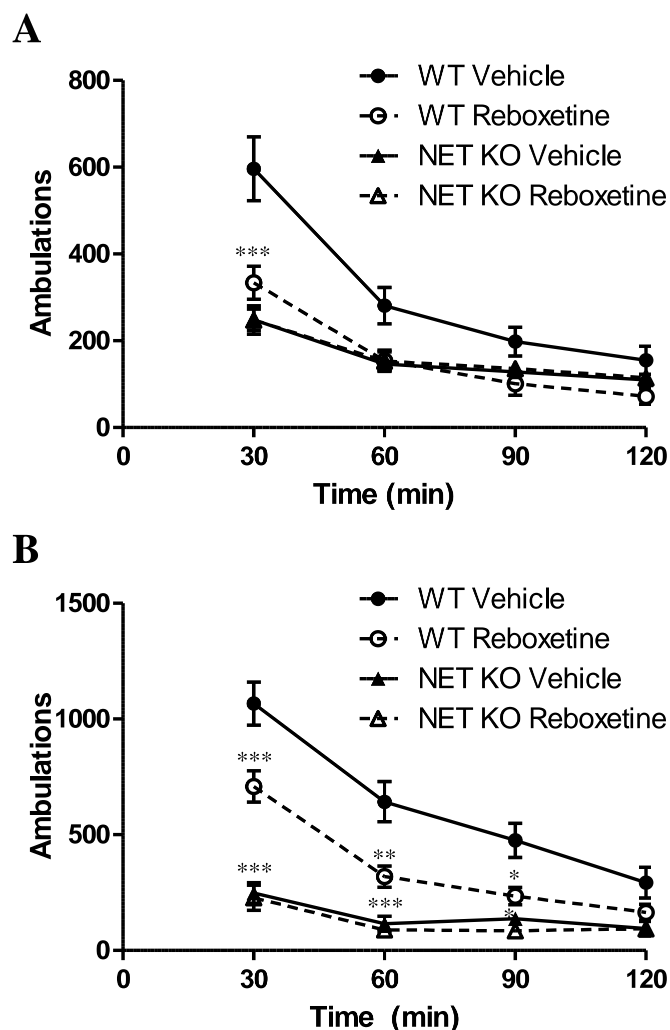


Figure 2.5 Effects of reboxetine administration and NET genotype on spontaneous locomotor activity in a novel environment. Shown is mean ± SEM ambulations (consecutive beam breaks) of WT and NET KO mice administered vehicle or reboxetine (**A**) acutely (20 mg/kg, i.p. injection 30 min prior to test, n = 6 – 8 per group) or (**B**) chronically (20 mg/kg/d for 18-20 days via osmotic minipump, n = 5 – 9 per group). *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ compared to WT vehicle.

Figure 2.6 Effects of bupropion administration and NET genotype on spontaneous locomotor activity in a novel and a familiar environment

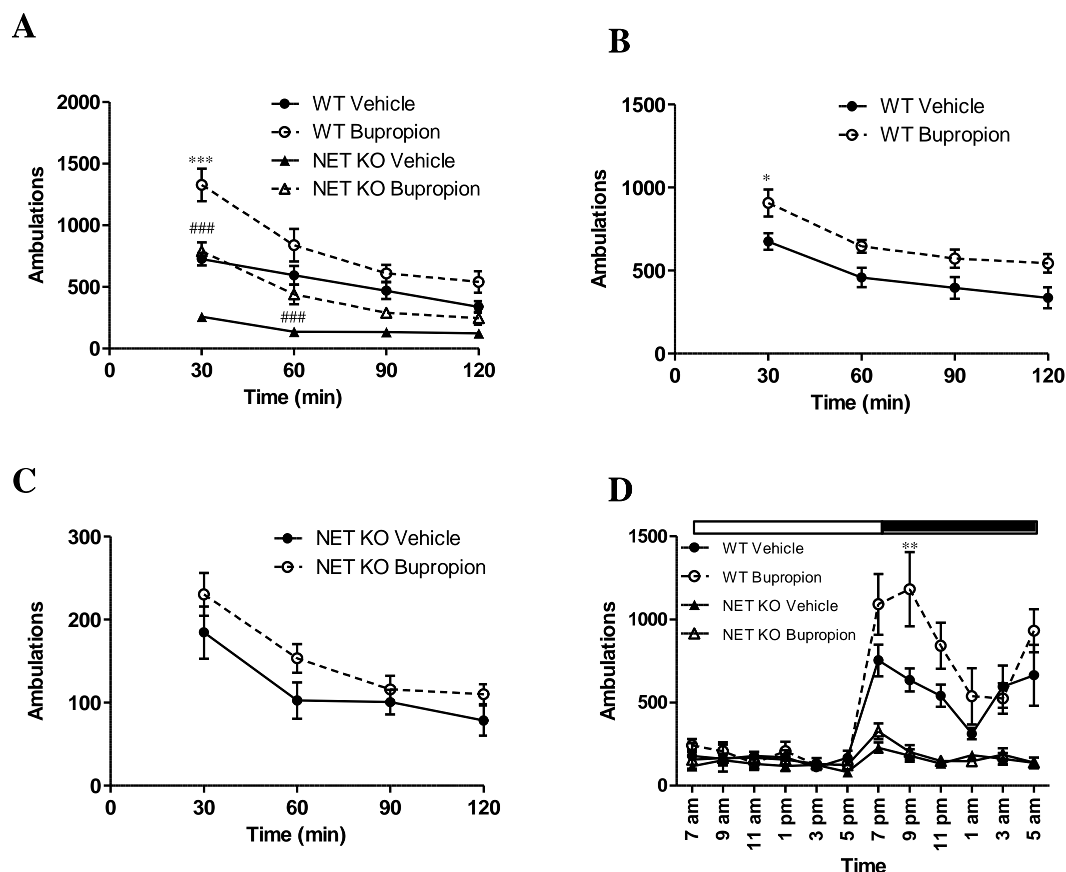


Figure 2.6. Effects of bupropion administration and NET genotype on spontaneous locomotor activity in a novel and a familiar environment. Shown is mean ± SEM ambulations (consecutive beam breaks) of WT and NET KO mice administered vehicle or bupropion acutely (**A**), or chronically (20 mg/kg/d for 18-20 days via osmotic minipump, n = 5 – 8 per group) and tested in (**B, C**) a novel environment for 2 hours or (**D**) a familiar environment for 24 hours. *** P < 0.001, ** P < 0.01, * P < 0.05 compared to WT given vehicle, ### P < 0.001 compared to NET KO given vehicle.

CHAPTER 3:

BEHAVIORAL RESPONSES OF DOPAMINE β-HYDROXYLASE KNOCKOUT MICE TO MODAFINIL SUGGEST A DUAL NORADRENERGIC-DOPAMINERGIC MECHANISM OF ACTION

Adapted from: Mitchell HA, Bogenpohl JW, Liles LC, Epstein MP, Bozycz-/--Coyne D, Williams M, Weinshenker D. Behavioral responses of dopamine beta-hydroxylase knockout mice to modafinil suggest a dual noradrenergic-dopaminergic mechanism of action. Pharmacol Biochem Behav. 2008 Dec;91(2):217-22.

3.1 Abstract

Modafinil is approved for use in the treatment of excessive daytime sleepiness. The precise mechanism of modafinil action has not been elucidated, although both dopamine (DA) and norepinephrine (NE) systems have been implicated. To explore the roles of DA and NE in the mechanism of modafinil-induced arousal, dopamine β-hydroxylase knockout (*Dbh* -/-) mice were examined in behavioral paradigms of arousal (locomotor activity measured by photobeam breaks and behavioral scoring of sleep latency). *Dbh* -/- mice completely lack NE but have hypersensitive DA signaling. It was hypothesized that *Dbh* -/- mice would be unresponsive to modafinil if the compound acts primarily via NE, but would be hypersensitive to modafinil if it acts primarily via DA. *Dbh* -/- mice had increased sensitivity to the locomotor-activating and wake-promoting effects of modafinil. Paradoxically, the α1-adrenergic receptor antagonist, prazosin, attenuated the effects of modafinil in control mice, but not in *Dbh* -/- mice. Blockade of DA receptors with flupenthixol decreased modafinil-induced locomotion and wake in both control and *Dbh* -/- mice. When the α1-adrenergic receptor antagonist terazosin was directly infused into the periaquaductal grey (PAG), the wake-promoting effects of modafinil were attenuated in both rats and mice. These results suggest that (1) both NE and DA are involved in the behavioral effects of modafinil in control mice, but the requirement for NE can be bypassed by hypersensitive DA signaling, and (2) modafinil-induced wakefulness requires α1-adrenergic receptor stimulation of PAG DA neurons.

3.2 Introduction

It has been estimated that 50-70 million Americans suffer from chronic disorders of sleep and wakefulness that adversely affect health and longevity, making sleep disorders second only to pain in the number of patients seeking medical attention (Colten and Altevogt, 2006). Accordingly, the development of medications to treat sleep disorders is a high priority. One such drug is modafinil, which is approved for use in the treatment of the excessive daytime sleepiness associated with narcolepsy and other sleep disorders. Modafinil has behavioral effects distinct from those of more traditional stimulants such as amphetamine or cocaine in that it does not produce rebound hypersomnolence and has limited dependence liability (Ballon and Feifel, 2006; Edgar and Seidel, 1997; Minzenberg and Carter, 2007). The mechanism of action(s) underlying the wake-promoting effects of modafinil remain to be elucidated (Minzenberg and Carter, 2007).

Early research from the 1990s implicated the catecholamines, NE and DA, in the mechanism of action of modafinil but the animal data are confusing, with the role that each neurotransmitter plays yet to be clarified. Of more than 100 neurotransmitter and enzyme targets, the only consistent finding in vitro is a low affinity (Ki = 2-7 μM) inhibition of the DA transporter (DAT) (Mignot et al., 1994). While DAT knockout mice show decreased responsiveness to modafinil (Wisor et al., 2001) other data indicated that DA receptor antagonists were ineffective in blocking modafinil-induced arousal (Duteil et al., 1990; Lin et al., 1992; Simon et al., 1995). The compounds that consistently block the effects of modafinil are α1-adrenergic receptor (α1AR) antagonists (Duteil et al., 1990; Hermant et al., 1991; Lin et al., 1992) while α1bAR knockout mice show an attenuated response to the drug (Stone et al., 2002). However, modafinil does not bind α1ARs (Mignot et al., 1994). Modafinil is also a weak inhibitor of the norepinephrine transporter (NET) in vitro and in vivo (IC50 ~ 36 μM) (Madras et al., 2006). However the in vivo assessment of this interaction was limited as data from only one animal was available for the most effective modafinil dose (Madras et al., 2006). While many

neurotransmitters are involved in producing and maintaining arousal states, these transporters remain the only known biochemical targets of modafinil. Thus, the focus of these experiments was on the catecholaminergic systems and their interaction.

A novel tool for studying the relative roles of NE and DA in behavioral phenotypes is the dopamine β-hydroxylase knockout (*Dbh* -/-) mouse. These mice completely lack NE and epinephrine (EPI), but show a compensatory increase in high-affinity state DA receptors and are hypersensitive to the behavioral effects of both direct (e.g. quinpirole) and indirect (e.g. amphetamine, cocaine) DA agonists (Schank et al., 2006; Weinshenker et al., 2002a). Previous studies examining arousal behaviorally or by electroencephalgram (EEG) have revealed that exploratory activity in a novel environment, latency to sleep following handling or exposure to environmental stimuli, and wake bout duration were attenuated in *Dbh* -/- mice, suggesting that NE is important for maintaining vigilance (Hunsley and Palmiter, 2003; Hunsley and Palmiter, 2004). Thus, *Dbh* -/- mice were used to explore the role of catecholamines in modafinil-induced arousal.

It was hypothesized that if modafinil acts primarily via a noradrenergic mechanism, *Dbh* -/- mice should be non-responsive since they completely lack NE. In contrast, if modafinil acts mainly through DA systems, these mice should be hypersensitive. Modafinil was tested in *Dbh* -/- mice using both locomotor and sleep latency paradigms as behavioral readouts, and NE/DA interactions were further explored by examining the effect of systemic and intracranial NE and DA receptor antagonist pretreatments.

3.3 Methods

Animals and housing

Dbh -/- mice, maintained on a mixed 129/SvEv and C57BL/6J background, were developed and generated as described (Thomas et al., 1995, 1998). *Dbh* -/- males were bred to

Dbh +/- females. Pregnant *Dbh* +/- mice were given the AR agonists, isoproterenol and phenylephrine (20 µg/ml each) + vitamin C (2 mg/ml) from E9.5-E14.5, and L-3,4-dihydroxyphenylserine (DOPS; 2 mg/ml + vitamin C 2 mg/ml) from E14.5-birth in their drinking water to rescue the embryonic lethality associated with the homozygous *Dbh* -/- mutation. Because of this treatment, NE and EPI were present in *Dbh* -/- animals before but not after birth. *Dbh* -/- mice were identified by the delayed growth and ptosis phenotypes, which are 100% correlated with the *Dbh* -/- genotype. Genotypes were confirmed by PCR. *Dbh* +/- mice were used as controls as they had normal catecholamine levels and were indistinguishable from *Dbh* +/+ mice for all previously tested phenotypes, including locomotor activity (Bourdelat-Parks et al., 2005; Mitchell et al., 2006; Thomas et al., 1998; Thomas et al., 1995). Male and female mice aged 2-8 months were used in all experiments. No age or gender differences were found, and results were combined. All mice were reared in a specific pathogen-free facility with a 12 hour light/dark cycle (lights on - 7 am; lights off - 7 pm). Food and water were available ad libitum except during behavioral testing. All experiments were carried out in a quiet, isolated behavior room between 8:00 am and 3:00 pm. Mice were moved to this room at least 24 hours before testing.

Male Sprague-Dawley rats between 175 and 200 g were obtained from Charles River. Following surgical procedure, these rats were moved to the testing facility, and allowed to acclimate for at least 48 hours before experiments were conducted. The testing facility had regulated temperature and humidity and a 12 hour light/dark cycle (lights on - 7 am; lights off - 7 pm). Food and water were available ad libitum except during behavioral testing. Experimental protocols were approved by the Emory University IACUC and meet the guidelines of the American Association for Accreditation of Laboratory Animal Care.

Compounds

Modafinil (Cephalon, Inc., West Chester, PA) and the α1AR antagonist prazosin (0.5 mg/kg; Sigma-Aldrich, St. Louis, MO) were prepared by dissolving the compounds in warm 0.9% saline, 1.5% DMSO, and 1.5% cremophor EL. A 90 mg/kg dose of modafinil was reported to have identical effects in *Dbh* -/- and -/- control mice (Hunsley and Palmiter, 2004). Because *Dbh* -/- mice have altered responses to other stimulants that are most apparent at low to moderate doses (Schank et al., 2006; Weinshenker et al., 2002a), lower doses of modafinil (6.25 – 50 mg/kg) were selected for these experiments. For the rat experiment, modafinil was suspended in cyclodextrin (Sigma Aldrich, St. Louis, MO) and administered at 50 mg/kg, with an injection volume of 1.5 ml/kg. The dose of prazosin was selected based on published reports that it can block the behavioral effects of stimulants in mice (Drouin et al., 2001; Weinshenker et al., 2002a). The non-selective DA receptor antagonist, cis-(Z)-flupenthixol dihydrochloride (0.025 or 0.25 mg/kg; Sigma-Aldrich) was dissolved in 0.9% saline. These doses of flupenthixol were selected based on motor behavior dose-response experiments (Rommelfanger et al., 2007). All mouse compounds were administered i.p.in a volume of 10 ml/kg. Terazosin (3 µg/0.5µl; Sigma-Aldrich) was dissolved in 0.9% sterile saline, and the dose was selected based on the literature and our preliminary results indicating that it can blunt drug-induced behaviors, but does not impair motor behavior on its own ((Stone et al., 2003a); our unpublished data).

Sleep latency

Dbh +/- and *Dbh* -/- mice were individually housed in large plexiglass cages and allowed to acclimate for 4 hours. Vehicle or modafinil (6.25, 12.5, or 25 mg/kg) was then administered, and mice were observed by a trained experimenter for behavioral signs of sleep. During sleep, mice exhibit a distinctive posture and breathing pattern that allows the observer to determine onset. Sleep was defined as 2 minutes of uninterrupted sleep behavior, and 75% of the next 10 minutes spent asleep (Hunsley and Palmiter, 2004). This behavioral scoring paradigm has been shown to reliably correlate with onset of sleep using EEG measurements (Hunsley and Palmiter,

2003; Hunsley and Palmiter, 2004). In a subset of animals, saline or the DA receptor antagonist, flupenthixol, (0.25 mg/kg) was administered 30 minutes prior to modafinil (25 mg/kg) administration. This dose of modafinil was used for the antagonist experiment because it was the only dose tested that significantly increased sleep latency in both control and *Dbh* -/- mice.

Locomotor activity

Locomotor activity was assessed using an automated system (San Diego Instruments, La Jolla, CA, USA) with photobeams that recorded ambulations (consecutive beam breaks). Mice were placed individually in the chambers and allowed to acclimate for 4 hours, and were then administered vehicle or modafinil (6.25, 12.5, 25, or 50 mg/kg). Activity was recorded for an additional 2 hours. This time frame was selected because by 2 hours, locomotor activity had tapered off and was approaching baseline levels. In order to examine the effects of receptor antagonist pretreatment, vehicle, the α1AR antagonist prazosin (0.5 mg/kg), or the DA receptor antagonist, flupenthixol (0.025 or 0.25 mg/kg), were injected 30 minutes prior to modafinil (50 mg/kg) administration. This dose of modafinil was used because it produced comparable amounts of locomotor activity in control and *Dbh* -/- mice, thus making antagonist effects easier to compare between genotypes. All data were presented as total ambulations for the 2 hours following modafinil administration. To assess the effects of flupenthixol on exploratory activity in a novel environment, *Dbh* +/- and *Dbh* -/- mice were administered flupenthixol (0.25 mg/kg). Thirty minutes following injection, mice were placed in the locomotor chambers, and their activity was recorded for 2 hours.

Intracranial infusions in mice

Surgical procedure

To attempt to elucidate anatomical correlates of modafinil efficacy, the α1AR antagonist terazosin was infused into the PAG of *Dbh* +/- mice. Briefly, mice were anesthetized with inhaled

isoflourane, and stereotaxically implanted with an infusion cannula targeted at the PAG (AP -2.70, ML 0.3, DV -3.4). An incision was made to expose the skull, Bregma was located and used to determine the coordinates for the PAG. A dremel drill was used to drill a small hole in the skull directly over the implantation site, and then the infusion cannula was lowered to the correct position. One screw was placed into the skull to maintain headcap adherence. Dental acrylic (Ortho-Jet, Lang Dental Manufacturing Company) was applied around the cannula and screw, and used to build up a headcap. Mice were given the analgesic banamine (2.5 mg/kg, s.c.), and placed on a heating pad. The mice were allowed to recover for at least 4 days.

Infusions paired with sleep latency

On test day, mice were infused with vehicle or 3 μg/0.5μl of the α1AR antagonist terazosin. The infusion needle (made in the lab from 30G stainless steel tubing, Plastics One), connected to P20 tubing (Plastics One), connected to a 5 μl Hamilton syringe, connected to a Bee Hive syringe pump controller (Bioanalytical Sciences), was inserted into the guide cannula. A total of 0.5 μl of sterile saline or terazosin was infused over 165 sec, and the needle was left in place for an additional 60 sec. Modafinil (25 mg/kg, i.p.) was then injected, and latency to sleep was measured as described above.

EEG and intracranial infusions in rats

Surgical procedure

In order to confirm and extend the results found in the mice, infusion studies were combined with EEG recordings. Rats were used for these experiments to most easily combine intracranial infusions with EEG recordings in awake animals. Surgeries were conducted according to protocols used by Dr. David Rye, who has extensive experience with rodent EEGs. Briefly, animals were anesthetized with inhaled isofluorane and placed into a stereotax with a nose cone adapter for anesthesia. The skull was exposed, Bregma located and then used to

calculate placements for four EEG electrode screws (cortical: AP -1.5, ML 3.0; AP -6.3, ML 3.5; theta: AP 2.5, ML -1.5; AP -3.6, ML -1.5) and the infusion cannula targeted to the PAG (AP -7.3, ML -0.2, DV -5.4). Coordinates for the PAG were based on published observations from the Saper lab (Lu 2006). A dremel drill was used to make holes in the skull immediately above the desired coordinates. EEG electrode screws soldered to a 6-pin connector were implanted, and the stereotax was used to lower the cannula into place. For recording of neck EMG activity, fine wires (40 gauge Cooner Wire, Chatsworth, CA), also soldered to the connector, were inserted into the nuchal muscles. Sutures (6-0, Ethicon) were used to fix the muscle wires in place. Dental acrylic was then applied to the skull and allowed to harden. The skin over the muscle wires was sutured with 4-0 sutures. Rats were provided with ibuprofen in their drinking water (0.1 mg/ml), and placed on a heating pad to recover. Rats were given at least three days to recover before being moved to the experimental facility, and at least two days after being moved before experiments were conducted.

Infusions paired with sleep/wake recordings

During experiments, animals were placed into a sound attenuated, ventilated, and light-regulated environmental cubicle (BRS\LVE Davis, Maryland) and EEG/EMG wires were attached via a cable tethered to a counterbalanced and suspended commutator. Animals were placed in these chambers, and allowed to acclimate for 2 hours. They were next removed from the chambers, and infusions of either terazosin (3 μg/0.5 μl) or vehicle were conducted as described above. After completion of the infusion, modafinil (50 mg/kg, i.p., 1.5 ml/kg injection volume) or vehicle (cyclodextrin) was administered, and the animal returned to the EEG chamber. Collection of sleep-wake architecture continued uninterrupted for an additional two hours. The EEG, theta, and EMG signals were preamplified by Grass model 12A5 amplifiers; EEG and hippocampal theta signals were lo-pass filtered at 1 Hz and hi-pass filtered at 30 Hz while EMG signals were lo-pass filtered at 10 Hz and hi-pass filtered at 40 Hz. The amplified and filtered signals are outputted to a National Instruments analog-to-digital converter (PCI-MIO-16E-4), digitally

processed, and viewed on a real-time basis via Somnologica Science®, Medcare, Reykjavik, Iceland).

Scoring and analysis

Data was scored by a trained observer using Somnologica Science by categorizing 10-second bins as wake, sleep, or REM. Sleep statistics reports were generated by Somnologica Science, and included hypnograms as well amount of time spent in each state, both before and after treatment. Following completion of the experiment, animals were euthanized with chloral hydrate, and their brains were extracted and stored in 10% formalin. After at least 2 days in formalin, brains were transferred to 30% sucrose for at least 1 day, following which time they were sliced on a freezing microtome. Sections were then stained with thionin to determine cannula placement. Two animals were found to have incorrect placements and were subsequently excluded from analysis.

Statistical analysis

All data is presented as mean ± standard error of the mean. Student's t-tests were used when comparing 2 groups with equal variance, Mann-Whitney tests were used when comparing 2 groups with unequal variance, and two-way ANOVA followed by Bonferroni post hoc tests were used when comparing more than 2 groups. ANOVA analysis assumes normally distributed data, and in two cases (Fig. 3.1, Fig. 3.2), the data was not normally distributed. Natural log transformation was performed, which resulted in normally distributed data, and statistics were conducted on the transformed data. Statistical analysis was conducted using Graphpad™ Prism 4.0c for Macintosh or PC (San Diego, CA).

3.4 Results

***Dbh* -/- mice are hypersensitive to modafinil-induced locomotion and wake.**

Modafinil dose-dependently increased locomotor activity in both control (*Dbh +/-*) and *Dbh -/-* mice (Fig. 3.1). There was a main effect of modafinil dose ($F(4,67) = 65.08$, $p < 0.0001$), genotype ($F(1,67) = 38.21$, $p < 0.0001$), and a dose x genotype interaction ($F(4,67) = 3.87$, $p < 0.007$). Post hoc tests revealed a significant response to modafinil compared to vehicle in both *Dbh -/-* and control mice at all doses tested. *Dbh -/-* mice tended to have a greater response at all doses, and the genotype difference was significant at the 12.5 and 25 mg/kg doses (Fig. 3.1). The results of the sleep latency experiments mirrored those of the locomotor activity experiments. Vehicle-treated *Dbh -/-* mice had a shorter sleep latency than vehicle-treated *Dbh +/-* mice, as previously reported (Hunsley and Palmiter, 2003) (Fig. 3.2). Modafinil dose-dependently increased sleep latency in both *Dbh +/-* and *Dbh -/-* mice, and *Dbh -/-* mice were hypersensitive to the wake-promoting effects of modafinil (Fig. 3.2). There was a significant effect of dose ($F(3,66) = 65.53$, $p < 0.0001$) and a dose x genotype interaction ($F(3,66) = 8.800$, $p < 0.0001$). All doses of modafinil tested significantly increased sleep latency in *Dbh -/-* mice, whereas only the highest dose tested (25 mg/kg) increased sleep latency in *Dbh +/-* mice. Furthermore, the highest dose of modafinil produced significantly longer sleep latency in *Dbh -/-* mice compared *Dbh +/-* mice.

Blockade of α1AR or DA receptors attenuates modafinil-induced locomotor activity and wake.

Antagonists of α1ARs can attenuate the locomotor-activating and wake-promoting effects of modafinil in rodents and non-human primates (Duteil et al., 1990; Hermant et al., 1991; Lin et al., 1992). Consistent with these results, pretreatment of control (*Dbh +/-*) mice with the α1AR antagonist, prazosin (0.5 mg/kg), attenuated modafinil-induced (50 mg/kg) locomotor activity (Fig. 3.3). In contrast, prazosin pretreatment had no effect on modafinil-induced locomotor activity in *Dbh -/-* mice (Fig. 3.3).

To determine whether DA signaling was critical for the effects of modafinil, mice were pretreated with the non-selective DA receptor antagonist, flupenthixol (0.025 or 0.25 mg/kg), 30 minutes prior to modafinil (50 mg/kg for locomotor activity, 25 mg/kg for sleep latency). While the lower dose of flupenthixol had no effect (data not shown), the higher dose decreased modafinil-induced locomotor activity in both *Dbh* +/- and *Dbh* -/- mice (Fig. 3.4). This dose of flupenthixol decreased exploratory locomotor activity (main effect of treatment; $F(1,28) = 7.74$, $p < 0.01$), but to a lesser extent than modafinil-induced locomotor activity (49% and 46% decrease in exploratory activity in *Dbh* +/- and *Dbh* -/- mice versus 76% and 83% decrease in modafinil-induced activity) (Fig. 3.5). Flupenthixol also attenuated modafinil-induced wake in mice of both genotypes (Fig. 3.6).

Direct infusion of terazosin into the PAG attenuates the wake-promoting effects of modafinil.

When *Dbh* +/- mice were infused with terazosin into the PAG prior to modafinil administration, their latency to onset of sleep was significantly shorter than that of mice administered saline pretreatment (Fig 3.7a). EEGs were conducted in rats to support and extend these findings, and sample EEG traces are shown (Fig 3.8). Modafinil significantly decreased the amount of time spent asleep, and this effect was partially reversed by a pretreatment of terazosin infused into the PAG ($F(3,15) = 7.592$, $p < 0.01$) (Fig 3.7b).

3.5 Discussion

Although the effects of modafinil appear to involve both NE and DA, the exact contribution of these two monoamines to the mechanism of modafinil action remains unclear. *Dbh* -/- mice completely lack NE but have hypersensitive DA signaling. Thus, it was hypothesized that if modafinil acts primarily via NE, then the behavioral effects of modafinil

would be attenuated in these mice, while if modafinil acts primarily via DA, *Dbh* -/- mice would be hypersensitive. As *Dbh* -/- mice were hypersensitive to both the wake-promoting and locomotor activating effects of modafinil, it is tempting to conclude that modafinil acts primarily via the dopaminergic system. However, this finding requires reconciliation with the reported effects of α1AR antagonists in attenuating the effects of modafinil. Lesioning of the locus coeruleus (LC), the major brainstem noradrenergic nucleus, using the selective noradrenergic neurotoxin N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4), had no effect on modafinil-induced wake behavior (Wisor and Eriksson, 2005). Because α1AR blockade attenuated the effects of modafinil in both intact and LC-lesioned animals, these authors proposed that modafinil acts by blocking DAT and increasing extracellular DA, which then directly stimulates α1ARs to promote wake (Wisor and Eriksson, 2005). However, there are a number of caveats to this model. Firstly, the potency of DA at cloned α1ARs is approximately 100-fold lower than that of NE (Zhang et al., 2004). Secondly, DSP-4 does not completely eliminate LC neurons, and in fact leaves ventral brainstem adrenergic and noradrenergic nuclei (e.g. A1, A2, C1, C2) intact (Fritschy and Grzanna, 1991). This is an important point, as projections from these nuclei provide the majority of the noradrenergic innervation to dopaminergic areas (i.e., ventral tegmental area, nucleus accumbens, PAG) and supplies NE and EPI to the hypothalamus (Delfs et al., 1998; Jones and Moore, 1977; Woulfe et al., 1990) which is a likely site of the wake-promoting effects of modafinil (Engber et al., 1998a; Lin et al., 1996; Scammell et al., 2000).

This hypothesis was tested in the present study by examining the effects of the α1AR antagonist, prazosin, in *Dbh* -/- mice. If modafinil acts by facilitating the ability of DA to directly stimulate α1ARs, then blocking α1ARs should attenuate the behavioral effects of modafinil whether or not NE is present. However, while prazosin attenuated modafinil-induced locomotor activity in control mice, it failed to do so in *Dbh* -/- mice. In contrast, the DA receptor antagonist, flupenthixol, attenuated the effects of modafinil in both control and *Dbh* -/- mice. Thus an alternate mechanism for modafinil-induced arousal may be proposed that partially depends on

NE/DA interactions (Fig. 3.9, “right” pathway). The noradrenergic system provides excitatory drive onto DA neurons via α1ARs, which are critical for DA release and responses to dopaminergic drugs like psychostimulants (Weinshenker and Schroeder, 2007). This is consistent with the hypothesis that modafinil produces its behavioral effects via weak blockade of both DAT and NET (Gallopin et al., 2004; Madras et al., 2006). NET blockade increases extracellular NE, which in turn activates α1ARs and promotes the firing of DA neurons (possibly in the PAG) and DA release in terminal regions. DAT blockade prevents the reuptake of the released DA, which then promotes the behavioral effects of modafinil by activating DA receptors. NET blockade also increases NE in other brain regions involved in sleep-wake regulation, such as the hypothalamus (Fig. 3.9, “left” pathway). Although *Dbh* -/- mice lack NE, they can bypass the requirement for α1AR stimulation because of hypersensitive DA receptors.

Blockade of DAT and NET by modafinil

Early studies showed that modafinil interacts with DAT with low affinity (IC50 ~ 2-6 μM), but had no effect on NET (Mignot et al., 1994). However, subsequent studies showed that modafinil increased extracellular levels of both DA and NE in vivo, and in fact only NE was elevated in the hypothalamus (de Saint Hilaire et al., 2001). Modafinil was reported to inhibit catecholamine uptake via cloned human DAT and NET in human embryonic kidney cells, and displaced both DAT and NET PET ligands from primate brain in vivo (Madras et al., 2006) although there are concerns about the robustness of this effect of modafinil on NET as in vivo data were only reported for one animal. In an in vitro study, modafinil suppressed the activity of sleep-promoting neurons in the hypothalamus in a NE-dependent manner, and its effects were mimicked by the selective NET blocker, nisoxetine (Gallopin et al., 2004). These results support an integral part of the present model, that modafinil blocks DAT and NET and increases extracellular DA and NE. Moreover, modafinil has several similarities with bupropion, an existing NET/DAT blocker used both as an antidepressant as an anti-smoking pharmacotherapy

(Wilkes, 2006). Bupropion increases locomotor activity in rodents (Mitchell et al., 2006), and a common side effect of bupropion therapy is insomnia (Wilkes, 2006). Future studies directly comparing the wake-promoting effects of modafinil and bupropion might yield interesting information, as specific affinities for each drug at DAT and NET could underlie differences in clinical efficacy. Validation of our model will require a more complete understanding of modafinil-transporter affinities and interactions across species.

Activation of α1ARs receptors is involved in modafinil-induced arousal

It has previously been reported that modafinil-induced arousal is reduced by α1AR antagonists and in α1b knockout mice (Duteil et al., 1990; Hermant et al., 1991; Lin et al., 1992; Wisor and Eriksson, 2005; Wisor et al., 2001), and this was confirmed in our experiment with prazosin in control mice (Fig. 3.3). However, prazosin failed to block modafinil-induced locomotion in *Dbh* -/- mice. This result is reminiscent of the pattern of response to typical dopaminergic agents; *Dbh* -/- mice have an increase in high affinity-state DA receptors and are hypersensitive to amphetamine, cocaine, and direct DA agonists, and these behavioral responses cannot be blocked by α1AR antagonists as they are in control mice (Schank et al., 2006; Weinshenker et al., 2002a). Thus, α1AR signaling appears critical for modafinil-induced arousal in normal animals via its effect on DA neuron activity and DA release, but the requirement for NE can be bypassed by hypersensitive DA receptors. We believe this is because NE, acting primarily via α1ARs, can provide excitatory drive onto midbrain DA neurons and facilitate DA release (Weinshenker and Schroeder, 2007). This supports the idea that the activation of α1ARs is important for DA transmission, as proposed in our model (Fig. 3.9, “right” pathway). These results suggest that the increase in extracellular NE following modafinil administration promotes arousal by activating α1ARs and facilitating DA transmission (Fig. 3.9, “right” pathway).

Activation of DA receptors is involved in modafinil-induced arousal

Early data indicated that DA antagonists could not block the behavioral effects of modafinil. For example, it was reported that D1 and D2 antagonists failed to prevent modafinil-induced locomotor activity in rodents (Duteil et al., 1990; Simon et al., 1995). However, additional examination of the actual data indicates that DA antagonists can attenuate modafinil responses under certain conditions. Both the D1 antagonist, SCH23390 (Simon et al., 1995) and the D2 antagonist, haloperidol (Duteil et al., 1990; Simon et al., 1995) partially inhibited modafinil-induced locomotor activity. It was argued that the antagonists did not suppress modafinil-induced locomotor activity to a greater extent than baseline locomotor activity. An additional caveat was that neither of these studies simultaneously examined D1 and D2 inhibition or examined the effects of DA antagonists on the wake-promoting effects of modafinil. More recently, it was shown that activation of D2 autoreceptors with quinpirole, which can inhibit DA release, attenuated the wake-promoting effects of modafinil (Wisor and Eriksson, 2005). In the present study, the D1/D2 antagonist flupenthixol had a larger suppressive effect on modafinil-induced locomotor activity than it did on exploratory activity. Furthermore, when the confound of DA antagonist effects on locomotor activity were controlled for by examining sleep latency, flupenthixol still attenuated the effects of modafinil. The fact that flupenthixol was effective in *Dbh* -/- mice indicates that the DA antagonist was acting downstream of NE signaling. These results are consistent with the final part of the proposed model, that the increased extracellular DA (brought about by dual DAT blockade and α1AR activation) promotes arousal by activating DA receptors. Although DA is not traditionally thought of as a regulator of the sleep-wake cycle, recent evidence indicates that DA can influence sleep states and promote wake (Berridge, 2006; Dzirasa et al., 2006; Isaac and Berridge, 2003; Lu et al., 2006; Wisor et al., 2001).

Anatomical correlates of the model

Where in the brain might these noradrenergic-dopaminergic interactions be occurring? Lu and colleagues recently identified a population of wake-active DA neurons in the ventral

periaqueductal gray (vPAG) that receive noradrenergic and adrenergic innervation (Lu et al., 2006). α1ARs are expressed in the vPAG (Jones et al., 1985; Pieribone et al., 1994), and α1AR agonists depolarize nearly all vPAG neurons (Vaughan et al., 1996). Thus, modafinil may increase NE in the vPAG and activate the wake-promoting DA neurons, which innervate other brain regions implicated in arousal such as the hypothalamus and prefrontal cortex. DAT blockade by modafinil in these regions could further amplify the wake-promoting signal (Fig. 3.9, "right" pathway). We tested this hypothesis by infusing the α1AR antagonist terazosin into the PAG prior to systemic modafinil administration, and then measuring behavioral sleep latency (mice) or EEG sleep architecture (rats). We found that, as predicted, blocking α1ARs in the PAG attenuates the wake-promoting effects of modafinil in both mice and rats. While more animals are needed to increase the power of these experiments, and more modafinil and terazosin doses should be tested, these results nevertheless suggest that α1ARs in the PAG are important for the mechanism of action of modafinil.

Limitations of the model

The primary caveat of our model is that we do not know the extent of direct NET blockade by modafinil in vivo. Modafinil can elevate extracellular NE in rats, and its electrophysiological effects can be mimicked by a selective NET blocker in brain slices (de Saint Hilaire et al., 2001; Gallopin et al., 2004). However, there are conflicting data sets on in vitro NET blockade (Madras et al., 2006; Mignot et al., 1994), and only one report of in vivo NET blockade (measured in a single monkey (Madras et al., 2006)), and species differences may exist (our unpublished data). It is also possible that modafinil cannot block NET in vivo at physiological doses but can increase extracellular NE via indirect pathways.

Although the proposed model can explain many of the previous findings on the role of catecholamines in modafinil-induced arousal, it cannot explain all of them. Most prominently, it fails to account for some of the observed behavioral, neurochemical and molecular differences

between modafinil and typical psychostimulants like amphetamine. For example, while *Dbh* -/- mice are hypersensitive to all doses of modafinil and high doses of amphetamine, they are actually resistant to the wake-promoting effects of low amphetamine doses (Hunsley and Palmiter, 2003). Canonical psychostimulants induce robust c-Fos expression in the striatum, while modafinil may not (Engber et al., 1998b; Lin et al., 1996). Furthermore, modafinil increased extracellular NE, but not DA, in the hypothalamus, which appears to be an important site of action for modafinil (de Saint Hilaire et al., 2001). Finally, modafinil suppressed sleep-promoting neurons in the hypothalamus in a NE-dependent manner, and its effects were mimicked by the selective NET blocker, nisoxetine (Gallopin et al., 2004). These data are consistent with previous studies indicating that NE potently increased wake via α1ARs in the hypothalamus and other brain regions (Berridge et al., 2003; Berridge and O'Neill, 2001). Taken together, these results indicate that NE plays a dual role in modafinil-induced arousal. Firstly, acting via α1ARs it facilitates DA transmission and promotes wake. Secondly, there appears to be a noradrenergic component of modafinil action that is independent of its effects on DA transmission and may involve suppression of sleep-promoting neurons in the hypothalamus. Third, blockade of α1ARs specifically in the PAG decreases the wake-promoting effects of modafinil. Further experiments will be needed to confirm various aspects of this model. There is also growing evidence that histamine release is an important mediator of modafinil-induced wakefulness. Because NE and histamine can reciprocally facilitate each other's release, a positive feedback loop may exist between these two neurotransmitters (Bealer, 1993; Prast et al., 1991). Thus, histamine may be acting in conjunction with the catecholamines to produce modafinil-induced arousal, but whether it occurs in parallel to or downstream of DAT/NET blockade remains to be elucidated (Ishizuka et al., 2003; Lin et al., 2008; Minzenberg and Carter, 2007).

Disclosure/conflict of interest

D. Bozyczko-Coyne and M. Williams are employees of Cephalon, Inc., and provided research funds for this study and partial support of D. Weinshenker and H. Mitchell.

Acknowledgements

We thank Sumitomo Pharmaceuticals (Osaka, Japan) for generous donation of the DOPS needed to maintain our Dbh mouse breeding program. This study was supported in part by Cephalon, Inc. Heather Mitchell has also been supported by NIH grant T32 GM 008602.

Figure 3.1 Effect of modafinil on locomotor behavior

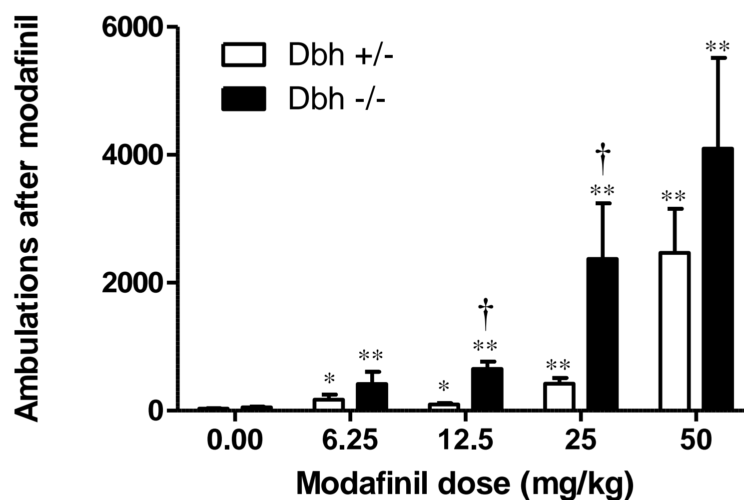


Figure 3.1 Effect of modafinil on locomotor behavior. *Dbh* +/- and *Dbh* -/- mice were placed in locomotor activity chambers (n = 6 – 12 per group). Four hours later, mice were injected with vehicle or modafinil (6.25, 12.5, 25, or 50 mg/kg, i.p.), and ambulations (consecutive beam breaks) were recorded for an additional 2 hours. Shown are the total ambulations for the 2 hours following modafinil administration. All data is presented as mean ± SEM. * $p < 0.01$, ** $P < 0.001$, compared to vehicle control for that genotype. † $p < 0.001$ compared to *Dbh* +/- mice for that dose.

Figure 3.2 Effect of modafinil on sleep latency

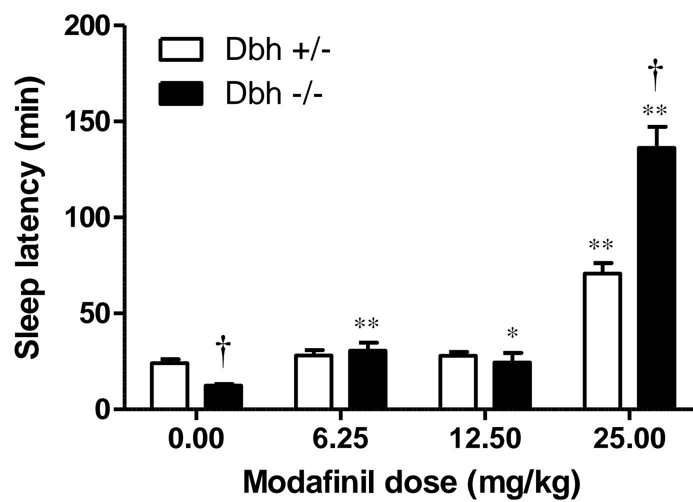


Figure 3.2 Effect of modafinil on sleep latency. *Dbh* +/- and *Dbh* -/- mice were placed in observation chambers, injected with vehicle or modafinil (6.25, 12.5, or 25 mg/kg, i.p.) 4 hours later, and observed until the onset of sleep (n = 8 – 13 per group). Shown is latency to sleep following the injection. * $P < 0.05$, ** $P < 0.001$ compared to vehicle control for that genotype. † $P < 0.01$ compared to *Dbh* +/- mice for that dose.

Figure 3.3 Effect of α1AR blockade on modafinil-induced locomotion

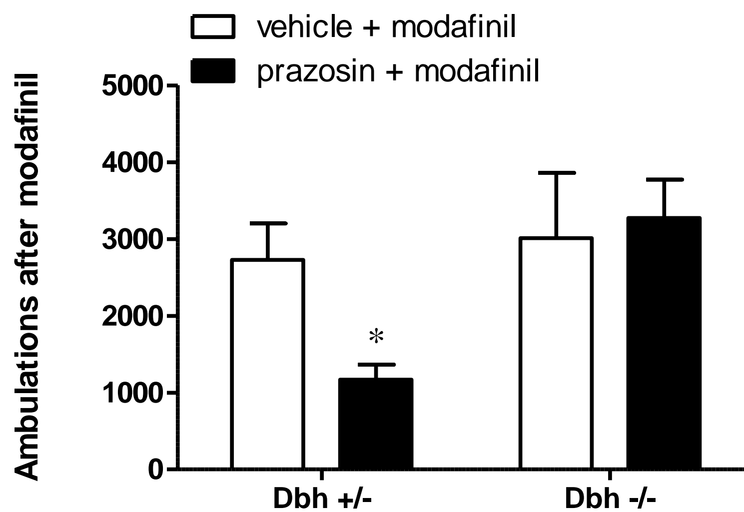


Figure 3.3 Effect of α1AR blockade on modafinil-induced locomotion. *Dbh* +/- and *Dbh* -/- mice were placed in locomotor activity chambers and injected with vehicle or prazosin (0.5 mg/kg, i.p.) 3.5 hours later (n = 8 – 11 per group). Thirty minutes following the pretreatment, mice were injected with modafinil (50 mg/kg, i.p.), and ambulations (consecutive beam breaks) were recorded for an additional 2 hours. Shown are the total ambulations for the 2 hours after modafinil administration. * $P < 0.05$ compared to vehicle control for that genotype.

Figure 3.4 Effect of DA receptor blockade on modafinil-induced locomotion.

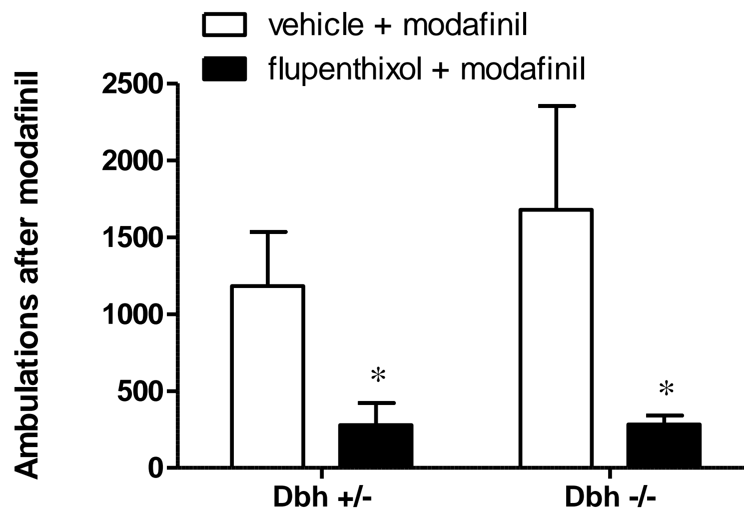


Figure 3.4 Effect of DA receptor blockade on modafinil-induced locomotion. *Dbh* +/- and *Dbh* -/- mice were placed in locomotor activity chambers and injected with vehicle or flupenthixol (0.25 mg/kg, i.p.) 3.5 hours later (n = 8 – 10 per group). Thirty minutes following the pretreatment, mice were injected with modafinil (50 mg/kg, i.p.), and ambulations (consecutive beam breaks) were recorded for an additional 2 hours. Shown are the total ambulations for the 2 hours after modafinil administration. * $P < 0.05$ compared to vehicle control for that genotype.

Figure 3.5 Effect of DA receptor blockade on locomotor behavior in a novel environment

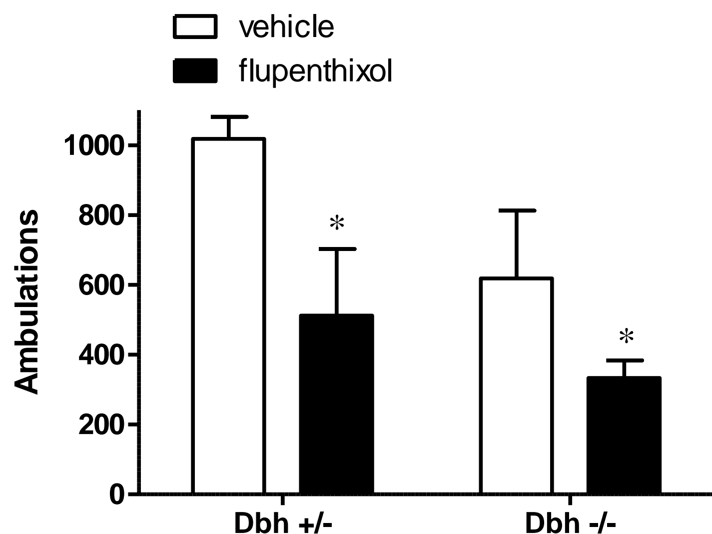


Figure 3.5 Effect of DA receptor blockade on locomotor behavior in a novel environment. *Dbh* +/- and *Dbh* -/- mice were injected with flupenthixol (0.25 mg/kg, i.p.), placed in locomotor activity chambers 30 minutes later, and ambulations (consecutive beam breaks) were recorded for an additional 2 hours (n = 8 per group). Shown are total ambulations for the 2 hours after being placed in the chambers. * $P < 0.05$ compared to vehicle control for that genotype.

Figure 3.6 Effect of DA receptor blockade on sleep latency in modafinil-treated mice

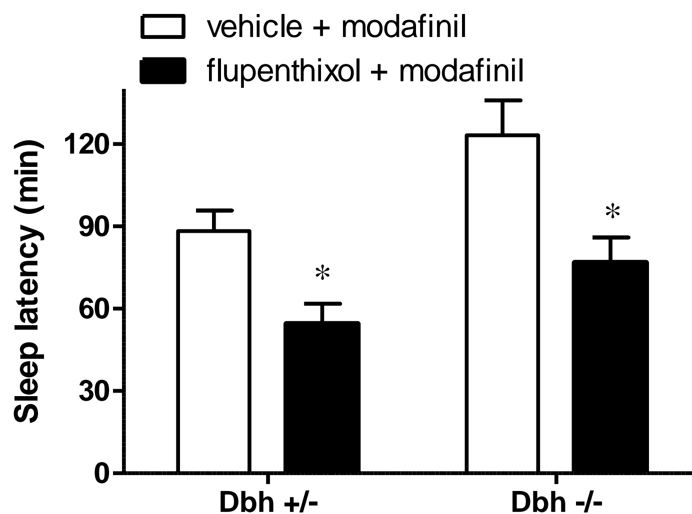


Figure 3.6 Effect of DA receptor blockade on sleep latency in modafinil-treated mice. *Dbh* +/- and *Dbh* -/- mice were placed in observation chambers, and injected with vehicle or flupenthixol (0.25 mg/kg, i.p.) 3.5 hours later (n = 7 – 8 per group). Thirty minutes following the pretreatment, mice were injected with modafinil (25 mg/kg, i.p.) and observed until the onset of sleep. Shown is latency to sleep following modafinil injection. * $P < 0.05$ compared to vehicle control for that genotype.

Figure 3.7 Infusion of terazosin into the PAG attenuates the affects of modafinil.

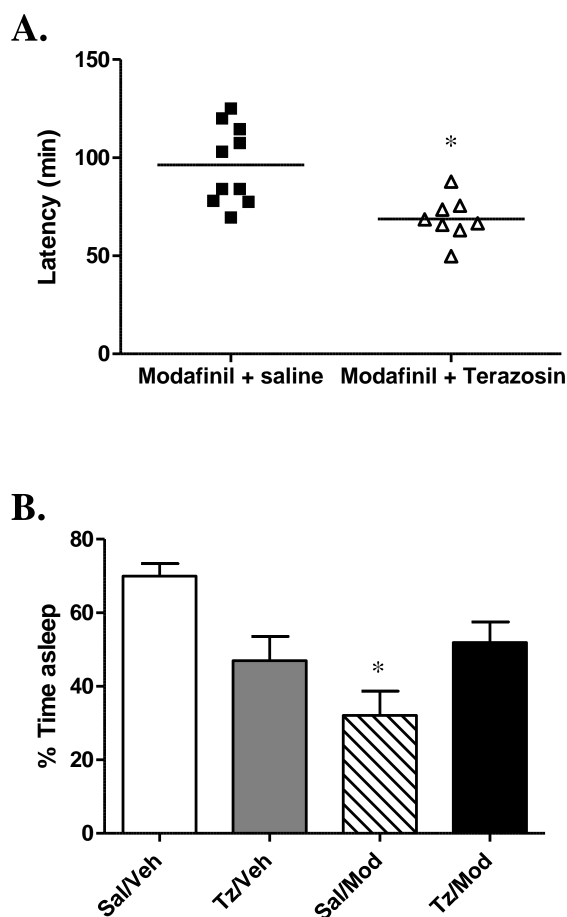
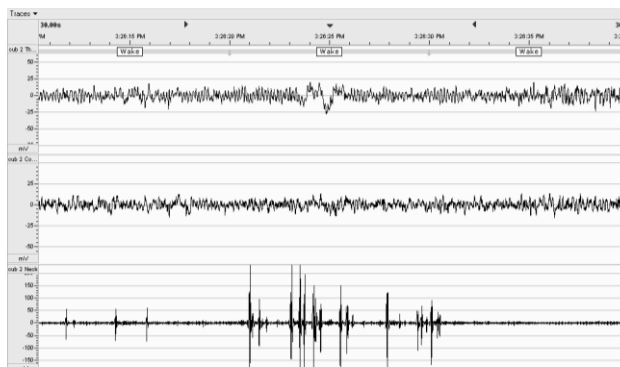


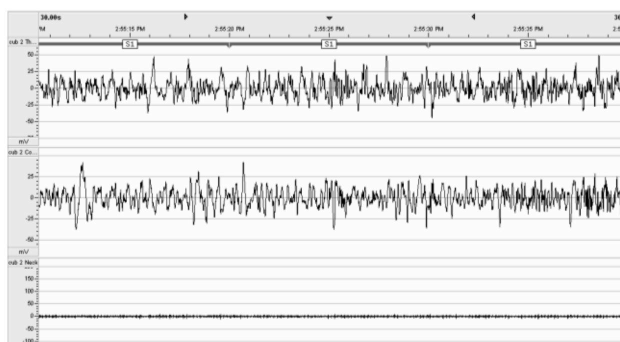
Figure 3.7 Infusion of terazosin into the PAG attenuates the affects of modafinil. (**A**) *Dbh* +/- mice were infused with vehicle (saline) or the α1AR antagonist terazosin (3 µg/0.5µl, intra-PAG) in their home cage prior to modafinil (25 mg/kg i.p.) or vehicle administration, and mice were observed until onset of sleep (n = 8 – 10 per group). Shown is latency to behavioral signs of sleep following modafinil injection. * $P < 0.005$ compared to vehicle control. (**B**) Rats were infused intra-PAG with saline or terazosin (3 µg/0.5µl, intra-PAG) prior to modafinil (50 mg/kg, i.p.) or vehicle (cyclodextrin), and placed in monitoring chambers (n = 3 – 6 per group). EEGs were recorded for a subsequent 2 hours. Shown is percent time spent asleep for 2 hours following modafinil injection. * $P < 0.01$ compared to saline pretreatment with a vehicle i.p. injection. Sal, saline; Veh, vehicle; Tz, terazosin; Mod, modafinil.

Figure 3.8 Representative EEG traces

A.



B.



C.

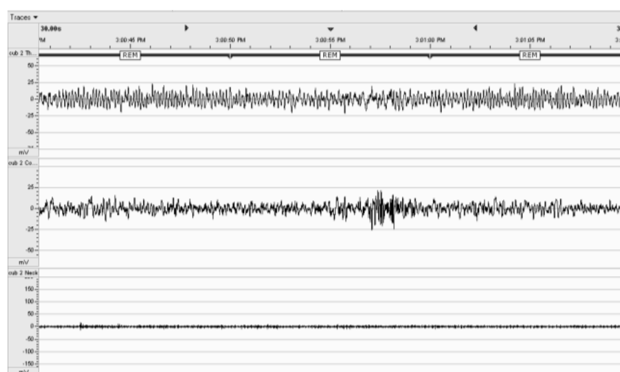


Figure 3.8 Representative rat EEG traces. Traces portraying wake (A), sleep (B), and REM (C) collected from rats used in Fig. 3.7 are shown. Individual traces are theta waves, EEG cortical waves, and muscle activity for each panel.

Figure 3.9 A hypothetical parallel pathway wiring diagram for modafinil-induced arousal.

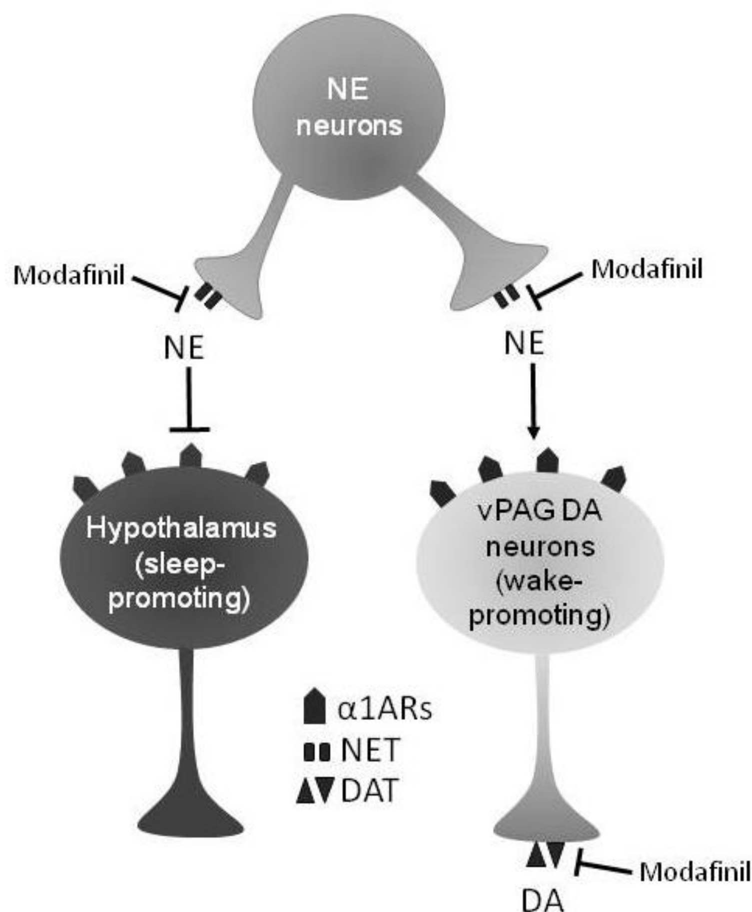


Figure 3.9 A hypothetical parallel pathway wiring diagram for modafinil-induced arousal.
Modafinil blocks NET and DAT. In the wake-promoting pathway (right), the increased extracellular NE signals via α1ARs to activate wake-promoting DA neurons in the ventral periaqueductal gray (vPAG). The DAT blockade prevents the uptake of the released DA, thus facilitating DA transmission in vPAG projection areas. Simultaneously, NE inhibits sleep-promoting neurons in the hypothalamus (and perhaps other brain regions). Arrow to DA neurons signifies excitation, and bar to hypothalamic neurons signifies inhibition. NE, norepinephrine; DA, dopamine; α1ARs, α1-adrenergic receptors; NET, norepinephrine transporter; DAT, dopamine transporter.

CHAPTER 4:

BEHAVIORAL ANALYSIS OF A MOUSE MODEL FOR LESCH-NYHAN DISEASE

4.1 Abstract

Lesch-Nyhan disease (LND) is a debilitating disorder caused by dysfunction of the hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene. Disruption of this gene in humans causes hyperuricemia, motor deficits, mental retardation, and self-injurious behavior. The mechanism underlying the neurological manifestations of this disorder remains unclear, as mice that lack HPRT do not display any behavioral phenotype of this disease. Mice lack another gene in the HPRT family that is normally expressed in humans, phosphoribosyl transferase domain containing protein (PRTFDC1), and it has been hypothesized that an interaction effect between loss of HPRT and presence of PRTFDC1 is required for the phenotypic expression of LND. In order to test this, transgenic mice that are knockout for HPRT and express human PRTFDC1 were created and tested for LND-like phenotypes using paradigms measuring locomotor activity, amphetamine-induced stereotypy, and aggression. Mice that both lacked HPRT and possessed the transgene for PRTFDC1 displayed a normal pattern of locomotor activity, both circadian, and in a novel environment. However, they exhibited a unique form of amphetamine-induced stereotypy reminiscent of finger biting behavior observed in LND patients. In addition, the knockout-transgenic mice had both a shorter latency to attack, and a greater frequency of attacks on intruder mice. These results indicate that mice lacking HPRT and expressing human PRTFDC1 display a hyperaroused state, characterized by a unique stereotypy response to amphetamine and increased aggression, which may recapitulate the self-injurious behavior and aggression seen in human LND patients.

4.2 Introduction

LND is a rare X-linked genetic condition that has severe behavioral and physiological pathology. The disease is caused by a deficit of HPRT, an enzyme in the purine metabolic pathway. Loss of this protein leads to a variety of symptoms affecting multiple systems, including metabolism, motor control, neurological function, and behavior (Jinnah et al., 2006; Schretlen et al., 2005). LND is a spectrum disorder, with severity of symptoms directly related to the degree of HPRT deficit. Loss of HPRT leads to a build up of uric acid and crystalization in the joints, which can be treated with pharmacological intervention (Torres and Puig, 2007). However, while medications can block production of uric acid and prevent gout, these medications do not alleviate the motor impairments and neurological symptoms of the disease. These symptoms include a variety of motor disabilities, but the hallmark of this disease and its most debilitating symptom is self-injurious behavior. In the most severe form of the disease patients frequently bite their own lips and fingers (often to such a degree that they bite their fingers off), bang their head against walls, doors, tables, etc, and poke their fingers into their eyes or into the spokes of a wheelchair. This self-mutilation is not due to lack of sensation, as patients do report feeling pain (Torres and Puig, 2007). While patients frequently suffer from mental impairments, they are still aware that they are inflicting the pain upon themselves, and often ask for physical restraints and act more comfortable when restrained and prevented from harming themselves or others (Torres and Puig, 2007). In addition to harming themselves, LND patients also display aggressive behaviors toward others. Along with the behavior, patients also exhibit a 70-90% depletion of brain dopamine (DA), particularly in the basal ganglia (Lloyd et al., 1981).

As LND is known to be caused by an almost complete loss of HPRT, it was an ideal candidate for a knockout (KO) mouse model. Two groups independently created HPRT KO mice, which exhibited a 19% reduction in DA levels but did not display any of the behavioral signs of the disease (Finger et al., 1988; Hooper et al., 1987; Kuehn et al., 1987). The HPRT KO mice did

show enhanced amphetamine-induced locomotion and stereotypy (Jinnah et al., 1991). While this is an interesting finding, it is not especially informative, as behavior was only measured on a numeric scale, with 0 representing sleeping, 8 representing a tonic-clonic seizure, and numbers in between indicating arousal level or a degree of stereotypy. No specific stereotypy behaviors were described, and therefore the analysis was rather vague. Furthermore, the doses of amphetamine administered were extremely high (up to 32 mg/kg), and beyond the typical range of amphetamine doses in behavioral studies (normally 0.5-5 mg/kg; e.g. (Weinshenker et al., 2002a)).

The fact that HPRT KO mice did not exhibit the behavioral signs of LND suggests that deletion of HPRT alone does not cause LND, and that other intereacting genes/proteins might be involved. One candidate for a potential interaction is PRTFDC1, a gene with an unknown function in the HPRT gene family (Nicklas, 2006). Keebaugh and colleagues characterized this gene in vertebrates, and discovered that it is functional in all vertebrates except the mouse (Keebaugh et al., 2007). This led to the hypothesis that an interaction between the loss of HPRT and the presence of PRTFDC1 causes LND; thus, the lack of PRTFDC1 in mouse may prevent the expression of LND-like behavioral symptoms in the HPRT KO mice. In order to test this hypothesis, Alaine Keebaugh and Jim Thomas (Department of Human Genetics, Emory University) created a transgenic mouse that expressed the human PRTFDC1 gene, and then crossed that mouse to the HPRT-deficient mouse to create a double mutant mouse that lacks HPRT but expresses human PRTFDC1 (HPRT KO/Tg). If the hypothesis is correct, then both lack of HPRT and expression of PRTFDC1 should recapitulate the behavioral symptoms of LND to a greater degree than HPRT KO alone.

The performance of 2 independent lines of HPRT KO/Tg mice were compared to several different control mice (HPRT KO alone, PRTFDC1 Tg alone, wildtype) in a battery of behavioral tests to look for any phenotypes that might parallel behaviors seen in human LND patients. As motor deficits are one of the defining characteristics of the disease, assays to examine overall

locomotor activity (both arousal in a novel environment and total circadian locomotor activity) were conducted. Amphetamine-induced stereotypy was of particular interest because (1) it can include behaviors such as nail biting that is reminiscent of LND, (2) previous studies revealed a vague hypersensitivity of the HPRT KO mice to amphetamine, and (3) amphetamine is an indirect DA agonist and thus acts on a neurotransmitter system that is altered in LND. Finally, the intruder-aggression paradigm was used to determine whether the HPRT KO/Tg mice show heightened aggression, similar to LND patients.

4.3 Methods

Animals and housing

PRTFDC1 Tg mice were created by Alaine Keebaugh in the lab of Dr. James Thomas, in collaboration with the Emory Mouse Transgenic Core (Keebaugh, 2009). The BAC clone RP11-129O07 containing the human PRTFDC1 transgene was inserted into fertilized FVB eggs via pronuclear injection. Presence of the PRTFDC1 gene was verified by PCR, and two independent lines were created using different founders. These mice were then backcrossed onto the C57BL6/J background for 5 generations. Once on the C57BL6/J background, the mice were crossed with HPRT KO mice obtained from Jackson Laboratories. Presence of the PRTFDC1 transgene and absence of HPRT were verified by PCR. In total, six genotypes were analyzed: 1) wildtype for both HPRT and the PRTFDC1 transgene (WT), 2) HPRT knockout, WT for PRTFDC1 transgene (HPRT KO), 3) WT for HPRT, expression of PRTFDC1 transgene line 9 (HPRT WT/Tg), 4) WT for HPRT, expression of PRTFDC1 transgene line 13 (HPRT WT/Tg), 5) HPRT KO, expression of PRTFDC1 transgene line 9 (HPRT KO/Tg), 6) HPRT KO, expression of PRTFDC transgene line 13 (HPRT KO/Tg). As LND is X-linked and therefore predominately exhibited by male patients, only male mice between 3 and 9 months of age were used for the analysis. All behavioral tests were conducted by an experienced investigator blind to mouse

genotype. Following completion of analysis, statistics revealed no differences between transgenic lines 9 and 13, and results from these two lines were combined.

All mice were reared in a specific pathogen-free facility with a 12 hour light/dark cycle (lights on - 7 am; lights off - 7 pm). Food and water were available ad libitum except during behavioral testing. All experiments were carried out in a quiet, isolated behavior room between 8:00 am and 5:00 pm. Mice were moved to this room at least 24 hours before testing. Experimental protocols were approved by the Emory University IACUC and meet the guidelines of the American Association for Accreditation of Laboratory Animal Care.

Locomotor activity

Because human LND patients display motor deficits, locomotor activity was tested, both in a novel environment, and over 24 hours. Locomotor activity was assessed using an automated system (San Diego Instruments, La Jolla, CA, USA) with photobeams that recorded ambulations (consecutive beam breaks). In order to test for exploratory behavior in a novel environment, mice were placed individually in the chambers and allowed to explore for two hours. All ambulations over the two hours were recorded. To examine circadian locomotor activity, mice were kept in the chambers following their novel activity test, in order to eliminate the activity spike following exposure to a new environment. Mice were kept in these chambers for an additional 24 hours, and food and water was provided. Total ambulations were recorded.

Sleep latency

Because DA transmission has been linked to arousal, sleep latency tests were conducted. Mice were placed individually in large plexiglass cages and allowed to acclimate for four hours. An i.p. saline injection was then administered, and mice were observed for behavioral signs of sleep. During sleep, mice exhibit a distinctive posture and breathing pattern that allows the observer to determine onset. Sleep was defined as 2 minutes of uninterrupted sleep behavior, and

75% of the next 10 minutes spent asleep (Hunsley and Palmiter, 2004; Mitchell et al., 2008). This behavioral scoring paradigm has been shown to reliably correlate with onset of sleep using EEG measurements (Hunsley and Palmiter, 2003; Hunsley and Palmiter, 2004).

Amphetamine-induced stereotypy

Stereotypical behaviors following amphetamine administration were analyzed to determine if the HPRT KO/Tg animals displayed any unique behaviors in response to a dopaminergic drug. Mice were placed individually in locomotor-monitoring chambers for two hours to acclimate to their environment, and then injected with amphetamine (5 mg/kg, i.p., 10 ml/kg injection volume) (Sigma-Aldrich). Thirty minutes after injection, mice were videotaped for an additional 30 minute test. The test was then scored for stereotypy by breaking the 30 minutes into 10 second bins, and recording the predominant behavior the mouse was engaged in during each bin. Behaviors included ambulating, rearing, sniffing, head-bobbing, nail-biting, and freezing.

Resident-Intruder aggression

As human LND patients frequently display aggressive behavior, a resident-intruder paradigm was used to assess aggressive behavior in the mice. Mice were individually housed for at least two weeks before the onset of this test. An intruder male mouse with a different coat color than the resident mouse was introduced into the cage of the resident mouse for a 5 minute videotaped session. Events scored included (1) whether the resident mouse attacked the intruder mouse, (2) the latency to the first attack, and (3) number of attacks. This test was then repeated twice, with two days in between each test, for a total of three trials.

Statistical analysis

All data is presented as mean ± standard error of the mean. Exploratory locomotor behavior and nail-biting stereotypy were analyzed using a one-way ANOVA. Circadian locomotor activity, aggression latency, and aggression attack number were analyzed with a two-way repeated measures ANOVA. Stereotypy behavior was analyzed with a one-way ANOVA. Bonferroni post hoc tests were conducted following ANOVA analysis. Percentage of attackers was assessed with a Chi square contingency table, followed by Bonferroni post hoc tests, adjusted for multiple comparisons. Statistical analysis was conducted using Graphpad™ Prism 4.0c for Macintosh or PC (San Diego, CA).

4.4 Results

Locomotor activity

Absence of HPRT and presence of PRTFDC1 transgene did not affect exploratory or circadian locomotor activity (Fig. 4.1). When the mice were exposed to a novel environment for two hours, mice of all genotypes displayed similar levels of exploratory locomotion (Fig. 4.1A). Examination of activity over 24 hours revealed that all mice displayed a normal circadian pattern of behavior, with high levels of activity during the dark period, and low levels during the light period (Fig. 4.1B). While post hoc tests revealed minor differences between HPRT WT/Tg and HPRT KO/Tg at the onset of the dark phase, there was no main effect of genotype on locomotor behavior.

Sleep latency

No significant differences were seen between the genotypes in behavioral sleep latency studies (data not shown).

Amphetamine-induced stereotypy

Amphetamine induced a combination of locomotor behavior and stereotypy in all animals tested (Fig. 4.2A). A two-way ANOVA revealed a significant interaction effect between genotype and type of behavior ($F(15,312) = 2.36$, $p = 0.0032$), and a significant effect of behavior ($F(6,312) = 108.2$, $p < 0.001$). WT, HPRT KO, and HPRT WT/Tg animals spent the majority of time ambulating, but also spent a considerable amount of time engaged in sniffing stereotypy. Some rearing stereotypy and freezing behavior were also observed. On average, HPRT KO/Tg mice were the only group to spend significantly more time engaged in stereotypy than locomotor behavior. In addition, the HPRT KO/Tg mice frequently displayed a peculiar behavior; they had a distinctive hunched posture, with their forepaws off the ground, and their head angled downwards. The mice brought their forepaws toward their mouths in a repetitive motion, while bobbing their heads toward the paws, and appeared to be biting or chewing. This behavior is henceforth referred to as nail biting, although subsequent examination of the paws and nails did not reveal any physical damage. HPRT KO mice occasionally displayed this behavior, but it was only seen in a few mice and the bouts were much shorter. Nail biting was not observed in mice of any other genotype.

Resident-intruder aggression

HPRT KO/Tg displayed a pattern of aggressive behavior in the resident-intruder paradigm that increased with each successive trial (Fig. 4.3). By the third trial, HPRT KO/Tg mice had the highest percentage of mice that attacked the intruder (A), had the shortest latency to onset of the first attack (B), and displayed the most number of attacks (C). A two-way repeated measures ANOVA of attack latency revealed a significant interaction effect between genotype and trial ($F(6,106) = 3.981$, $p = 0.0012$), as well as main effects of both genotype ($F(3,106) = 4.533$, $p = 0.0067$) and trial ($F(2,106) = 10.66$, $p < 0.001$). The HPRT KO/Tg mice had a significantly shorter latency to attack than any of the other genotypes by trial 3 (Fig. 4.3B). They also had a shorter latency to attack than the HPRT WT/Tg animals at trial 2. Additionally, the

HPRT KO mice had a shorter latency to attack than the HPRT WT/Tg mice at trial 2. When number of attacks were analyzed, there was a main effect of both genotype ($F(3,106) = 2.942$, $p = 0.0413$) and trial ($F(2,106) = 4.459$, $p = 0.0138$), in addition to a significant interaction between genotype and trial ($F(6,106) = 2.375$, $p = 0.0342$). Similar to attack latency, by the third trial, the HPRT KO/Tg mice attacked the intruder mouse more times than any of the other genotypes, and the HPRT KO mice attacked more times than the HPRT WT/Tg mice during trial 2.

4.5 Discussion

LND is characterized by a number of behavioral symptoms, which include injurious behavior, both self-directed and towards others, locomotor deficits, and mental deficits. When the mice were tested for gross locomotor activity, they displayed normal patterns of locomotion, both short-term exploratory, and over 24 hours. However, as reviewed by Jinnah, motor symptoms of LND in humans are varied and on a spectrum (Jinnah et al., 2006). Furthermore, while the mice displayed no obvious differences in arousal, tests were not conducted to look at more specific motor parameters. In addition, Jinnah reports that in humans, most extrapyramidal signs are minor at rest, and only become more pronounced under stress (Jinnah et al., 2006). It is possible that subjecting the HPRT KO/Tg mice to stress might affect their motor capabilities. In addition, locomotor paradigms that are more sensitive might reveal some differences from controls. Tests like stride length, challenging beam traversal, or the rotorod could show some motor abnormalities in the HPRT KO/Tg.

While no differences were seen in locomotor behavior, the HPRT KO/Tg did display a set of behaviors that could be considered a hyperaroused state. They have a unique response to amphetamine, which is of particular interest as the type of stereotypy displayed is similar to nail and finger biting common in LND human patients. While the HPRT KO mice also displayed a small amount of this behavior, the effect was much more robust in the HPRT KO/Tg mice,

indicating that the presence of the PRTFDC1 transgene facilitates this phenotype in the absence of HPRT. It is of note that none of the WT or HPRT WT/Tg controls exhibited this behavior at all, indicating that it is specific to loss of HPRT. In addition to demonstrating a unique type of stereotypy that may correlate to self injurious behavior in LND patients, the HPRT KO/Tg mice also displayed a pattern of aggressive behavior, similar to LND patients. HPRT KO/Tg mice were more likely to attack, had a shorter latency to attack, and attacked more frequently than mice of any other genotype.

Based on these studies, we posit that the HPRT KO/Tg mice recapitulate the behavioral signs of LND better than any of the currently existing models. These mice are unique from previous models in that they display both the genetic alterations and some of the behavioral symptoms of LND. Some behavioral signs of LND have been evoked in wildtype mice using pharmacological treatments. For example, chronic methylxanthine treatment was able to produce self-injurious behavior in Fischer rats, but much more rarely in Wistar rats, and also increased catecholamine turnover (Lloyd et al., 1981; Minana and Grisolia, 1986). When a variety of compounds (caffeine, clonidine, methamphetamine and others) were tested in C57BL/6 wild type mice and in HPRT KO mice, the degree of self-injurious behavior was found to be dependent on strain background, not genotype (Kasim et al., 2002). Thus, while it is possible to create self-injurious behavior pharmacologically, it is unclear if this approach would be successful for testing treatments or for identifying the underlying neurobiological cause of LND. Conversely, creating a model by replicating the genetic source of the disease instead of recreating the behavioral symptoms has not been successful either, as HPRT KO mice do not show behavioral signs of the disease (Finger et al., 1988; Hooper et al., 1987). No self-injurious or aggressive behavior was evident under general observation, HPRT KO mice were found to be comparable to WT in a battery of motor/behavioral tests conducted (locomotor activity, crossing a narrow bridge, open field, hindlimb reflex, foot slipping on a metal grid, passive avoidance of footshock, and swimming), and they had a normal response to a range of doses of the non-selective dopamine

agonist apomorphine (Finger et al., 1988). The only behavioral test that the HPRT KO mice had an altered response in was response to amphetamine, but as previously mentioned, amphetamine doses used in this study were abnormally high and the stereotypy was not described in detail (Jinnah et al., 1991). The HPRT KO/Tg mice appear to be superior, as they have the genetic (absence of HPRT), neurochemical (striatal dopamine depletion), and some of the behavioral symptoms (heightened aggression, amphetamine-induced stereotypy reminiscent of finger biting) of LND.

While results from these mice are indicative of a physiological interaction between HPRT and PRTFDC1, the mechanism by which this occurs is unclear. The two proteins have overlapping expression patterns, and these patterns in the mouse brain are reasonably consistent with patterns in the human brain (Keebaugh, 2009). Modeling studies predict that the proteins interact in a normal system, and possibly form a heterotetramer ((Rual et al., 2005); unpublished observations from the Thomas lab). In the absence of HPRT, PRTFDC1 protein levels decreased in the brain and the testes, both of which are affected in LND (Keebaugh, 2009). It is possible that HPRT acts somehow to stabilize PRTFDC1, and in its absence, PRTFDC1 becomes dysregulated, leading to downstream effects causing the behavioral symptoms of LND (Keebaugh, 2009). Additionally, it is possibly that a destabilized or altered PRTFDC1 is toxic to the DA system and causes the loss of DA in the basal ganglia. Further research is required to elucidate if these or other mechanisms are responsible for an interaction between HPRT and PRTFDC1 that may lead to behavioral signs of LND. Regardless of the mechanism, the results from these experiments clearly indicate that further study into PRTFDC1 is warranted to illuminate its potential role in the behavioral symptoms of LND and as a possible avenue for development of new treatment options.

Acknowledgements

We thank James Thomas, Alaine Keebaugh, and Jamie Davis for creation and maintenance of the mouse strains used in this study. Additionally, we thank Meriem Gaval, Kimberly Freeman and Galen Edwards for assistance with HPLC. This study was primarily funded by NIH grant 1R21NS060935. Heather Mitchell has been supported by NIH grants T32 GM 008602 and DA017963, and a grant from Cephalon Pharmaceuticals.

Figure 4.1 Lack of HPRT and presence of PRTFDC1 do not affect locomotor behavior

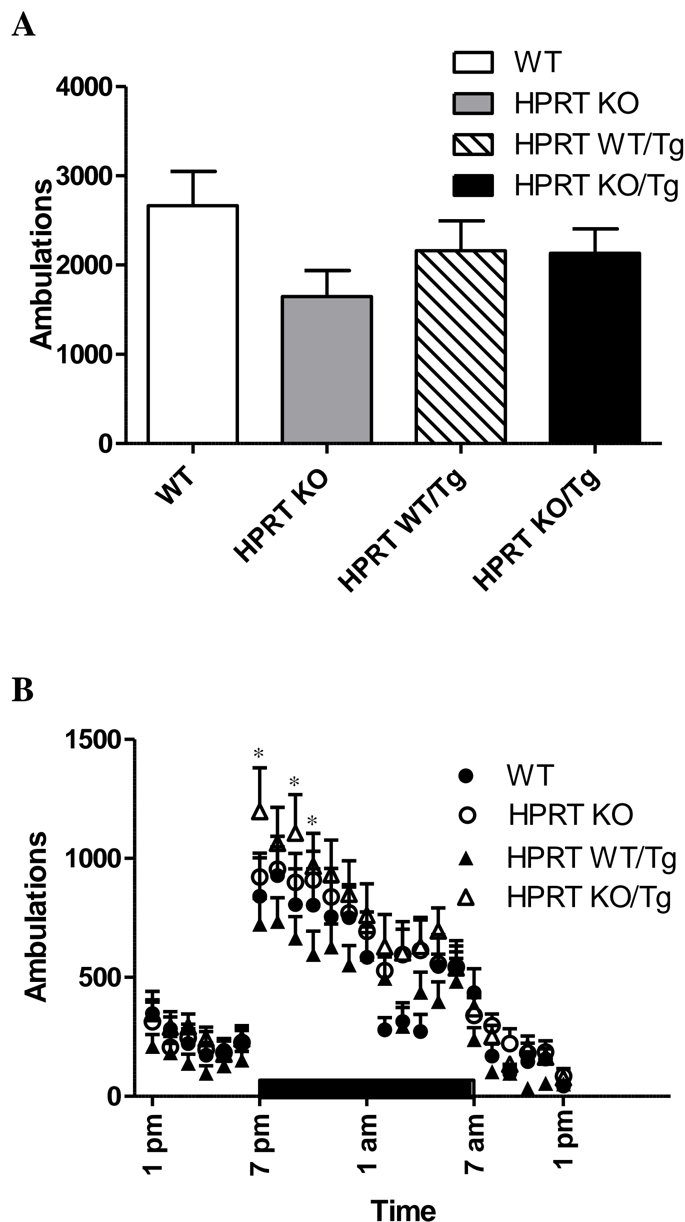


Figure 4.1 Lack of HPRT and presence of PRTFDC1 do not affect locomotor behavior. Mice were placed in locomotor-monitoring chambers and ambulations were recorded for 2 (**A**) or 24 (**B**) hours (n = 11 – 18 per group). Shown are mean ± SEM ambulations (consecutive beam breaks); * $P < 0.05$ between HPRT WT/Tg and HPRT KO/Tg.

Figure 4.2 Amphetamine-induced stereotypy

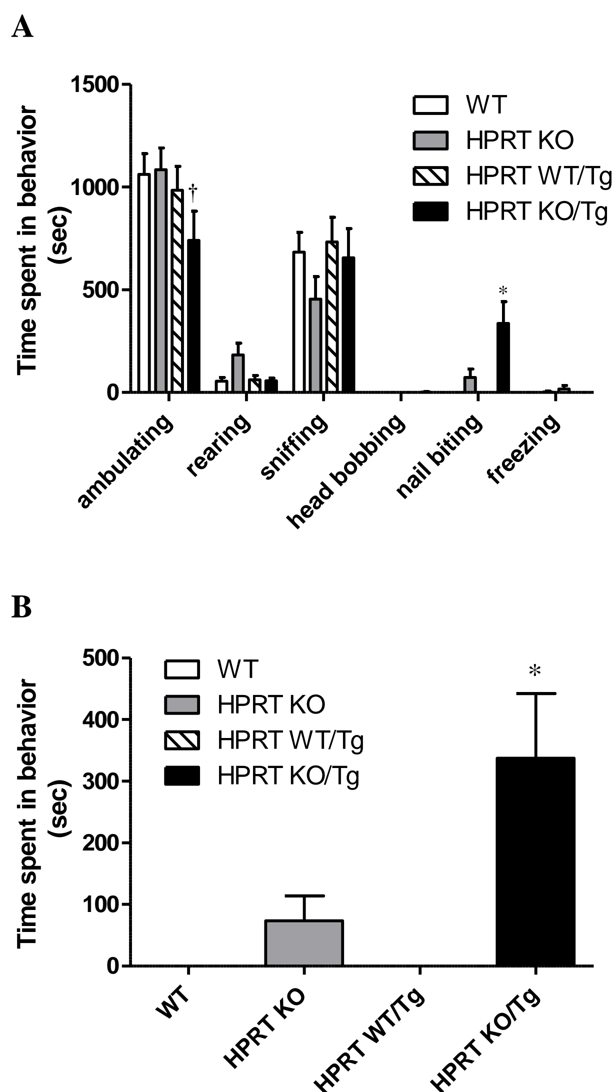


Figure 4.2 Amphetamine-induced stereotypy. Mice were scored for stereotypy behaviors following 5 mg/kg amphetamine (n = 11 – 18 per group). Shown is mean time in behavior ± SEM. A 30 minute videotaped test was split into 10 second bins, and behavior for each bin was recorded. (**A**) Total stereotypy behaviors observed. † $P < 0.05$ comparing HPRT KO/Tg to WT and HPRT KO. * $P < 0.05$ comparing HPRT KO/Tg to WT and HPRT WT/Tg. (**B**) Nail-biting stereotypy. * $P < 0.05$ comparing HPRT KO/Tg to all other genotypes.

Figure 4.3 Resident-Intruder aggression

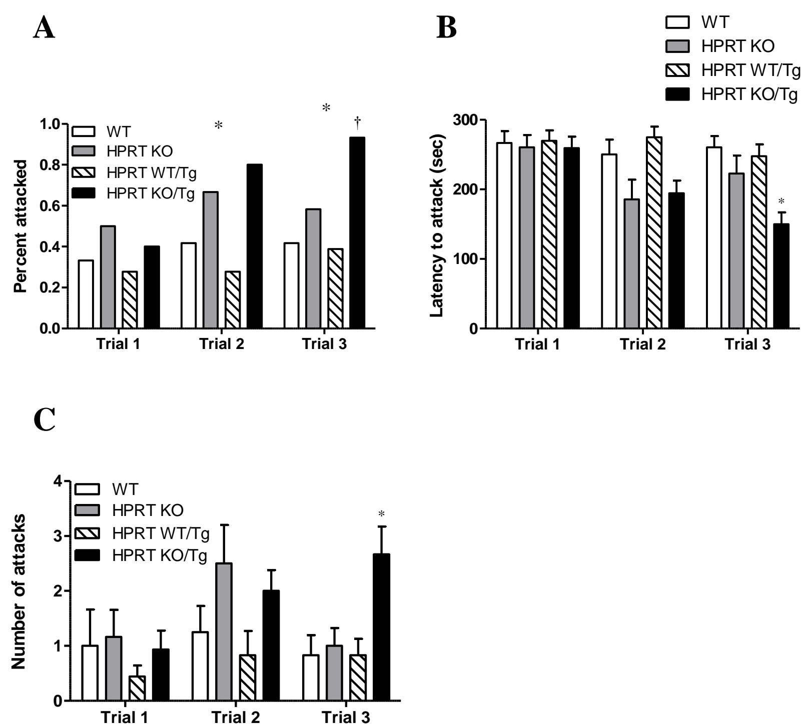


Figure 4.3 Resident-Intruder aggression. Mice were tested for intruder aggression in three separate trials, with two days in between trials (n = 11 – 18 per group). (**A**) Percentage of animals that attacked the intruder at each trial. * P < 0.05 for overall differences between groups within the same trial trial. † P < 0.05 compared to WT and HPRT WT/Tg within the same trial. (**B**) Mean latency to attack ± SEM for each genotype during each trial; * P < 0.05 compared to all other genotypes within the same trial. (**C**) Number of attacks ± SEM for each genotype during each trial; * P < 0.05 compared to all other genotypes within the same trial.

CHAPTER 5:
CONCLUSIONS AND FUTURE DIRECTIONS

We have demonstrated herein that catecholamines profoundly impact arousal-related disease states, and that pharmacological alterations of the catecholamine systems have a substantial effect on various types of arousal. We utilized a wide array of paradigms including spontaneous locomotor activity, ultradian hour locomotor activity, behavioral sleep latency, EEG sleep architecture, amphetamine-induced stereotypy, and resident-intruder aggression. Knockout mice are a particularly useful tool for studying the importance of various endogenous biological mediators, and in addition to wildtype (WT) mice and rats, we used knockout mice with alterations in their catecholamine systems including NET knockout (KO) mice that have elevated extracellular NE, *Dbh -/-* mice that cannot synthesize NE, and mice lacking HPRT that were also transgenic for PRTFDC1.

5.1 Antidepressants and locomotor behavior

First, we analyzed the impact of catecholamines in antidepressant-induced locomotor behavior. Many antidepressants are known to affect arousal in humans and locomotor behavior in animals. Because NE and DA are important regulators of arousal and locomotor behavior, we explored the impact of antidepressants that affect catecholamine levels on locomotor behavior. We administered reboxetine (selective NET blocker), desipramine (tricyclic NET blocker), imipramine (tricyclic NET/SERT blocker), venlafaxine (selective SERT/NET blocker), and bupropion (NET/DAT blocker) either acutely (i.p. injection) or chronically (via osmotic minipump), and then measured locomotor activity using an automated photobeam system. For the chronic experiments, we used doses of the antidepressants that produced serum levels comparable to therapeutic serum levels in human patients, so as to maximize clinical relevance (Ahern et al., 2006).

When the drugs were administered acutely, they suppressed locomotor activity, with the exception of venlafaxine, which had no effect on locomotion, and bupropion, which increased locomotion. Combining reboxetine with the DAT blocker GBR12909 increased locomotor behavior to a much higher level than vehicle, indicating that (1) DAT blockade can rescue the locomotor-attenuating effects of the NET blocker, and (2) the locomotor activating effects of bupropion are likely mediated by DAT blockade. After 18-21 days of administration via osmotic minipump, all antidepressants decreased locomotor activity, with the exception of bupropion, which had no effect. In addition, chronic reboxetine decreased home cage activity in a 24 hour circadian test, particularly in the dark phase when mice are usually most active.

To determine the specific role of the NET, we conducted further experiments in NET KO mice. NET KO mice have previously been shown to display decreased locomotor activity in response to a novel environment (Xu et al., 2000), and we replicated this finding. In addition, acute reboxetine in WT mice mimicked the effects of genetic ablation of NET, but had no effect in NET KO mice, indicating that the effect of reboxetine on locomotor behavior is due specifically to NET blockade. Chronic reboxetine also suppressed locomotor behavior in WT animals. Either acute or chronic administration of bupropion increased locomotor activity in WT and NET KO mice, indicating that its arousing effects are not dependent on NET and are likely due to DAT blockade.

This was the first study that examined the impact of NET-acting antidepressants on locomotor behavior using therapeutically-relevant doses. Through this study, we were able to ascertain that chronic NET blockade decreases locomotor behavior in either a novel or familiar environment. Combination with DAT blockade was able to reverse this effect, as seen with bupropion and GBR12909. Furthermore, utilization of NET KO mice enabled us to determine the specific role of the NET in these effects, as antidepressant drugs are notorious for off-target effects. These results provide important new data on the catecholamines and arousal behavior. Increases in NE typically increase exploratory behavior; however, antidepressants that block

NET, and thereby increase extracellular NE, decreased spontaneous locomotor activity in our model. This can be reconciled by considering that increased levels of NE also activate inhibitory α2-autoreceptors, resulting in decreased noradrenergic neuron firing and NE release. Long-term administration of NET blockers also resulted in a decrease of locomotor activity, most likely through other mechanisms such as down-regulation of adrenergic receptors or internalization of NET. However, simultaneous blockade of both NET and DAT by bupropion had the opposite effect and increased locomotor behavior. This suggests that increasing extracellular DA by acute inhibition of DAT does not diminish the ability of DA to increase locomotor behavior, and thus DA may be under less negative feedback control than NE. Additionally this could indicate that when both transporters are blocked, as in the case of bupropion or combination of reboxetine and GBR12909, compensatory mechanisms occur in an attempt to normalize levels of arousal (Fig. 5.1).

These studies are important because many depressed patients experience alterations in arousal, either as a symptom of their depression, or as a result of their antidepressant pharmacotherapies. Learning more about which antidepressants increase arousal, which medications decrease it, and how these alterations occur, will provide better information for physicians prescribing antidepressants, and allow them to better customize treatment for all symptoms of depression.

5.2 Catecholamines in the mechanism of action of modafinil

We also developed and tested a novel model for the mechanism of action of the widely-used wake-promoting medication, modafinil. Previous research had shown that the catecholamines were important for the mechanism of action of modafinil, but the role that each played, and the manner by which they interact, was unclear. In order to address these questions, we conducted experiments in *Dbh -/-* mice with the hypothesis that if modafinil acts primarily

through NE, the mice would be nonresponsive, whereas if modafinil acts primarily through DA, the mice would be hypersensitive. We found that the *Dbh -/-* mice were hypersensitive to the wake- and locomotor-promoting effects of modafinil, indicating an important role for DA in its mechanism. However, this must be reconciled with the finding that α1AR antagonism attenuates the effects of modafinil. To test the hypothesis that DA was acting by binding and activating α1ARs directly, we administered the α1AR antagonist prazosin prior to modafinil and measured locomotor behavior. We observed that prazosin blocked the effects of modafinil in control, but not in *Dbh -/-* mice. Furthermore, the DA receptor antagonist flupenthixol was able to suppress the wake- and locomotor-promoting effects of modafinil in both control and *Dbh -/-* mice. Combined, these results indicate that (1) both DA and α1AR signaling is necessary for modafinil-induced arousal in normal mice, (2) excessive DA signaling can bypass the requirement for NE transmission, and (3) DA is acting through DA receptors and not α1ARs.

Based on these findings and existing literature, we developed a model of modafinil action that includes both catecholamines. We theorized that modafinil blocks DAT and NET, thereby increasing extracellular concentrations of DA and NE, which then act downstream. In this model, the NE plays a dual role, both to inhibit sleep-promoting neurons in the hypothalamus, and also to excite wake-promoting neurons via α1ARs. One promising candidate for these wake-promoting neurons is the dopaminergic neurons of the vPAG that were recently identified by Saper's group (Lu et al., 2006). We investigated this hypothesis by infusing the α1AR antagonist terazosin directly into the PAG, and then administering modafinil systemically. In both mice and rats, terazosin in the PAG attenuated the wake-promoting effects of modafinil, providing support for this branch of our proposed model (Fig. 5.2).

Modafinil has been used to treat conditions of the arousal system for over 10 years, yet its precise mechanism has yet to be determined. These studies provide novel and important evidence about the role of catecholamines in the mechanism of action of modafinil. Our experiments

showed that interactions between NE and DA are necessary for modafinil's mechanism of action, and proposed a new model for how these interactions may be occurring. The intracranial infusion studies are especially valuable as they demonstrate that blocking α1AR transmission in a specific population of wake-promoting DA neurons can attenuate modafinil's effects. Additionally, by showing that flupenthixol could block the wake-promoting effects of modafinil in a sleep-latency paradigm, we provided further support for the concept that DA is an important mediator of sleep states. While further studies would provide additional support to our proposed model, these experiments demonstrate new evidence for how modafinil may be exerting its wake-promoting effects. These and future studies may form the basis for better treatment of arousal disorders, and aid in the development of the next generation of sleep/wake pharmacotherapies.

5.3 A novel mouse model for Lesch-Nyhan Disease

Finally, we characterized a new animal model for Lesch-Nyhan Disease, created by crossing HPRT KO mice with transgenic mice expressing the human form of PRTFDC1. While loss of HPRT in humans leads to LND, characterized by a host of neurological and behavioral symptoms, deletion of HPRT in mice has no discernable behavioral phenotype. It was hypothesized that this discrepancy was due to the fact that mice lack PRTFDC1, a protein in the HPRT family that is expressed in most vertebrate species, including humans. In order to test this, PRTFDC1 transgenic mice were created and crossed to HPRT KO mice, and then behaviorally characterized for phenotypes reminiscent of LND. While the mice showed no obvious deficits in basal locomotor activity, they did demonstrate other behaviors consistent with aspects of hyperarousal that correlate with the symptoms of LND. HPRT KO/Tg mice were more aggressive than any of the control genotypes, by all parameters examined. In addition, the HPRT KO/Tg mice exhibited a unique type of stereotypy reminiscent of finger biting behavior seen in human LND patients.

No treatment exists for the most debilitating symptoms of LND, and no animal model has been able to fully recapitulate the genetic, behavioral, and neurochemical impairments of this condition. While the HPRT KO/Tg mouse model does not perfectly replicate the human phenotype, it is the only model that includes loss of HPRT protein, reduction of DA in the caudate putamen, and the emergence of behavioral signs of the disease such as aggression and an amphetamine-induced stereotypy reminiscent of the finger biting behavior seen in human patients. This is extremely valuable because it provides a new animal model to further study the disease and also indicates a novel interaction between HPRT and PRTFDC1 that may underlie symptoms of LND. Learning more about PRTFDC1 and how it interacts with HPRT may provide an important new direction in understanding the mechanisms behind the behavioral signs of this condition and a platform for the development of new treatment possibilities.

While the source of the behavioral symptoms of LND is unclear, it is possible that they may be a result of dopaminergic deficits (Fig. 5.3). When challenged with amphetamine, the HPRT KO/Tg mice exhibited more stereotypy overall, and the emergence of a type of stereotypy unique to the HPRT KO/Tg. The HPRT KO also displayed this nail biting behavior, although to a much lesser extent. Since amphetamine induces DA release, and DA receptor antagonists can blunt amphetamine-induced stereotypies, it is conceivable that the dysregulated DA systems in HPRT KO/Tg mice underlie the expression of this unusual stereotypical behavior. However, it should be pointed out that the presence of the PRTFDC1 transgene facilitates stereotypy without significantly altering the degree of striatal DA reduction seen in the HPRT KO mice, suggesting the engagement of additional mechanisms. Nevertheless, the increased aggression and stereotypy may be considered deficits in arousal – in fact hyperarousal – which has been shown, in this document and others, to be regulated in part by DA. While the mechanism underlying these dopaminergic deficits is not yet known, it appears that DA is a crucial component for the phenotypic expression of these symptoms, and learning more about the interaction between HPRT and PRTFDC1and how they impinge on the dopaminergic system warrants further study.

5.4 Summary

The goals of this report were to learn more about how catecholamines impact both natural and pharmacologically-induced arousal. We wanted to explore how catecholamines are involved in modulation of arousal states through pharmacological treatments, and to gain a better understanding of how diverse diseases and their pharmacotherapies that impact catecholaminergic systems affect arousal. We achieved this through the use of catecholaminergic drugs such as antidepressants that block NET, modafinil, which likely blocks NET and DAT, and amphetamine, which induces catecholamine release. We administered these drugs to rats or WT and mutant mice and examined behavioral paradigms for arousal. In addition, we characterized a novel mouse model for LND, a disorder which is known to have losses in DA, and behavioral symptoms that include motor disturbances and aggression, both of which represent forms of arousal.

Through these experiments we have been able to increase our understanding of several aspects of arousal and its pharmacology. We learned that inhibition of the NET by antidepressant medications decreases locomotor behavior unless combined with inhibition of DAT. Our studies also demonstrated evidence that the mechanism of action of modafinil, a key wake-promoting medication, requires complex interactions between NE and DA. This particular set of findings allowed us to formulate a new, testable model for modafinil-induced wakefulness. Finally, we conducted behavioral characterization of a novel mouse model for LND, and found that this mouse model had aspects of hyperarousal, as measured by increased aggression and a unique stereotypical response to amphetamine. The source of this altered arousal may be related to the reduction of striatal DA in these mice. These findings all indicate that the catecholamines NE and DA interact to produce arousal patterns, and that manipulation of either or both catecholamines can induce either heightened or reduced arousal.

5.5 Future Directions

While these results address several outstanding questions in arousal literature, further experiments could be conducted to extend these findings. Our study was the first to analyze the impact of a range of chronically-administered antidepressants on locomotor behavior, but further experiments should examine the same antidepressants on sleep. While we have made valuable contributions toward elucidating the mechanism of action of modafinil, questions remain to be answered, including the location of the critical DA receptors and their subtype and the degree to which modafinil binds NET in vivo. Finally, we have characterized a new mouse model for LND based on a novel proposed interaction between HPRT and PRTFDC1, but the manner in which these two proteins interact and how they regulate DA levels has yet to be determined.

Noradrenergic antidepressants and sleep

While we learned that antidepressants that block the NET decrease arousal as measured by locomotor activity, a systematic analysis of chronic antidepressant administration on sleep behavior should also be conducted. As mentioned in Chapter 1 of this document, many antidepressants alter sleep behavior, and others are actually used to treat insomnia. Surprisingly, a study examining chronic administration of multiple noradrenergic antidepressants on sleep behavior in an animal model has not been done. Performing this study would greatly enhance knowledge about antidepressants and sleep, and provide important information for treatment of both depression and sleep disorders. These experiments should use the same antidepressants as used in the locomotor study, administered via minipump, but instead of measuring locomotor behavior, EEG activity should be measured. This would allow for direct comparison of noradrenergic antidepressants on sleep activity, conducted in the same animal model, using clinically relevant doses. As decreased or fragmented REM sleep is the most frequent disruption

of sleep that occurs with antidepressant administration, it will be important to conduct EEGs so as to observe the impact of these medications on REM sleep. Chronic drug administration would best mimic the human therapeutic dosing regimen for antidepressants, particularly since antidepressants typically require three to four weeks to alleviate depressive symptoms. Additionally, changes occur in the catecholaminergic systems following long-term antidepressant administration that may affect sleep differently than acute administration.

Modafinil and catecholamines

We have developed and provided support for a new model of modafinil efficacy. One of the strengths of the model is that essentially all of its aspects are testable. We have proposed that modafinil blocks the DAT and NET to increase extracellular concentrations of DA and NE, but the evidence that modafinil blocks these transporters is still controversial. Early studies indicated that modafinil binds the DAT, albeit with low affinity, and more recent work shows evidence of NET binding, though with even lower affinity than the DAT (Madras et al., 2006; Mignot et al., 1994). While these low binding affinities may be the reason modafinil only increases wake and lacks the reinforcing effects or abuse potential of other NET and DAT blockers such as methylphenidate or cocaine, further studies should be conducted to confirm that modafinil does indeed block both transporters. In addition to traditional in vitro binding studies, this possibility could be explored with in vivo PET studies similar to the Madras studies (Madras et al., 2006), but utilizing more doses and animals, or even conducting PET studies in humans. Some of this would depend on the development and availability of a good NET PET ligand. Furthermore, experiments could be conducted in knockout mice. While studies have administered modafinil to both DAT and NET KO mice ((Wisor et al., 2001); our own observations, Appendix 1), mice that have lacked these transporters since birth have developed compensatory mechanisms that may alter their responses to modafinil. Using conditional knockouts to eliminate one or both of these transporters immediately prior to experiments would remove this confound.

The next part of our proposed model deals with activation of specific regions of the brain. We proposed that excess NE from blockade of NET activates α1ARs on the dopaminergic PAG neurons, and found support for this from our local infusion studies. We also proposed that NE has an inhibitory effect on sleep promoting neurons in the hypothalamus, possibly the VLPO. The next set of experiments should explore this possibility by infusing terazosin into the VLPO, providing systemic modafinil, and then measuring EEG activity. It is possible that VLPO neurons are inhibited by α2ARs instead of α1ARs, so we could additionally infuse an α2AR antagonist such as yohimbine. We also postulated certain populations of wake-promoting neurons, such as the anterior hypothalamus and the prefrontal cortex, that are a target for the downstream DA branch of the pathway. Both of these regions are involved in sleep/wake maintenance and contain DA receptors. Cannulae could be implanted into these regions, and a dopaminergic antagonist such as flupenthixol (or selective D1 or D2 antagonists) infused prior to systemic modafinil administration. Again, EEGs should be used to measure wake and sleep. In these ways, discrete aspects of our model could be tested systematically, and a clearer picture of the role of catecholamines in modafinil-induced wakefulness will emerge.

Interactions between HPRT and PRTFDC1

We have helped to characterize a novel mouse model for LND, based on an interaction between HPRT and PRTFDC1, but many outstanding questions about this interaction still exist. In order to address these questions, several experiments could be conducted to characterize the interaction between these proteins. First, while the two proteins have overlapping expression patterns, it is not known whether they actually exist in the same cells. Double label immunocytochemistry at the electron microscopic level could be conducted to determine if the proteins are located in the same cells, and if they are localized in a manner that predicts direct contact. Next, coimmunoprecipitation studies should be conducted to determine if they bind each other, and if they are found to interact, site-directed mutagenesis based on sequence analysis

should be conducted to determine the binding site. Knowledge of the crystal structure of these proteins could also be a useful tool. Furthermore, comparison of mRNA expression in the brains of HPRT KO mice compared to HPRT KO/PRTFDC1 transgenic mice could lead to discovery of interesting differences in pathways.

The primary rationale behind the creation of HPRT KO/PRTFDC1 transgenic mice was to test the hypothesis that HPRT KO mice do not show signs of LND because mice lack PRTFDC1. However, most other vertebrate animal species do possess PRTFDC1, and it would be interesting to remove HPRT in a different model species, such as a rat, and conduct the same behavioral and neurochemical studies. Knockout rat technology is just coming online, and replicating these experiments in this model that innately expresses PRTFDC1 would enable us to learn more about the interactions between HPRT and PRTFDC1. Furthermore, the same behavioral tests that were conducted on the mice could be easily conducted in rats with only minor modifications. It is also possible that some of the neurological LND symptoms are unique to the primate lineage. The first transgenic monkey model of a human neurological disease was created here in the Emory Department of Human Genetics and Yerkes by Dr. Anthony Chan (Yang et al., 2008), and the future of LND work may require this type of effort.

Finally, we hypothesized that the behavioral changes seen in the HPRT KO/Tg mice may be due to their dopaminergic deficits. It is possible that losses in DA in the caudate putamen result in upregulation of DA receptors or their downstream signaling components, and when these pathways are stimulated, it causes the extreme behaviors. The DA systems of the HPRT KO/Tg mice should be better characterized, including the DA biosynthetic and metabolic pathways, DA receptors and their coupling to second messenger systems, and transporter levels. It would be interesting to determine whether dopaminergic antagonists could attenuate the aggressive or stereotypical behavior observed in the HPRT KO/Tg mice. In addition, as DA frequently interacts with NE, it is possible that noradrenergic systems are also dysregulated in these mice. While HPLC did not reveal any gross differences, there still could be changes in adrenergic receptors or

signaling. Other studies of interest would include further research into the normal function of PRTFDC1, and development of inhibitors with a goal towards clinical use for treatment of LND.

Completion of these proposed studies would enhance the findings generated in this report and contribute greatly to the understanding of catecholamines and arousal. This would allow for an increased understanding of how antidepressants affect sleep, which would improve treatment for both sleep disorders and depression. Learning the mechanism of action of modafinil would greatly aid in the development of new drugs for sleep and wake disorders, a major problem health problem. Finally, understanding the interaction between HPRT and PRTFDC1, and how this interaction contributes to the behavioral symptoms of LND, may enable the development of treatments for this incapacitating condition.

Figure 5.1 Effect of antidepressants on locomotor activity

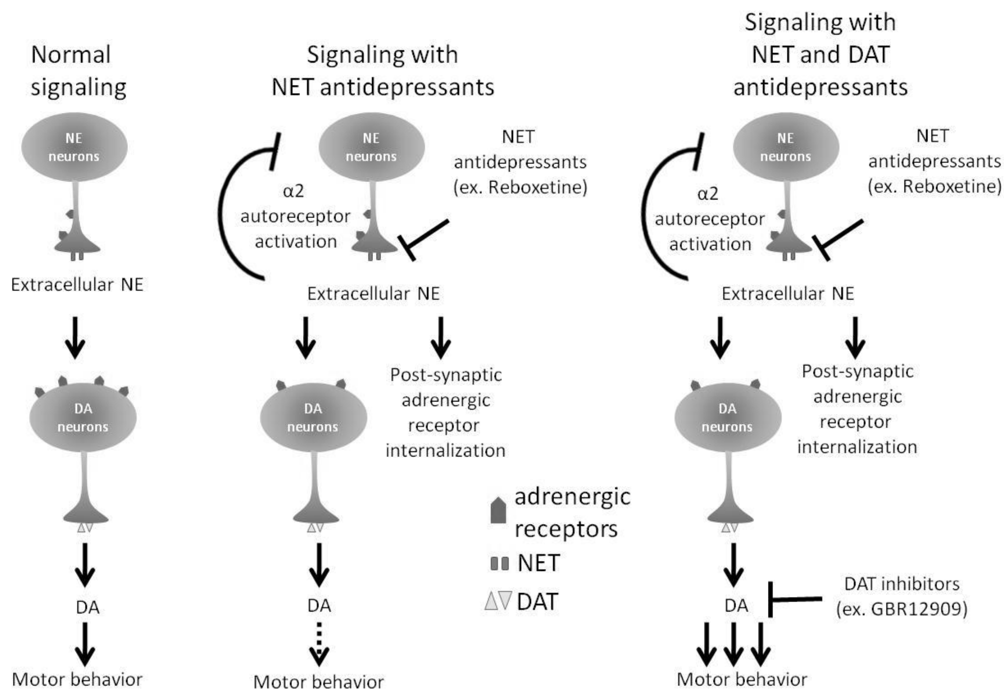


Figure 5.1 Effect of antidepressants on locomotor activity. Under normal signaling, released NE acts through adrenergic receptors on DA neurons to help increase locomotor behavior. NET inhibitors block the NET, preventing reuptake of NE, thereby increasing extracellular NE. This increased NE can cause receptor internalization in the long-term, and activation of inhibitory alpha2 autoreceptors in the short-term. Activation of autoreceptors causes decreases in NE release, which prevents the stimulatory effects of NE onto DA neurons. Without this stimulation, less DA is released, decreasing motor behavior. In contrast, DAT inhibitors increase extracellular DA, promoting motor behavior. When DAT and NET are blocked simultaneously, as in the case of bupropion or reboxetine in combination with GBR12909, the degree of DAT blockade is enough to overcome the locomotor inhibitor effects of NET blockade.

Figure 5.2 A hypothetical parallel pathway wiring diagram for modafinil-induced arousal.

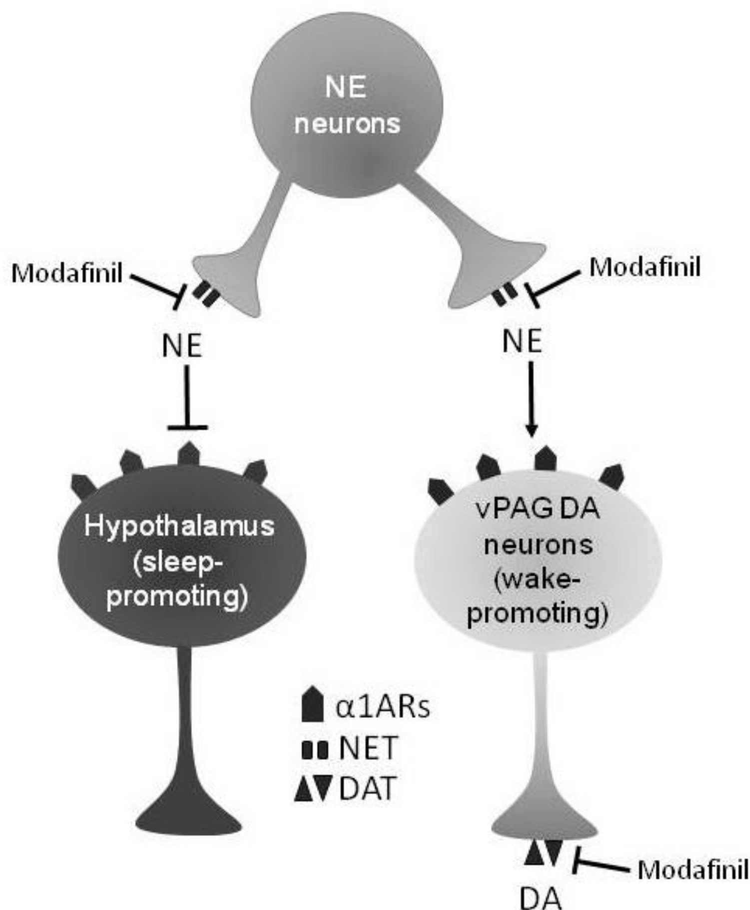
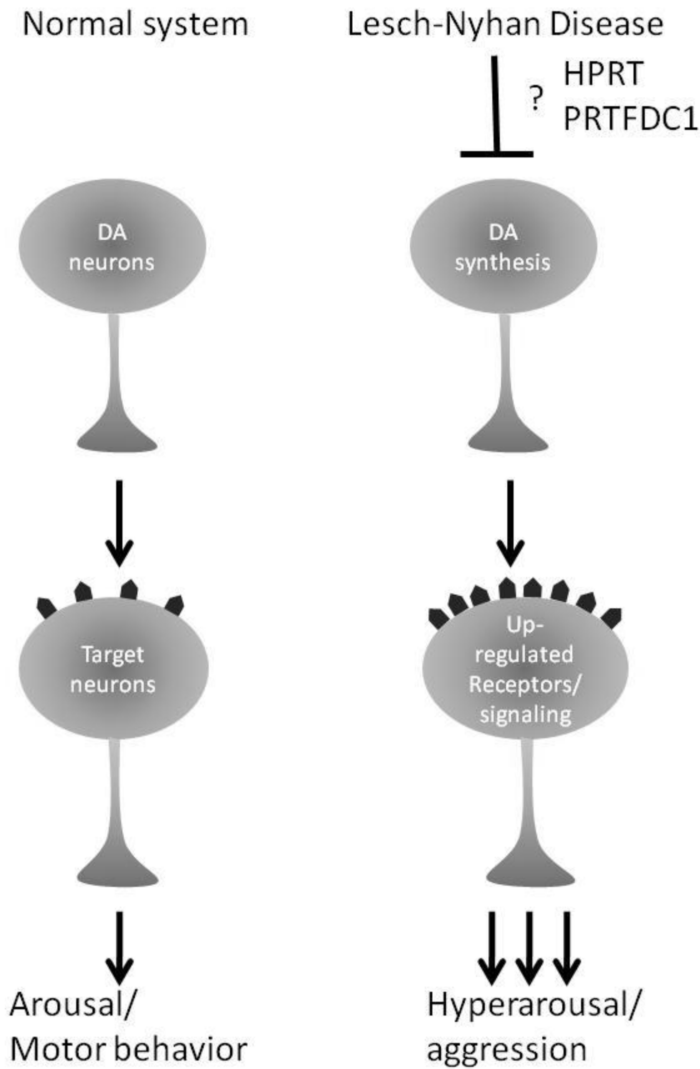


Figure 5.2 A hypothetical parallel pathway wiring diagram for modafinil-induced arousal.
Modafinil blocks NET and DAT. In the wake-promoting pathway (right), the increased extracellular NE signals via α1ARs to activate wake-promoting DA neurons in the ventral periaqueductal gray (vPAG). The DAT blockade prevents the uptake of the released DA, thus facilitating DA transmission in vPAG projection areas. Simultaneously, NE inhibits sleep-promoting neurons in the hypothalamus (and perhaps other brain regions). Arrow to DA neurons signifies excitation, and bar to hypothalamic neurons signifies inhibition. NE, norepinephrine; DA, dopamine; α1ARs, α1-adrenergic receptors; NET, norepinephrine transporter; DAT, dopamine transporter.

5.3 Proposed mechanism for hyperarousal phenotype in Lesch-Nyhan Disease



5.3 Proposed mechanism for hyperarousal phenotype in Lesch-Nyhan Disease. Lesch-Nyhan Disease causes losses in striatal DA by an unknown mechanism. We hypothesize this results in an upregulation of DA receptors. In a situation where DA would normally be released (i.e., amphetamine injection, or intruder threat), this causes overstimulation of these receptors, leading to heightened signaling down this pathway, resulting in a hyperarousal-like phenotype.

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APPENDIX 1:

EFFECT OF NOREPINEPHRINE TRANSPORTER INACTIVATION ON MODAFINIL-INDUCED LOCOMOTION

A1.1 Abstract

Modafinil is the most commonly-prescribed medication in the United States for excessive sleepiness associated with narcolepsy and other arousal disorders. Despite its prevalence of use, the precise molecular mechanism of modafinil has yet to be elucidated. Research has indicated that norepinephrine (NE), and specifically the norepinephrine transporter (NET), may play a key role. In order to explore the role of the NET in the mechanism of action of modafinil, locomotor activity experiments were conducted using a NET inhibitor as a pretreatment for modafinil, and administering modafinil to both wildtype (WT) and NET knockout (NET KO) mice. Multiple trials of these experiments were conducted, with conflicting results. Different lots of modafinil were tested to determine if expired drug was the underlying cause of the discrepancy, as were WT mice of a different strain background. Ultimately it was determined that the NET blocker nisoxetine had no effect on modafinil-induced locomotor behavior, and that both WT and NET KO mice responded normally to modafinil. While this could indicate that the NET is not required for the mechanism of action of modafinil, it is more likely that other systems are compensating for the blockade of NET, most notably, the dopaminergic system.

A1.2 Introduction

Modafinil is a novel wake-promoting drug approved for the treatment of excessive daytime sleepiness associated with narcolepsy and other sleep disorders. It is particularly useful because compared to traditional stimulants used to treat these disorders (i.e., amphetamine), modafinil has few side effects, is non-habit-forming, and does not cause sleep rebound (Ballon and Feifel, 2006; Edgar and Seidel, 1997; Minzenberg and Carter, 2007). However, despite considerable research, the mechanism of action of modafinil remains elusive. Because of its ability to increase wake and locomotor activity, a dopaminergic mechanism similar to amphetamine and cocaine was originally investigated. It was found that modafinil acts by a mechanism unique from traditional stimulants, and there are several pieces of evidence to support this. First, while modafinil's only known biochemical activities are blockade of both the dopamine transporter (DAT) and the NET, its affinity for the DAT is low, and affinity for the NET is even lower (Madras et al., 2006; Mignot et al., 1994). Secondly, modafinil and amphetamine have distinct patterns of brain activation, with amphetamine causing profound and diffuse c-fos staining, and modafinil causing a much more moderate and restricted pattern with activation localized to regions of hypothalamus, with some staining in the amygdala (Engber et al., 1998a; Engber et al., 1998b; Lin et al., 1996; Scammell et al., 2000). In addition, blocking DA synthesis via tyrosine hydroxylase inhibition prevents the increase in locomotor activity caused by amphetamine, but not that caused by modafinil (Simon et al., 1995).

As a result of this, the noradrenergic system has been investigated for a role in the mechanism of action of modafinil. Several lines of evidence support a noradrenergic mechanism. The only compounds capable of reliably blocking the locomotor-promoting effects of modafinil are α1 adrenergic receptor (α1AR) antagonists (Duteil et al., 1990; Hermant et al., 1991; Lin et al., 1992; Mitchell et al., 2008), and modafinil-induced increases in gross motor activity are attenuated in α1bAR knockout mice (Stone et al., 2002). Furthermore, evidence indicates that the

NET may be important in the wake- and locomotor-promoting effects. Gallopin demonstrated in rat brain slices that modafinil inhibits the activity of neurons in the sleep-promoting ventrolateral preoptic area (VLPO), but only in the presence of NE (Gallopin et al., 2004). When tested in combination with histamine or serotonin, modafinil had no impact on discharge rate of the VLPO cells. When the NET reuptake blocker nisoxetine was applied to the cells in combination with NE, the change in discharge rate was similar to the change induced by modafinil and NE. No potentiation occurred when modafinil and nisoxetine were coadministered. These results indicate that 1) modafinil can impact the activity of the NET, 2) NE is required for the action of modafinil, and 3) that modafinil acts similarly to a known NET blocker in the sleep-promoting neurons of the VLPO. Additionally, a PET study in rhesus monkeys was published showing that at therapeutically-relevant doses, modafinil can displace both NET and DAT ligands (Madras et al., 2006). While data specific to the NET was limited in this study, and based only on one monkey, it provides an interesting new direction for a noradrenergic role in modafinil's mechanism of action. Based on this evidence, we conducted studies examining the impact of both genetic and acute blockade of the NET on modafinil-induced locomotor activity in mice.

A1.3 Methods

Animals and housing

NET WT and KO mice on a pure C57BL6/J background were used in these experiments, generated from NET heterozygote (+/-) breeders obtained from Marc Caron (Duke University, Durham, NC), in addition to WT mice on a different strain background (mixed C57BL6/J and 129SvEv). Males and females between 3 and 8 months of age were used and no gender or age differences were found. Mice were housed in a colony room with food and water available ad libitum and lights on from 0700 to 1900 hours. All animals were moved to an isolated behavior

room at least 24 hours before testing. All experiments were conducted in accordance with Emory University IACUC approval.

Compounds

Drugs used in this study were modafinil (Cephalon, West Chester, PA), and the selective NET inhibitor nisoxetine (Sigma-Aldrich, St. Louis, Missouri). The dose of 25 mg/kg modafinil was based on previous studies from this lab, indicating that it is the lowest dose capable of increasing locomotor behavior in WT mice. Doses of nisoxetine with behavioral effects vary widely in the literature (between 5 mg/kg and 45 mg/kg), so the dose of 20 mg/kg was chosen as a mid-range dose capable producing behavioral effects (Darmani, 1998; Rommelfanger et al., 2004; Wisor and Eriksson, 2005).

Locomotor activity

We measured locomotor activity following administration of modafinil in these mice in the presence and absence of a nisoxetine pretreatment. Experiments were conducted between 8 am and 3 pm. Ambulations (consecutive beam breaks) were measured in clear plexiglass cages (40 x 20 x 20 cm) connected to an automated photobeam monitoring apparatus (San Diego Instruments Inc, La Jolla, CA). We individually housed the mice in the locomotor chambers for 3.5 hours, and then administered either a saline or nisoxetine (20 mg/kg; dissolved in 0.9% saline; injected i.p.) pretreatment. 30 minutes later, we administered modafinil (25 mg/kg; dissolved in warm 0.9% saline, 1.5% DMSO, 1.5% cremophor EL; injected i.p.), and recorded ambulations for an additional 2 hours. All data are presented as total ambulations in the 2 hours after modafinil administration.

Multiple trials

Initial experiments were conducted in the summer of 2005. Upon replication of the initial experiments in the fall of 2006, conflicting results were obtained. As a result of this, new modafinil was acquired (lot number CEP-1538) and tested in comparison with the previous modafinil (lot number 97C168) in WT mice. Following this experiment, the nisoxetine experiment was completed using only the new modafinil. Finally, the new modafinil was tested in WT mice of a different strain background (mixed C57BL6/J and 129SvEv). As a negative control, the effect of nisoxetine (20 mg/kg) alone on locomotor activity was tested.

Statistics

Group differnces were analyzed by one way analysis of variance testing using Graphpad Prism for Macintosh.

A1.4 Results

Initially, 25 mg/kg modafinil increased locomotor activity in both NET WT and KO mice. Furthermore, 20 mg/kg nisoxetine administered 30 minutes prior to modafinil suppressed the locomotor effects of modafinil only in NET WT mice ($F(3,28) = 2.952$, $p < 0.05$) (Fig. A1.1). However, upon replication, nisoxetine pretreatment led to a non-significant increase in modafinil-induced locomotion in WT animals ($F(3,36) = 1.433$, $p = 0.25$) (Fig. A1.2). In an attempt to reconcile these conflicting results, modafinil from a new lot was obtained and tested to determine if this change in results was due to altered efficacy of an old batch of modafinil. The new modafinil was compared to the modafinil used in the previous studies, using only WT mice, and no differences were found in the modafinil-induced locomotion between the old and the new batches ($p = 0.85$) (Fig. A1.3). Despite the lack of significant difference between lots of modafinil, the new modafinil was used in a third trial of 20 mg/kg nisoxetine as a pretreatment for 25 mg/kg modafinil in NET WT and KO mice. No significant differences were found between

groups (Fig. A1.4). In order to determine whether these results were strain-specific, the effect of the nisoxetine pretreatment on modafinil-induced locomotor activity was tested in wildtype mice of a different strain (mixed C57BL6/J and 129 SvEv). Nisoxetine had no significant effect on modafinil-induced locomotion in these mice ($p = 0.61$) (Fig. A1.5). Finally, in order to determine the effects of all experiments done in NET mice using nisoxetine and modafinil, the data from Experiments 1,2, and 4 was pooled and no significant effect of nisoxetine or genotype on modafinil-induced locomotor activity was found ($F(3, 84) = 0.4164$, $p = 0.74$) (Fig. A1.6). Nisoxetine, given alone, had no effect on locomotor activity (Fig. A1.7).

A1.5 Discussion

To determine whether NET inhibition could affect modafinil-induced locomotor activity, nisoxetine and NET knockout mice were used in a behavioral paradigm. The sum of all the experiments included in this study indicate that neither acute pharmacological NET blockade nor genetic inactivation of the NET has any effect on modafinil-induced locomotion. The simplest interpretation of these results is that NET blockade is not involved in modafinil-induced locomotor activity. However, this interpretation is unlikely given that modafinil displaces a NET PET ligand in vivo and that activation of α1ARs is required for modafinil-induced locomotor activity (Duteil et al., 1990; Hermant et al., 1991; Lin et al., 1992; Madras et al., 2006). Our working model is that modafinil is a combination NET/DAT blocker, and that blockade of both transporters is required for its efficacy. Under normal circumstances, modafinil inhibits both transporters, causing an increase in extracellular NE and DA. The increased NE provides excitatory drive onto DA neurons via α1ARs, causing increased DA release. The increased DA, which is a combined consequence of increased DA neuron firing and DAT blockade, causes increases in arousal and locomotor activity.

There are a number of possible explanations for why these results indicate no effect of NET inhibition on modafinil-induced locomotion. First is that knocking out the NET results in many compensatory changes. These changes include alterations to multiple aspects of their noradrenergic systems, and secondary changes to their dopaminergic systems, leading to resultant changes in behavior. The NET KO mice display decreased tissue concentrations of NE, but clearance rates are also decreased, leading to increased extracellular NE (Xu et al., 2000). Their receptor concentrations are also altered, with downregulated α1 receptors, but upregulated α2a and α2c receptors in the brain, and heightened responses to clonidine, a α2AR agonist (Gilsbach et al., 2006; Xu et al., 2000). It is unclear whether these α2ARs are pre- or post-synaptic, but the fact that activating them with clonidine decreases locomotor activity could indicate that they are pre-synaptic autoreceptors that act to decrease release of further NE. Furthermore, both tissue concentrations and extracellular concentrations of DA are decreased, while post-synaptic D2 and D3 DA receptors are upregulated (Xu et al., 2000).

Reponses of these mice to stimulants have been varied and contradictory (reviewed by (Rocha, 2003)). In an initial study characterizing these mice, acute cocaine and amphetamine both produced an enhanced locomotor response in the NET KO mice and a more robust place preference for cocaine compared to the WT mice; however the NET KO mice were unable to sensitize to effects of cocaine (Xu et al., 2000). Subsequent studies have found decreased locomotor activity in a novel environment and that acute cocaine decreases locomotor activity, but that chronic cocaine restores drug-induced activity levels to those of WT mice.

With all these compensatory changes in the NET KO mice, it is perhaps not surprising that they have normal responses to modafinil. If modafinil acts according to our hypothesis, and blocks both DAT and NET in normal mice, leading to increased extracellular concentrations of DA and NE, then the increased basal extracellular NE in the NET KO mice can compensate for the inability to block the transporter. These increased catecholamines then act on receptors,

which have also been altered in the NET KO mice. While α1ARs are decreased, and α2 adrenergic autoreceptors are increased, leading to a decrease in the downstream effects of NE, DA receptors are hypersensitive, allowing for the increased downstream effects of DA to compensate for the lack of NE effects. These results are also compatible with our other work indicating that mice lacking NE (*Dbh -/-* mice) are hypersensitive to the effects of modafinil, due to hypersensitive DA systems (Mitchell et al., 2008).

Therefore, our hypothesis is consistent with these results and some of the literature. Gallopin and colleagues found that in the presence of NE, modafinil increased inhibition of sleep-promoting neurons, but has no effect on its own (Gallopin et al., 2004). Furthermore, nisoxetine also inhibited these neurons, but when given in combination with modafinil did not show synergistic inhibition. Thus, their experiments also indicate that modafinil requires increased extracellular NE for its efficacy. However, NET blockade alone is not sufficient to cause the increase in locomotor effects seen with modafinil (Fig. A1.7), indicating that another molecular action is also required, most likely DAT blockade. One limitation of this study is that we examined only locomotor activity. It is possible that the wake-promoting effects of modafinil would be altered by nisoxetine pretreatment or in NET knockout mice.

Acknowledgements

We thank Marc Caron for providing NET KO breeding pairs, and Cephalon, Inc for providing modafinil, and funding in support of David Weinshenker and Heather Mitchell. Heather Mitchell has also been supported by NIH grant T32 GM 008602.

Figure A1.1 Effect of nisoxetine pretreatment on modafinil-induced locomotion (Trial 1)

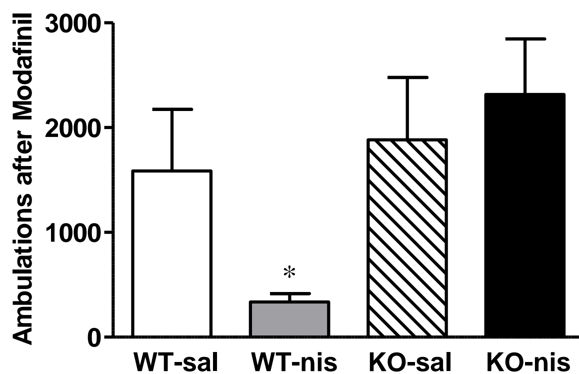

Figure A1.1 Effect of nisoxetine pretreatment on modafinil-induced locomotion (Trial 1). Mice (n = 8 per group) were placed in activity chambers and allowed to acclimate for 3.5 hours before injection of vehicle or a 20 mg/kg nisoxetine pretreatment. 30 minutes after pretreatment, mice were injected i.p. with 25 mg/kg modafinil, and ambulations were recorded for 2 hours. Shown are total ambulations following modafinil. Nisoxetine suppressed modafinil-induced locomotion in control animals (*$p < 0.05$ compared to vehicle control). WT, wildtype; sal, saline; nis, nisoxetine; KO, knockout.

Figure A1.2 Effect of nisoxetine pretreatment on modafinil-induced locomotion (Trial 2).

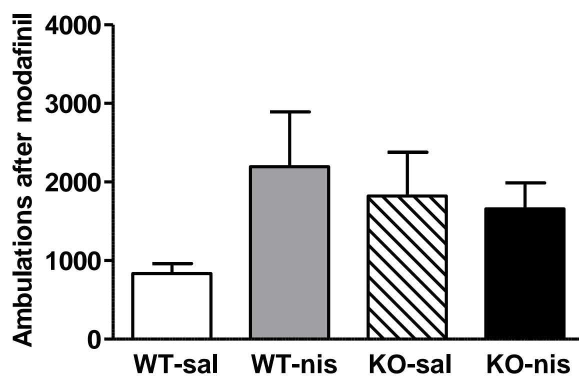

Figure A1.2 Effect of nisoxetine pretreatment on modafinil-induced locomotion (Trial 2). Mice (n = 10 per group) were placed in activity chambers and allowed to acclimate for 3.5 hours before injection of vehicle or a 20 mg/kg nisoxetine pretreatment. 30 minutes after pretreatment, mice were injected i.p. with 25 mg/kg modafinil, and ambulations were recorded for 2 hours. Shown are total ambulations following modafinil. No significant differences were found between groups. WT, wildtype; sal, saline; nis, nisoxetine; KO, knockout.

Figure A1.3 Comparison of new and old modafinil

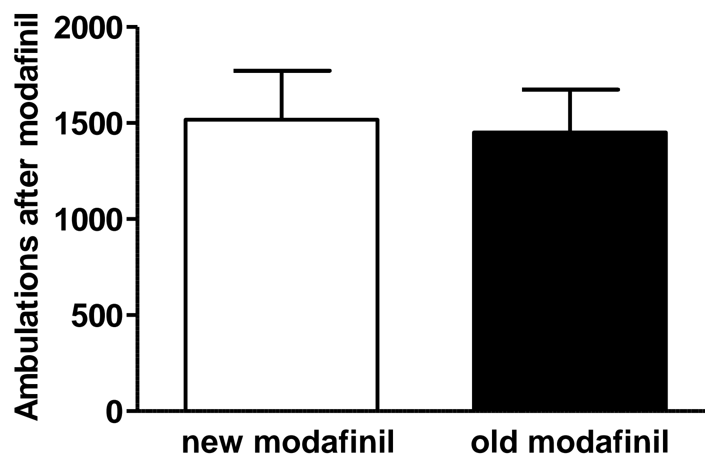

Figure A1.3 Comparison of new and old modafinil. Mice (n = 4 per group) were placed in activity chambers and allowed to acclimate for 4 hours before injection of 25 mg/kg modafinil, and ambulations were recorded for 2 hours. Shown are total ambulations following modafinil. No significant differences were found between groups.

Figure A1.4 Effect of nisoxetine pretreatment on modafinil-induced locomotion (new lot modafinil)

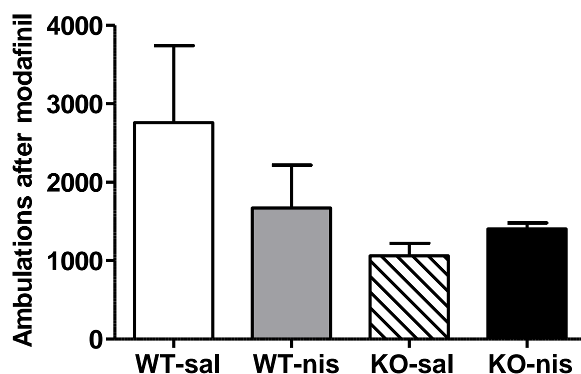

Figure A1.4 Effect of nisoxetine pretreatment on modafinil-induced locomotion (new lot modafinil) Mice (n = 4 per group) were placed in activity chambers and allowed to acclimate for 3.5 hours before injection of vehicle or a 20 mg/kg nisoxetine pretreatment. 30 minutes after pretreatment, mice were injected i.p. with 25 mg/kg of the new modafinil, and ambulations were recorded for 2 hours. Shown are total ambulations following modafinil. No significant differences were found between groups. WT, wildtype; sal, saline; nis, nisoxetine; KO, knockout.

Figure A1.5 Effect of nisoxetine pretreatment on modafinil-induced locomotion in mixed background WT mice

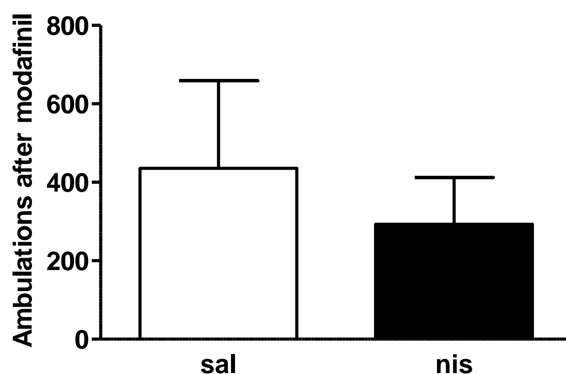

Figure A1.5 Effect of nisoxetine pretreatment on modafinil-induced locomotion in mixed background WT mice. Mice of a mixed C57BL6/J and 129SvEv background (n = 4 per group) were placed in activity chambers and allowed to acclimate for 3.5 hours before injection of vehicle or a 20 mg/kg nisoxetine pretreatment. 30 minutes after pretreatment, mice were injected i.p. with 25 mg/kg modafinil, and ambulations were recorded for 2 hours. Shown are total ambulations following modafinil. No significant differences were found between groups. sal, saline; nis, nisoxetine.

Figure A1.6 Effect of nisoxetine pretreatment on modafinil-induced locomotion (combined trials)

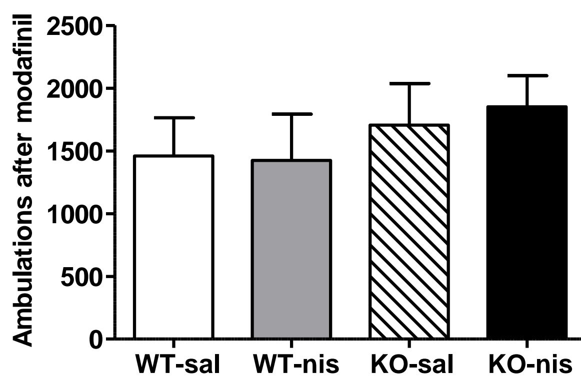

Figure A1.6 Effect of nisoxetine pretreatment on modafinil-induced locomotion. Data from experiments utilizing the 20 mg/kg nisoxetine pretreatment for 25 mg/kg modafinil in both WT and NET KO mice (experiments 1, 2, and 4) were combined for a total n = 22. No significant differences were found between groups. WT, wildtype; sal, saline; nis, nisoxetine; KO, knockout.

Figure A1.7 Effect of nisoxetine on basal locomotion

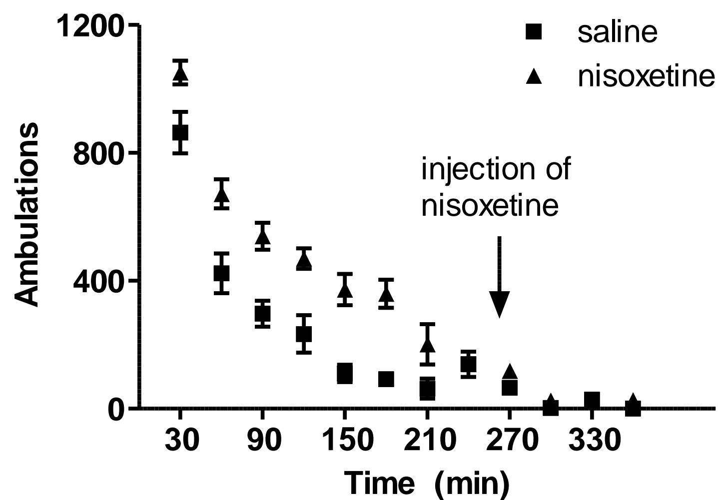

Figure A1.7 Effect of nisoxetine on basal locomotion. Mice (n = 8 per group) were placed in activity chambers and allowed to acclimate for 4 hours before injection of 20 mg/kg nisoxetine, and ambulations were recorded for 2 additional hours. Shown is a time course of ambulations over the course of the experiment. There are no significant differences between groups following injection of nisoxetine.

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APPENDIX 2:

A GENETIC ASSOCIATION STUDY BETWEEN SINGLE NUCLEOTIDE POLYMORPHISMS IN THE DOPAMINE β-HYDROXYLASE GENE AND EXCESSIVE SLEEPINESS ASSOCIATED WITH NEURODEGENERATIVE DISEASE

A2.1 Abstract

Sleep disorders are often comorbid with neurodegenerative disorders such as Parkinson's disease (PD) and Alzheimer's disease (AD). One potential reason for this relationship is that the catecholamine system is altered in both AD and PD, and sleep is partially regulated by the catecholamines norepinephrine (NE) and dopamine (DA). Thus, it is possible that alterations in the catecholamine system are responsible for some of the sleep abnormalities in patients with AD or PD. Because catecholamine levels are regulated by the activity of dopamine β-hydroxylase (DBH), which converts DA to NE in noradrenergic neurons, and DBH activity is under strong genetic control, we hypothesized that sleep disturbances in patients with AD or PD may be associated with variants in the DBH gene. In order to investigate this, a genetic association study was conducted exploring 4 single nucleotide polymorphisms (SNPs) in the DBH gene and their impact on sleep parameters in control and neurodegenerative disease patients from the Clinical Research in Neurodegeneration (CRIN) database. No significant associations were found between DBH SNPs and any of the sleep parameters tested. This was a pilot study, with small sample sizes and incomplete phenotypic data, and despite the negative results, may be worth exploring with greater numbers of patients and more robust data on sleep phenotypes.

A2.2 Introduction

Sleep is a necessary and highly conserved biological process. It has been shown to be necessary for life (Rechtschaffen et al., 1989) and has a profound impact on quality of life (Colten and Altevogt, 2006). Because it is such a fundamental process, sleep affects, and is affected by, many other biological states, both basal and pathological (Knutson et al., 2007). Sleep is regulated through a complex network, involving multiple neurotransmitter and hormone systems (Saper et al., 2005). The catecholamines NE and DA are key components of this network. Neurons in the locus coeruleus (LC), the main noradrenergic nucleus in the brain, fire in a wake-dependent manner; they are highly active during wake, slow-firing during slow wave sleep, and almost completely quiescent during rapid eye movement sleep (REM) (Aston-Jones and Bloom, 1981). Furthermore, adrenergic receptor agonists have robust wake-promoting actions, while blockade of these receptors can result in sedation (Berridge, 2008). While DA has not traditionally been associated with sleep, recent evidence indicates that it also plays an important role (Berridge, 2006). A population of dopaminergic wake-active neurons exists in the ventral periaquaductal grey (Lu et al., 2006) and mice with altered DA display irregular sleep patterns (Dzirasa et al., 2006). Finally, drugs such as psychostimulants that increase DA in the brain also increase arousal and wakefulness (Berridge, 2006; Dzirasa et al., 2006). Because both catecholamines are clearly involved in sleep, it follows that disruption of the catecholamine system might lead to sleep disturbances.

This is of particular interest, as the pathological signs of both PD and AD impact the DA and NE systems. The hallmark of PD is the loss of DA neurons in the substantia nigra, and more recent work has shown that degeneration of the LC and deficits in NE also contribute to the progression of the disease (reviewed in (Rommelfanger et al., 2007)). LC degeneration occurs early in the progression of AD and likely contributes to the cognitive deficits (reviewed by (Weinshenker, 2008)). The precise impact of DA on AD is less clear, mainly due to interactions

of the DA system with the cholinergic system, which is known to have a strong role in the pathology of AD. However, research has shown that DA receptors are altered in AD (Reeves et al., 2010), and that dopaminergic drugs have a positive impact in patients suffering from this disease (Martorana et al., 2009).

Patients with these neurodegenerative diseases frequently suffer from comorbid sleep concerns (reviewed by (Dauvilliers, 2007)). In fact, the original description of PD by James Parkinson included a reference to sleep disturbances (Comella, 2006). Exact percentages of PD patients that suffer from sleep problems are variable, and range from 30% to 98% (Comella, 2006; Dauvilliers, 2007; Thorpy and Adler, 2005). PD patients experience significantly more nocturnal awakening and sleep fragmentation, and also have significantly shorter total sleep time with decreased sleep efficiency, in addition to a greater incidence of restless legs syndrome than healthy controls (Comella, 2006; Dauvilliers, 2007). Because of difficulty sleeping at night, these patients also experience excessive daytime sleepiness, which can strongly impact social interactions and performance at work, as well as the ability to drive (Thorpy and Adler, 2005). Most PD patients take dopaminergic medications to ameliorate their motor symptoms, though these often make sleep disturbances worse (Comella, 2006; Thorpy and Adler, 2005). For this reason, learning more about the mechanisms and genetic factors that might impact these conditions is important, as it may provide better treatment directions.

Approximately 25-35% of AD patients suffer from sleep disturbances, the most common of which is “sundowning” (Dauvilliers, 2007). Sundowning is a form of nocturnal agitation, and is categorized as a sleep disorder because symptoms are exacerbated in the evening and involve nocturnal wandering. Sleep apnea and other sleep-related breathing problems are also common in AD, which can seriously impact daytime cognitive ability (Dauvilliers, 2007). In addition to the negative impact on the patient, sleep disorders in an AD patient can also negatively affect the patient’s caregiver, and are a frequent factor in institutionalizing these patients (Dauvilliers, 2007). According a study by Park et al (Park et al., 2006), there is also some overlap between PD

and AD; AD patients with sleep disorders also tended to have a greater incidence of PD-like motor symptoms, indicating that similar mechanisms may be responsible for the sleep difficulties.

As catecholamines are important both in the disease processes and in sleep regulation, altered levels of catecholamines may impact sleep variations in patients. Catecholamine levels are regulated, in part, by DBH, the enzyme that converts DA to NE in noradrenergic neurons. The DBH gene is under strong genetic control, making it an excellent candidate for genetic analysis (Cubells et al., 1998). A C→T SNP at position -1021 is of particular interest, as it is strongly associated with plasma DBH activity in European American, African American, Eastern Indian, and Japanese populations, and accounts for more than one third of all variance in plasma DBH activity (Bhaduri et al., 2010; Zabetian et al., 2001). Individuals with the TT genotype have extremely low levels of DBH activity (<10% of CC), and the CT heterozygous genotype confers intermediate DBH activity, indicating autosomal codominant inheritance of the low DBH trait. This SNP is also reasonably common (T allele frequency ~ 0.2), indicating that approximately 4% of the population is homozygous and 32% are heterozygous for the low activity (T) allele.

In addition to the C→T SNP at -1021 (rs1611115), there are three other SNPs in the DBH gene that are of interest as possible functional polymorphisms (Fig. A2.1). There is an A→G SNP (rs6271) at position 1603 in exon 11 that has been found to significantly contribute to the variance of plasma activity levels (Tang et al., 2007; Zabetian et al., 2001). A SNP in the promoter region (rs2519148) has been shown to associate with some social behaviors in autistic patients (Yrigollen et al., 2008). A final SNP in intron 4 (rs1611125) is in linkage disequilibrium with a SNP in intron 5 that may be associated with attention deficit hyperactivity disorder (ADHD) (Cubells and Zabetian, 2004).

These SNPs have been found to have significant effects on psychiatric and neurological conditions. The SNP at -1021C→T is of particular interest, as it has the strongest impact on plasma activity levels. A study explored whether this polymorphism significantly associated with PD risk itself, but no association was found (Ross et al., 2008). An additional study found that the

T allele did not impact risk or age of onset of PD (Chun et al., 2007). The TT genotype of this SNP was found to significantly associate with adult attention deficit/hyperactivity disorder (ADHD) (Hess et al., 2009). While no genotype frequency differences were found between healthy control patients and patients diagnosed with affective disorders or personality disorders, patients with personality disorders with the TT genotype were found to score higher on tests for neuroticism and novelty-seeking behavior, and were more likely to display impulsiveness and aggression (Hess et al., 2009). DBH genotype at -1021 was also found to associate with migraine in two independent cohorts, though no association with genotype at the SNP in exon 11 was found (Fernandez et al., 2009). No association was seen between genotype at -1021 and cocaine addiction (Guindalini et al., 2008), but low plasma levels of DBH have been associated with cocaine induced paranoia (Cubells and Zabetian, 2004), so it is possible that DBH SNPs may affect specific aspects of cocaine-taking behavior. Additionally, a SNP in exon 2 of the DBH gene associates with “interpersonal sensitivity” and paranoia in depressed patients (Wood et al., 2002). This indicates that alterations in DBH may make an individual more prone to paranoia or psychosis, if afflicted by other psychiatric conditions.

The impact of variation in DBH on sleep has yet to be examined. Catecholamines have a significant role in regulating sleep; therefore, functional polymorphisms in the DBH gene may impact sleep parameters, particularly in patients who already have disrupted levels of catecholamines, such as patients suffering from AD or PD.

A2.3 Methods

Subjects

Subjects were 345 healthy controls, 450 with a PD diagnosis, and 377 with an AD diagnosis of European American descent. It was not possible to match subjects for age or gender.

Because medication status does not appear to impact plasma DBH levels, it was not used as a covariate in the analysis (Cubells et al., 1998).

Whole Genome Amplification

Whole genome amplification (WGA) was conducted on those samples with DNA concentrations of less than 10 ng/ml using a Qiagen REPLI-g WGA kit. Amplification was conducted as described in the Qiagen instructions for the kit. Briefly, denaturing buffer was added to the DNA and then incubated. The reaction was stopped with neutralization buffer, and then a reaction mix of water, reaction buffer and DNA polymerase was added to the denatured DNA, and incubated overnight at 30 degrees. The reaction was stopped by heating to 65°C, and then samples were quantified by the Center for Medical Genomics, Emory University.

Genotyping

Prior to genotyping for SNPs, all samples were normalized to the same concentration using a Biomek FX liquid handling robot to create stock plates. Calculations were performed as to the amount of water needed to add to each sample to normalize all samples to 5 ng/ml. Samples were added to 96 well plates, and then the Biomek added the desired amount of water to each well to create the stock plates. Negative controls (water), cross-plate positive controls, and within-plate positive controls were all included on each stock plate. Stock plates were then used to create working plates; samples from four 96 well stock plates were compiled to make 384 well plates, also performed on the Biomek. Commercially available pre-designed primers and buffers were added to each well using the Biomek, and these plates were then genotyped using the Taqman ABI 7900 system, using PCR with fluorescence detection. This system uses released fluorophores to analyze base pairs and which version of SNP is present, and then plots each sample on a scatterplot according to the emitted fluorophores.

Sleep phenotype

Sleep phenotype was assessed based on response to five questions, administered by a neurologist. Patients were asked about the average number of hours of sleep each night, whether or not they napped during the day, whether or not they snored, if they experienced symptoms of restless legs syndrome (RLS), and if they exhibited any unusual nighttime behavior. Of the patients with confirmed DBH genotypes, responses to these questions were available for 142 AD patients, 60 PD patients, and 160 controls.

Analysis

Chi square analyses were conducted for each SNP tested in comparison with each categorical parameter (snoring, napping, RLS, nighttime behavior), and one way ANOVAs were conducted for hours of sleep per night. Data was then broken down into disease group (AD, PD, healthy controls), and the same tests were conducted. P values were set at 0.05.

A2.4 Results

Sleep data was first compiled on healthy controls. Of the 345 patients in this group for which we had valid genetic information, we were able to obtain sleep data for approximately 30% of these patients. Chi square analyses were conducted on each SNP for each sleep parameter (snoring, napping, RLS, and nighttime behavior), and no significant associations were found. When patients with PD were analyzed for sleep parameters, only about 11% of those with genetic data also had sleep phenotype information. When Chi squares were conducted for each sleep parameter for each of the 4 SNPs, no significant associations were found. Finally, sleep data on AD patients was assessed, and of those patients with genetic information, sleep data was available for approximately 30%. When Chi square analyses were done for the AD population, examining each sleep parameter against each SNP, no significant associations were found.

A2.5 Discussion

This study attempted to address the importance of putative functional SNPs in the DBH gene on sleep parameters in patients with AD or PD. The impact of DBH genotype on sleep has never been examined, but it is of particular interest due to the role that both NE and DA play in sleep regulation. As alterations in either of these neurotransmitters can cause sleep disturbances, it follows that disrupting their regulation through the enzyme that converts DA to NE might have a strong impact on sleep. While this is of interest in healthy populations, it has particular relevance to patients suffering from either AD or PD. Sleep disorders are common in these neurodegenerative conditions, and can profoundly impact quality of life in both the patients and their caregivers (Dauvilliers, 2007). Furthermore, both of these diseases are linked to degeneration of dopaminergic and/or noradrenergic brain regions (Rommelfanger et al., 2007; Weinshenker, 2008). For these reasons, we explored the impact of DBH polymorphisms on sleep phenotype in patients with AD or PD. It is possible that learning more about how sleep is regulated in these patients will provide better treatments for the sleep symptoms that accompany the neurodegeneration, and improve quality of life.

Unfortunately, it was not possible to draw any definitive conclusions from this study. This was a retrospective study, using DNA collected over a period of about 15 years, and DNA from many of these samples was not quantifiable. In addition, information on sleep phenotype was scarce, with only 427 subjects out of over 1500 having sleep data collected, and of those, only 376 had both sleep data and quantifiable DBH genotype. These numbers are simply not sufficient for a genetic analysis of enough power to draw any meaningful conclusions. For example, the association study examining the -1021 SNP in DBH and PD had a sample size of almost 3000 (Ross et al., 2008), and the Hess (Hess et al., 2009) study exploring the same polymorphism and ADHD had a sample size of about 1500. In addition, the sleep data that was

gathered was very rudimentory, asking only five general questions about sleep habits. Four of these were simple yes or no questions, with no follow-up information, and the fifth asked for average number of hours slept per night. These questions are of interest, but it would be helpful to have more quantitative information about sleepiness.

Despite a lack of positive findings in this study, an in-depth exploration of the impact of DBH genotype on sleep behavior in patients with neurodegenerative diseases may be warranted. An ideal study would be prospective, and involve approximately 1000 subjects for each of the three conditions, AD, PD and healthy controls. In addition, more detailed sleep data would be collected on all these patients, using the Epworth questionnaire in addition to the questions asked in this study. This questionnaire has 8 items that determine how likely a patient would be to fall asleep while engaged in activities from a list. In this way, it is possible to obtain a numerical value for sleepiness in the patient, making data more quantifiable. In addition to the Epworth, if patients answer yes on any of the previous questions, follow-up information should be gathered as to the type and degree of impairment.

While association studies have been performed examining the relationship between DBH SNPs and AD and PD, these have looked at overall incidence of the disease, and not the impact of DBH on specific symptoms. Despite not finding a significant association between incidence of PD or AD and DBH variations, it is still possible that functional SNPs may affect sleep behavior in patients with these diseases. While it has been found that the -1021 SNP does not affect cocaine addiction rates, it does associate with an increase in cocaine-induced paranoia during self-administration (Kalayasiri et al., 2007). Additionally, the Hess study found no association with incidence of affective disorders, but found that patients with personality disorders with the TT genotype were more likely to have significant behavioral differences (Hess et al., 2009). Therefore, while incidence of a particular disorder is likely to be influenced by many factors, both genetic and environmental, it is possible that a particular SNP may impact certain characteristics of the disorder. If a more thorough study examining DBH genotype and sleep in patients with

neurodegenerative diseases were to be carried out, interesting links between sleep, DBH and these conditions may be elucidated. This could allow clinicians to better customize medical treatment for their patients, and thereby improve their quality of life.

Acknowledgements

We thank Joseph Cubells, Donald Bliewise, and Yilang Tang for technical assistance and guidance in this study. Heather Mitchell has also been supported by NIH grant T32 GM 008602.

Figure A2.1 Map of DBH with analyzed SNPs

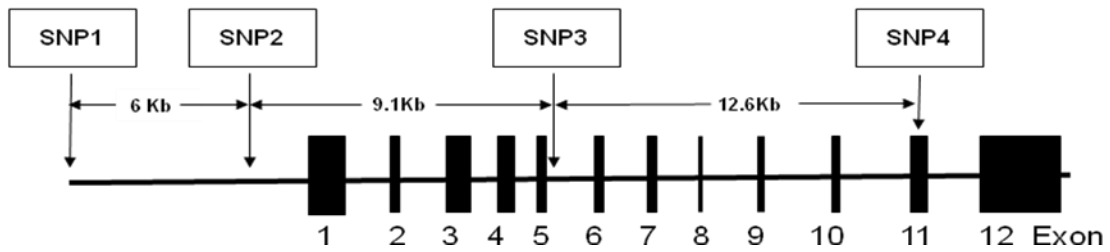

Figure A2.1 Map of DBH with analyzed SNPs. SNPs of interest are marked on the DBH gene. SNP1 is rs2519148, SNP2 is rs1611115, SNP3 is 1611125, and SNP 4 is rs6271.

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