A 5HT2A INVERSE AGONIST REVERSES NICOTINE WITHDRAWAL EFFECTS ON SLEEP STAGES

by

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Dedication

This thesis is dedicated to my family and friends whose invaluable support carried me throughout this project.

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ABSTRACT

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Sleep disturbances are common in nicotine withdrawal, increasing risk for relapse to smoking. This study determined if the selective 5-HT2A serotonin receptor inverse agonist MDL 100907 (volinanserin) can increase time spent in restorative NREM sleep and reduce sleep fragmentation. All surgery was conducted under isoflurane anesthesia. Male Sprague-Dawley rats (N = 34) were implanted with EEG and EMG electrodes and

with osmotic minipumps continuously infusing either 9 mg/kg/day s.c. nicotine bitartrate in saline or saline alone. After 7 days, pumps were removed to induce spontaneous nicotine withdrawal syndrome. Seventeen hours post-pump removal the rats were injected i.p. with 1 mg/kg MDL 100907 (volinanserin) in a vehicle of saline/DMSO/Tween80 or with a vehicle alone, 17 hours post-pump removal. The three treatment groups were: Saline infusion-Vehicle injection (n = 14), Nicotine infusion-Vehicle injection (n = 11) and Nicotine infusion-MDL injection 100907 (n = 9). Rats were monitored during peak withdrawal (18 to 22 hours post-pump removal) within the sleep-intensive, lights-on cycle. The EEG and EMG waves were scored by SleepSign software for the time spent in and number of separate bouts of Wake, NREM and REM. For accuracy, the resulting tracings were also scored manually under blind conditions. A one-way ANOVA revealed that nicotine withdrawal in the absence of volinanserin or (MDL100907) increased the average number of sleep bouts, decreased the percent time spent in NREM sleep, and increased EEG spectral band power across the spectrum. These findings are consistent with existing research on the effects of nicotine withdrawal on sleep. The nicotine withdrawal effect on average number of bouts, percent time spent in NREM sleep, and EEG spectral band power, was reduced by the injection of MDL100907. Inactivating 5HT2A receptors may favor NREM sleep and reduce sleep fragmentation, reversing the disruptive effects of nicotine withdrawal.

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CHAPTER I:

INTRODUCTION

Health Impacts of Tobacco Use and Withdrawal

Tobacco use is the leading cause of preventable disease, disability, and death in the United States (DiSilvio et al., 2021). The Centers for Disease Control and Prevention estimates that 34 million U.S. adults currently smoke (DiSilvio et al., 2021, p. 33). Each year, about 443,000 Americans die from smoking-related illnesses (Lucas et al., 2013). Cancer, cardiovascular disease, and non-cancerous pulmonary disease are the leading causes of death from tobacco use (Benowitz, 2010). In addition, cigarette smoking increases risks for recurring and chronic illnesses such respiratory tract infections, osteoporosis, reproductive disorders, peptic ulcers, and delayed wound healing (Benowitz, 2010). These tobacco and smoking-related illnesses are estimated to cost the United States 96 billion dollars in paid medical expenses every year (CDC, 2015). Although the number of Americans who smoke has decreased over the years, medical spending and deaths continue to pose tobacco usage as a significant public health concern.

Because of the financial and health costs of tobacco many smokers decide to abstain at some point in their lives. However, nicotine cessation can be difficult because of the lack of effective interventions. Of the 45 million Americans who smoke tobacco, 70% say they want to quit, but cannot (Benowitz, 2010). Eighty percent who attempt to quit on their own relapse within a month, and only 3% of smokers will quit permanently (Benowitz, 2010). Even with these percentages, the number of smokers in the United States has decreased. However, the reduction in smokers per year has been slow because the relatively small number of smokers that quit are replaced by people who start smoking (Benowitz, 2010). To decrease the overall number of smokers in the United

States, interventions have been implemented, such as raising the cost of tobacco products, antitobacco media campaigns, and smoke-free laws in public spaces (DiSilvio et al., 2021). Also, nicotine cessation treatments have aided many who decided to quit. Current treatments focus on nicotine replacement therapies such as the nicotine patch, spray, lozenge, gum, and non-nicotinic tablets like bupropion and varenicline (Patterson et al., 2019). While these are FDA-approved treatments that significantly improve the chances of six-month abstinence compared to placebos, less than one-quarter of smokers who use these treatments stay abstinent (Patterson et al., 2019). These statistics suggest that current treatments are partially ineffective and new treatments may be necessary. This raises questions about the effectiveness of current antitobacco campaigns and treatment.

Nicotine Dependence and Addiction

Despite current interventions and treatments, many Americans still choose to smoke. Studies have shown that even after individuals are diagnosed with tobacco-induced illnesses, such as chronic obstructive pulmonary disease (COPD), cancer, and metabolic diseases, they still have difficulties quitting smoking. (DiSilvio et al., 2021). The inability to quit despite negative impacts on health, demonstrates just how difficult it is for smokers to quit, once addicted. Tobacco addiction, like any addiction, "involves the interplay of pharmacology, learned or conditioned factors, genetics, and social and environmental factors" (Benowitz, 2010).

Smokers may struggle to permanently quit because they have become emotionally, psychologically, and physically dependent on the drug. While smoking-caused illnesses are a result of exposure to carcinogenic toxins in tobacco smoke, it is the addiction to nicotine that is the root cause of these illnesses (Benowitz, 2010). When smokers inhale, cigarette smoke particles are carried into the lungs and rapidly absorbed. From the lungs, nicotine moves quickly to the brain via arterial circulation. When

nicotine reaches the brain, it binds to the nicotinic cholinergic receptors that normally bind acetylcholine. Once the nicotine binds at the receptor, ligand-gated channels open and release dopamine into the mesolimbic area, the corpus striatum, and the frontal cortex (Benowitz, 2010).

Repeated exposures to nicotine can create tolerance. Desensitization, a ligand-induced closure and unresponsiveness of the nicotinic cholinergic receptors, may play a role in tolerance and dependence. Nicotine withdrawal symptoms and cravings begin when desensitized nicotinic cholinergic receptors become responsive again during periods of abstinence. Withdrawal causes anxiety, stress, restlessness, weight gain, trouble sleeping, urges to smoke, and difficulty concentrating. To avoid these symptoms, smokers continue to smoke causing the nicotinic cholinergic receptors to be completely saturated (Benowitz, 2010). Long-term saturation of the receptors to avoid a desensitized state makes the smoker physically dependent on nicotine.

The other aspect of nicotine addiction that prevents people from quitting is compulsive self-administration mediated by the underactivity of the dopaminergic reward system. Inhalation of nicotine also induces pleasure, elevates mood, and reduces withdrawal symptoms (Benowitz, 2010). These effects are caused by stimulating the dopaminergic neurons in the ventral tegmental area of the midbrain and the shell of the nucleus accumbens. Both regions are known to play a role in perceptions of pleasure and rewards (Benowitz, 2010). After long-term desensitization, even when someone who is addicted stops smoking, the urge to smoke remains. The association between smoking and smoking-related cues like moods, situations, and environmental factors, and the rewarding effects of nicotine are classified as a form of conditioning, which creates the urge to smoke (Benowitz, 2010).

Sleep Disturbances in Nicotine Withdrawal

One aspect of treatment in nicotine cessation that has not yet been adequately addressed is insomnia. Although sleep disturbance is clinically recognized as a symptom of nicotine withdrawal, it is not often assessed as a factor that contributes to the difficulties smokers may face during nicotine cessation (Patterson et al., 2019). Studies have shown that smokers take longer to fall asleep than nonsmokers (Patterson et al., 2019). Also, smokers, on average, report shorter sleep duration, more awakenings at night, and stronger feelings of daytime drowsiness compared to nonsmokers (Patterson et al., 2019). Polysomnography studies show that there is a negative association between cigarette smoking and sleep quality, further supporting smokers' claims of negative side effects of inadequate sleep (Dugas et al., 2017). The ramifications smokers face due to sleep disturbances poses an important issue, as those who report sleep disturbances and waking up at night to smoke are less likely to quit smoking (Fucito et al., 2017).

Sleep disruption in smokers can be explained by nighttime cravings and withdrawal symptoms caused by the sitmulation of various neurotransmittors and their effects on the receptors in the central nervous system. Receptors that were saturated and desensitized throughout the day will become more responsive during nighttime abstinence from smoking (Benowitz, 2010). Once blood nicotine decreases to low enough levels at night, and receptors become desaturated. Smokers awaken in order to resaturate receptors with nicotine via smoking. This disrupts their sleep continuity and architecture (Dugas et al., 2017).

While sleep disturbances are common amongst active smokers, these are exacerbated during smoking cessation. "Smoking cessation induces sleep fragmentation: more awakenings or arousals to lighter sleep stages or more stage changes" (Jahne et al., 2015). In a study where mice went through withdrawal after chronic oral nicotine

consumption, sleep bout duration decreased, causing sleep fragmentation (Matthews & Stitzel, 2019). The findings of this study suggests that is the nicotine in tobacco that causes sleep fragmentation in smoking cessation.

Fragmented sleep from smoking and nicotine cessation leads to negative cognitive and health consequences, such as decreased attention, increase in perceived stress, dysphoric mood, and a reduction in decision making skills and executive functioning (Fucito et al., 2014; Dugas et al., 2017; Hamidovic et al., 2009).Moreover, sleep plays an important role in information processing and memory consolidation, both of which can be hurt by insufficient sleep (McCooy & Strecker, 2011). Other consequences are decreased psychomotor performance on short-term memory, reaction time, and vigilance tasks (Bonnet, 2003). These negative consequences can help explain the likelihood of failure during cessation, as most individuals return to smoking to avoid unpleasant symptoms (Fucito et al., 2014; Dugas et al., 2017; Hamidovic et al., 2009). Since sleep is essential for healthy functioning, addressing poor sleep quality amongst smokers could reduce instances of relapse and improve the efficacy of current nicotine cessation treatments.

The Neurobiology of Sleep

Sleep is considered by researchers to be an engicmatic behavior, with much more to discover about how our neural systems help to regulate sleep and wakefulness. What is currently known is that sleep has “clear implications for cognition, performance, and overall well-being” (Schwartz & Kilduff, 2015). Dysregulation in one of the systems that regulate sleep can result in sleep disorders that affect these functions (Scammel et al., 2017; Schwartz & Kilduff, 2015). A deeper understanding of the neurobiological basis of sleep can lead to the development of treatments that can address disorders that

dsyregulate sleep and subject individuals to potential physical and neuropsychiatric disorders.

Sleep-Wake Stages

Sleep is divided into non-rapid eye movement (NREM) and rapid eye movement (REM) sleep (Moser et al., 2009; Carskadon & Dement, 2011). Light NREM sleep is characterized by unconsciousness, low sympathetic tone, and roving eye movements (Scammell et al., 2017). Light NREM sleep can be easily interrupted by environmental stimuli (Carskadon & Dement, 2011). As NREM sleep gets deeper, it becomes noticeably harder for sleep to be disrupted by environmental stimuli (Carskadon & Dement, 2011). In the deepest stage of NREM sleep, restorative slow-wave sleep (SWS) occurs, and a large external stimulus is necessary to disrupt sleep (Carskadon & Dement, 2011).

NREM sleep-promoting pathways consist of GABAergic neurons in the ventrolateral pre-optic area (VLPO) and median preoptic nucleus (MnPO) that inhibit wake-promoting neurons in the caudal hypothalamus and brainstem. Other sleep-promoting neurons innervate the basal forebrain and project directly to the cortex (Scammell et al, 2017). Recordings and analysis of Fos expression reveal that lesions of VLPO and MnPO nuclei result in significantly large and long-lasting reductions in sleep (Scammell et al., 2017; Alam et al., 2014; Sherin et al., 1996).

The other stage of sleep, REM, is distinguished by minimal movement, and the conservation of energy exerted for bodily functions (Bear, Connors, & Paradiso, 2016). The most notable features of REM sleep are intermittent, rapid movements of the eye and atonia, almost complete loss of muscle tone (atonia) (Bear, Connors, & Paradiso, 2016).

Certain pathways located in the pontine region play a crucial role in generating muscle atonia and inducing REM sleep. Glutamatergic neurons of the sublaterodorsal nucleus (SLD) in the pontine region generate muscle atonia in REM sleep by exciting

GABAergic neurons in the ventromedial medulla and spinal cord that hyperpolarize motor neurons (Scammell et al., 2017). Animals with lesions of the SLD or deletion of glutamate signaling in the SLD are often in a state like REM sleep, but without atonia (Scammell et al, 2017). REM sleep is also regulated by the inhibition of neurons in the brainstem. During wakefulness and NREM sleep, GABAergic neurons of the ventrolateral periaqueductal gray, lateral pontine tegmental nuclei, and monoaminergic neurons of the locus coeruleus inhibit the SLD (Scammell et al., 2017). In contrast, during REM sleep, the GABAergic neurons of the ventrolateral periaqueductal gray are inhibited by GABAergic neurons of the SLD and medulla (Scammell et al., 2017). Research on brain regions containing REM sleep-promoting nuclei indicates the necessary role the SLD plays in atonia and the generation of REM sleep.

After the onset of sleep, wakefulness follows. Wakefulness is defined as a "spectrum of behavioral states during which an animal exhibits voluntary motor activation and is responsive to internal and external stimuli" (Scammell et al., 2017). Motor activity and responsiveness to stimuli is regulated by wake-promoting pathways in the brain. These pathways ascend through the midbrain and divide into a dorsal pathway leading into the thalamus and a ventral pathway leading into the hypothalamus, basal forebrain, and cortex (Scammell et al., 2017). Both pathways are crucial for normal wakefulness, as they contain monoaminergic and cholinergic systems that are wake-promoting (Scammell et al., 2017: Shwartz & Kilduff, 2015). The dorsal pathway is responsible for generating consciousness during wakefulness through thalamocortical signaling that processes sensation, motor responses, and cognition (Scammell et al., 2017). Evidence to support the dorsal pathway's role in consciousness has been seen in patients with lesions to the thalamus. These patients were awake, but in an unresponsive vegetative state (Scammell et al., 2017; Giber et al., 2015; Lewis et al., 2015). In

comparison, the ventral pathway is responsible for the behavioral state of wakefulness. Studies with patients and animals that had injuries to the ventral pathway spent more time asleep than usual and had difficulties staying awake (Fuller et al., 2011; Ranson. 1939; Scammell et al., 2017).

The EEG Spectrum

The firing of neurons in sleep-promoting and wake-promoting pathways generates brain wave activity that can be detected using an electroencephalogram (EEG). EEG activity is conventionally classified into frequency bands that fixed ranges based on frequency and amplitude (Maloney et al., 1997). The frequency bands in the spectrum (from fastest to slowest) are gamma (30 – 60 Hz), beta (12 – 30 Hz), alpha (9 – 12 Hz), theta (5 – 9 Hz), and delta (0.5 – 4 Hz) (Silva – Pérez et al., 2020; Schwartz & Kilduff, 2015; Maloney, 1997). The dominant frequency band in a time-period is calculated using a quantitative technique known as spectral analysis (Silva-Pérez et al., 2020).

Sleep-wake stages (Wake, REM, and NREM) can be distinguished by the association of wave patterns with behaviors. Wake is distinguished by alertness and motor activity with relatively low amplitude and high frequency waves. The dominant wave patterns during wakefulness are gamma, beta, and alpha (Maloney et al., 1997). The highest mental activity during intense concentration is associated with gamma waves (Maloney et al., 1997). Beta waves increase with alertness and decrease with drowsiness and are associated with fairly high focused mental activity (Xavier et al., 2020). Alpha waves are higher in amplitude and lower in frequency than beta waves, and they are associated with a relaxed, yet awake state (Xavier et al., 2020; Silva-Pérez et al., 2020).

Rapid eye movement (REM) sleep shares similar wave activity with wakefulness with slight differences in wave patterns and a significantly different behavior profile. Like wakefulness, REM sleep is shown to have predominantly gamma and beta waves

but differs in that theta waves are also present in this stage (Maloney et al., 1997). In addition, REM sleep has hardly any movement detected by an electromyogram (EMG). This differs from the abundant motor activity present during wakefulness (Maloney et al., 2017). REM is called "paradoxical sleep" because of its high frequency-low amplitude EEG (characteristic of wake) and its low muscle tone (characteristic of deep sleep). The contradicting nature of REM sleep having low amplitude and high frequency wave activity with low muscle tone is why it is called "paradoxical sleep" (Maloney et al., 1997). NREM sleep, also called slow-wave sleep, is a restorative state of sleep distinguished by sleep spindles and high amplitude, low frequency waves (Silva-Perez et al., 2020; Xavier et al., 2020). Unlike REM and Wakefulness, delta waves are the predominant waves present during NREM.

Serotonin as a Modulator of Sleep

Serotonin (5HT) is a monoamine neurotransmitter, along with other monoaminergic neurotransmitters norepinephrine (NE) and dopamine (DA), that plays a role in regulating sleep-wake stages (Scammell et al., 2017). Serotonin occurs in nine groups of cell bodies in the pons and midbrain. Dorsal raphe nuclei (DRN) consist of serotonergic fibers that ascend and project to the forebrain and descend into the medulla and spinal cord (Monti & Jantos, 2008; Scammell et al., 2017). Serotonergic neurons of the DRN fire at a steady rate during wakefulness, decrease firing during NREM sleep, and nearly cease firing during REM sleep (Monti & Jantos, 2008). Research indicates that serotonin directly excites other wake-promoting neurons, and drugs that hyperactivate 5HT neurons increase wakefulness in humans and animals and fragments NREM sleep (Scammell et al. 2017; Ito et al., 2013). In addition, photostimulation of 5HT neurons in the nucleus accumbens or central nucleus of the amygdala has been shown to quickly awaken mice from sleep (Scammell et al., 2017).

The promotion of wake by stimulating 5HT neurons could depend on the type of stimulation. Other research has shown that the tonic stimulation of 5HT neurons can actually induce sleep, suggesting that it may be burst stimulation that specifically promotes wakefulness (Oikonomou et al., 2019). In one study, serotonergic raphe in zebrafish and mice were tonically stimulated using optogenetics, inducing sleep (Oikonomou et al., 2019). These results demonstrate that alternative approaches in stimulating 5HT neurons could potentially offer novel ways to promote sleep in animals and humans. In addition, differing subtypes of serotonin receptors might account for serotonin's varied roles in controlling sleep and wake.

5HT Inverse Agonists

Serotonin has many different receptors associated with different CNS pathways and differing behavioral functions (Nishitani et al., 2021). It appears to be involved in schizophrenia and is the main target of psychedelic drugs such as LSD (Bonhaus, 2011; Ansah et al., 2011; Preller et al., 2018). Previous research has demonstrated that 5HT2A serotonin receptor inverse agonists such as pimavanserin, volinanserin, pruvanserin and nelotanserin may have the potential to improve sleep quality by enhancing NREM sleep. Volinanserin (MDL100907) is a highly selective inverse agonist, antagonizing 5HT2A receptors 300 times more than 5HT2C receptors (Malin et al., 2019). Pimavanserin is also selective, antagonizing 5HT2A receptors 25 times more than 5HT2C receptors (Malin et al., 2019). The 5HT2A serotonin receptor displays increasing constitutive activity during nicotine withdrawal, due to changes in receptor expression levels (Weiner et al., 2001; Malin et al., 2019). Inverse agonists reduce both constitutive and stimulated receptor activity by binding to the receptor and creating effects opposite to the transmitter binding to the receptor.

Pimavanserin is FDA-approved to treat psychosis-like behaviors in Parkinson's disease (Bonhaus, 2011; Ansah et al., 2011). While this 5HT2A inverse agonist has been approved for treating Parkinson's Disease psychosis, it has not yet been investigated for the treatment of sleep disruption in individuals who have recently quit smoking. Some clinical studies in normal human participants have shown that 5HT2A receptor antagonists can increase restorative slow-wave sleep and reduce the REM sleep (Monti, 2011). Evidence of the role of 5HT2A in sleep can be seen in 5HT2A knockout mice. The knockout mice spent less time in REM sleep, had an increase in wakefulness, and a reduction of slow-wave sleep as compared with wild-type mice (Monti, 2011). In other experiments, 5HT2A receptor inverse agonists or antagonists (ritanserin, ketanserin, pruvanserin, and volinanserin) were administered to rodents at the beginning of the light period (rodents do most of their sleeping during the lights-on phase). This significantly increased slow-wave sleep and reduced REM sleep (Monti, 2011). These promising studies suggest that 5HT2A inverse agonists might be a novel nicotine cessation treatment targeting sleep disturbances.

Animal models of nicotine physical dependence & withdrawal

The relationship between smoking cessation and sleep is complex and not fully understood. Because rodent models of nicotine dependence and withdrawal can mirror human behavior during smoking abstinence, they can help reveal the underlying mechanisms that impact sleep during cessation (Malin & Goyarzu, 2009). Also, rodents and humans have similar neural circuitry and sleep EEG features making rodent models well suited for investigating the behavioral, genetic engineering, pharmacological effects of nicotine dependence and withdrawal on sleep (Mathews & Stitzel, 2019).

Studies have shown that serotonin can modulate complex biological functions such as sleep and may even regulate somatic nicotine withdrawal signs in rodents

(Nishitani et al., 2021). Optogenetic inhibition of 5HT neurons in the median raphe nucleus (MRN) induced nicotine withdrawal symptoms even in mice that were not exposed to nicotine. Conversely, optogenetic activation of 5HT neurons in the MRN almost eliminated the somatic withdrawal sign, ptosis, in mice dependent on nicotine (Nishitani et al., 2021).

Research indicates chronic nicotine administration in rats can increase the effects of 5HT2A/2C agonists (Batman et al., 2004). Inversely, withdrawal from chronic nicotine administration leads to the same effect. This contradictory effect was demonstrated in a study where the pharmacological activation of serotonin 5HT2A/2C receptors with DOI, an inverse agonist, weakened the reinforcing effects of nicotine and reduced locomotor activity (Batman et al., 2004). Rats treated with a selective serotonin receptor antagonist WAY 100635 during nicotine withdrawal did not show an increase in immobility time during a forced swim test. However, the rats did show a reduction in 5HT1A receptor expression (Mannucci et al., 2006). Since the forced swim test is an experimental model for depression, these results suggest that there could be a link between serotonin and mood disorders that arise during nicotine cessation. Another inverse agonist, volinanserin, reduced nicotine withdrawal signs in rats subcutaneously infused with nicotine bitartrate to approximately the same level as in non-dependent rats infused with saline (Malin et al., 2019). These results suggest that the 5HT2A receptor plays a role in mediating the symptoms of nicotine withdrawal syndrome (Malin et al., 2019).

Malin et al. (2022) developed a model of nicotine dependence and withdrawal in which rats underwent the peak stage of nicotine withdrawal around 22 hours after termination of nicotine bitartrate infusion via subcutaneous osmotic minipump. Using this model, later Malin et al. discovered that 5HT2A inverse agonists volinanserin (MDL100907) and pimavanserin reduced somatic nicotine withdrawal signs (2019). The

present study expands upon these findings by assessing whether the 5HT2A inverse agonist volinanserin can alleviate sleep disturbances during nicotine withdrawal. Based on previous research and the model developed by Malin et al., it can be hypothesized that:

MDL100907 will increase sleep consolidation (decrease the number of separate bouts of each sleep stage)

MDL100907 will increase the proportion of NREM sleep.

MDL100907 will decrease the proportion of time in Wake.

MDL100907 will significantly decrease the power of delta and gamma rhythms that are consistent with waking and inconsistent with NREM.

CHAPTER II:

METHODS

Experimental Methods

Animals and Housing

The subjects were 34 male Sprague-Dawley rats, approximately weighing 250 – 300 g. The animals were housed under a 12:12 hr light-dark cycle (light cycle from 10 am – 10 pm, followed by dark cycle 10 pm – 10 am), in a climate controlled facility with food and water available ad libitum. All procedures were approved by the the Institutional Animal Care and Use Committee (IACUC) of the University of Houston-Clear Lake, and were in accordance with the Guide for the Care and Use of Laboratory Animals, 8th ed.

Electrode (EEG/EMG) Implantation

Animals arriving to the University of Houston – Clear Lake Animal Research facility were habituated for seven days to the before starting experimental procedures. All animals were placed under general isoflurane anesthesia (1-3% in O2) administered through a nose cone extension. The first surgical procedure was the implantation of electroencephalogram and electromyogram (EEG/EMG) electrodes. The rats were shaved from the base of the neck up to the eyes. Next, the ears were prepped with 0.5% lidocaine cream and the surgical area cleaned with betadine. Rats were anchored to a stereotaxic apparatus by ear and tooth bars as well as a nose restraint. To numb the surgical area, rats were given a subcutaneous (s.c.) injection of 0.25% mg/kg bupivacaine. Once numb, a scalpel was used to make a single 4 cm incision 1 mm posterior to the eyes along the midline of the skull to the base of the neck. Skull sutures such as the bregma, lambda, the coronal and midsagittal sutures were exposed by removing tissue attached to the skull, and bulldog clips held the skin in place. In order to attach EEG electrodes, four holes

were drilled into the skull. They were located approximately 4 mm caudal and 2 mm rostral to bregma and 4 mm lateral to the midsagittal suture by a Dremmel electric drill with a 3/64 drill bit. Three 3.2 mm flexible stainless-steel electrodes were placed, one each in the left frontal region, right frontal region, and right parietal region. Each stainless-steel electrode was anchored to the skull using an 80 x 0.125 mm stainless-steel screw (36320XXXE, PlasticsOne Roanoke, VA). The fourth hole, in the left parietal region, only had an 80 x 0.125 mm stainless-steel screw anchor screw. Lastly, three flexible stainless-steel EMG electrodes were placed into the superior nuchal muscles (Mathews and Stitsel, 2019; Bautista, 2014; Shahin, 2016; Chapman, 2020). Any blood released during the surgery was cleared using hydrogen peroxide and a cauterizing pen.

All six EEG and EMG leads were gathered to the center of the head and threaded into a 6-channel nylon plastic electrode pedestal (MS363, PlasticsOne Roanoke, VA). The pedestal was secured above the head by a mounting holder (MH363, PlasticsOne Roanoke, VA) attached to the stereotaxic device. Once mounted, the entire area was covered with quick drying dental acrylic to ensure stability, and a nylon plastic dust cap (363DC, PlasticsOne Roanoke, VA) was screwed into the pedestal to keep it clean. Sutures were placed in front of and behind the pedestal and the surgical site was covered with topical 5% lidocaine analgesic. After the surgery, each animal was placed on a heating pad in a recovery cage before returning to single housing. Total procedure time was between 45 and 90 minutes. The recovery period following surgery was 7 days in a new home cage that doubled as a high walled recoding compartment. For 3 days following that, the rats were habituated to being connected to recording connection cables (363363, PlasticsOne Roanoke, VA The animal was then given an injection of the antibiotic Enrofloxain (5.0 mg/kg, SC). Finally, the entire wound area was covered with a topical antibiotic ointment (Neosporin). Each rat was individually housed to prevent any

harm to the new incision and to promote recovery. Each animal had 7 days to recover from surgery.

Habituation

Each rat was connected to an overhead EEG swivel after recovering from surgery and had 3 days to habituate to the overhead electrical swivel commutator (SL6C PlasticsOne Roanoke, VA). EEG and EMG activity was recorded using the Grass model 15 Physiodata 17 Amplification System and model 18LT amplifiers (Grass Technologies, West Warwick, RI).

Baseline Recording

A 24-hour baseline measurement of EEG and EMG activity was performed 4 days after electrode implantation surgery. This baseline measurement started during the 12-hour light cycle (10 am – 10 pm) and was completed at the end of the 12 hr dark cycle (10 pm – 10 am).

Osmotic Minipump Implantation

Under brief general isoflurane anesthesia, an incision was done in the scapular region of the rat perpendicular to the spine to create an approximately 2-inch pocket under the skin. A spatula was inserted to widen the pocket to implant the Azlet osmotic minipump (Model 2ML1, Durect Corp., Cupertino, CA, USA). The minipump was filled with either nicotine bitartrate in saline or 0.9% saline alone (Sigma Aldrich, St. Louis, MO) and placed inside the subcutaneous pocket. The nicotine pump infused approximately 9mg/kg of nicotine bitartrate per day. The saline pump released an equivalent volume of 0.9% saline alone. The minipump infusion period lasted 7 days (Malin et al., 1992; 2006; 2013).

Spontaneous Nicotine Withdrawal

After the 7-day infusion period, the minipumps were removed via a second incision made near the implantation incision under brief isoflurane general anesthesia. This gradually "induced" spontaneous nicotine withdrawal. The pump was pushed out from under the skin through the new opening. After explantation, 0.25% bupivacaine was injected and topical 0.5% lidocaine was applied. The minipump explantation procedure lasted about 5 minutes per animal. Leftover drug solution in the minipump was removed and measured to ensure overall pump efficiency and adequate drug administration.

Injections and Treatment Groups

Roughly 17 hours after minipump removal and immediately before the light phase (10 am), 22 animals were received an intraperitoneal injection (i.p.) of an injection vehicle [20% DMSO, 20% Tween-80, 60% saline]. The remaining 8 animal were injected with 1 mg/kg of MDL100907 (Acadia Pharmaceuticals, San Diego, CA, USA). As a result, there are 3 treatment groups: 10 rats infused with nicotine bitartrate and injected with the injection vehicle (Nic-Veh) to model spontaneous nicotine withdrawal, 12 rats infused with 0.9% saline alone and injected with the injection vehicle (Sal-Veh) to create a negative control group, and 8 rats infused with nicotine bitartrate and treated with 1 mg/kg of MDL100907 (Nic-MDL).

Twelve hours later, immediately before the dark phase (10 pm), the animals were given the same injections again. Polysomnography recording was only paused twice to administer the 1st and 2nd injections immediately before the light (10 am) and dark (10 pm) phases. The 1st injection before the light phase corresponds with onset of peak withdrawal symptoms 18 – 22 hours post-minipump removal. The light phase is also when the rats do much of their sleeping.

Polysomnography (EEG and EMG) Recordings

Polysomnography data was recorded using Grass Model 15 Physiodata Amplification system with model 18LT amplifiers (Grass Technologies, West Warwick, RI) connected to a pc computer installed with VitalRecorder program (Kissie Comtec Co., Nagano, Japan). The .kcd files were auto-scored in 30-second epochs, or episodes of brain wave activity, using SleepSign for Animals v.2.0 (KISSIE COMTEC CO., LTD.). Each epoch was then manually scored by hand to confirm accuracy of the SleepSign software. Sleep/wake behavior was categorized and scored into one of three stages: wake (W), rapid eye movement sleep (REM), or non-REM sleep (NREM).

Statistical Analysis

Although EEG and EMG data was collected and scored for the 24-hour baseline period before osmotic minipump implantation and the corresponding 24-hour withdrawal period post-minipump removal after 7-day infusion, statistical analyses will focus on the 18 – 22 hour peak withdrawal period. The data for the compete 24-hour baseline and complete 24-hour withdrawal periods was analyzed; however prevalence of EEG artifacts led to the decision to exclude these analyses.

The dependent variables measured for each comparison period are time spent in each stage, sleep fragmentation, and spectral band power. Total time spent in each stage is operationalized as the percentage of time spent in Wake, REM, NREM. Sleep fragmentation is operationalized as the number of bouts (defined as the shift from one sleep stage to another). Spectral band power is divided into five EEG rhythms (delta, theta, alpha, beta and gamma) measured in Hertz (Hz). The average of each band (Hz) is measure of voltage squared (v^2) per hour over each of the time-periods listed above. It will be reported as the percent change from baseline of total power within a given sleep-wake stage.

A one-way ANOVA was used to analyze the comparison measurements from baseline to withdrawal for the three treatment groups (Sal-Veh, Nic-Veh, Nic-MDL). Significant ANOVAs were followed by LSD post-hoc comparisons tests. Artifacts, defined as significant disturbance in EMG recorded movement, were removed during scoring. Rats that had greater than 30% EMG artifacts during either the 4-hour peak withdrawal period or the corresponding 4-hour withdrawal period were considered outliers and excluded from statistical analyses. In total, 5 rats were excluded due to artifacts, dropping the sample size from 34, to 29 for statistical analyses. Statistical analysis was conducted using SPSS Statistics for Windows (IBM Corp., Armonk, NY, USA), with an alpha level of .05.

CHAPTER III:

PEAK WITHDRAWAL RESULTS

The following data reflects changes or percent changes from each animal's baseline measurement to the corresponding measurement during nicotine withdrawal. Total time and number of bouts are reported as absolute changes. Alternatively, spectral band power is represented as percent change because research has shown that EEG spectra are unique to each brain (Lewandowski et al., 2013). Using percent change can give a more precise description as to how spectral band power is changing from baseline to withdrawal for each rat. Graphs with an asterisks over brackets denotes significant post-hoc comparisons, according to Fisher's LSD test.

Post-Minipump Removal Peak Nicotine Withdrawal Period

Data recorded between 18 – 22 hours post-minipump removal was analyzed separately in accordance with previous research by Malin (2015) that revealed behavioral withdrawal signs tend to peak around 22 hours post-minipump removal. The peak withdrawal period took place approximately in the middle of the 12-hour light phase when animals spend more time sleeping.

Percent Time in Sleep Stages

Figure 1 depicts the percentage of time in each sleep stage for the 4-hour peak withdrawal period and the corresponding baseline. Overall, nicotine withdrawal tended to decrease restorative NREM sleep while increasing the time spent in Wake. The one-way ANOVA revealed that for Wake, REM, NREM there were no significant effects of treatment during peak withdrawal. However, there was a trend that showed MDL100907 increased the percentage of time spent in NREM sleep during nicotine withdrawal. There was also a trend indicating MDL100907 tended to reduce the percentage of time spent in REM and Wake.

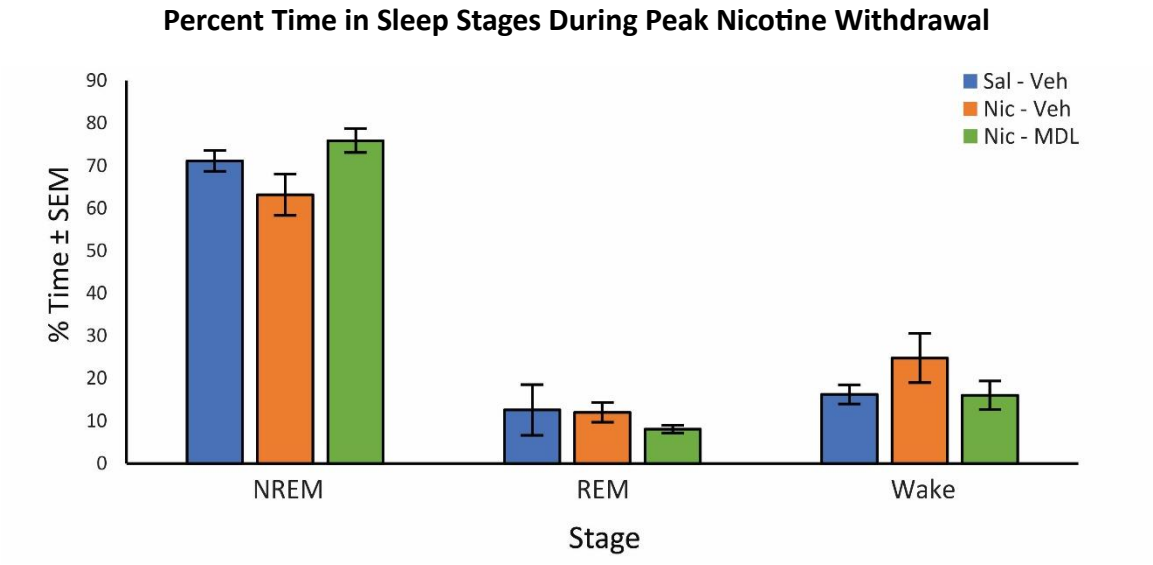

Figure 1:
Average ± SEM percent time in sleep stages during peak nicotine withdrawal (18 – 22 hours post pump removal).

Number of Bouts in Sleep Stages

Figure 2 depicts the number of bouts in each sleep stage for the 4-hour peak withdrawal period and the corresponding baseline. One-way ANOVAs revealed that there were significant effects of treatment in REM ($F(2, 27) = 5.254$, $p = .011$) and NREM ($F(2, 27) = 14.736$, $p = <.001$). There were no significant effects of treatment in Wake.

LSD post hoc comparisons revealed that MDL100907 significantly reduced sleep fragmentation, decreasing the total number of bouts in NREM, REM, and Wake stages. In NREM, bouts were significantly decreased for rats that were infused with nicotine bitartrate and treated with MDL100907 (Nic-MDL), compared to rats infused with nicotine bitartrate and injected with saline (Nic-Veh) ($p = <.001$). The number of bouts for nicotine bitartrate-infused and MDL100907 treated rats (Nic-MDL) significantly decreased below the negative control group (Sal-Veh) ($p = <.001$).

A similar significant reduction was also seen in REM. The number of bouts significantly decreased for rats that were infused with nicotine bitartrate and treated with MDL100907 (Nic-MDL) compared to rats infused with nicotine bitartrate and injected with saline (Nic-Veh) (p = <.016). The number of bouts for nicotine bitartrate-infused and MDL100907 treated rats (Nic-MDL) also significantly decreased below the negative control group (Sal-Veh) (p = <.004).

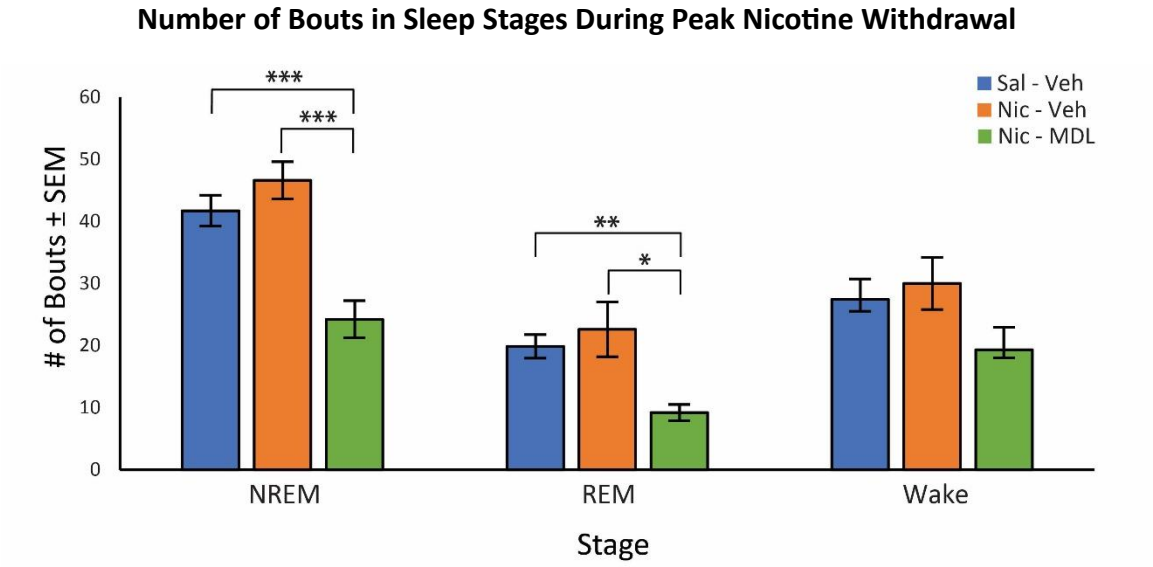

Figure 2:
*Average ± SEM number of bouts in sleep stages during peak nicotine withdrawal (18 – 22 hours post pump removal). * Indicates p <.05, ** Indicates p <.01, *** Indicates p < .001.*

EEG Spectral Band Power

One-way ANOVA was run for the change scores from pre-infusion baseline to 18 – 22 hours post-minipump removal during peak nicotine withdrawal**.** The analysis revealed that there were no significant effects of treatment across all EEG bands in the spectrum. However, there was a tendency for nicotine withdrawal to increase EEG power from pre-nicotine baseline across all bands in the frequency spectrum. There was also a trend for MDL100907 to revert spectral band power in nicotine withdrawn rats close to

pre-nicotine baselines. The propensity for nicotine bitartrate infusion and MDL100907 to have these effects on spectral band power was very apparent for delta frequency during wake and beta and gamma frequencies during NREM. This is very promising considering that delta frequency is inconsistent with wake, and beta and gamma frequencies are inconsistent with NREM sleep. This raises the possibility that both medications like MDL100907 might enhance both restorative NREM sleep and the waking state.

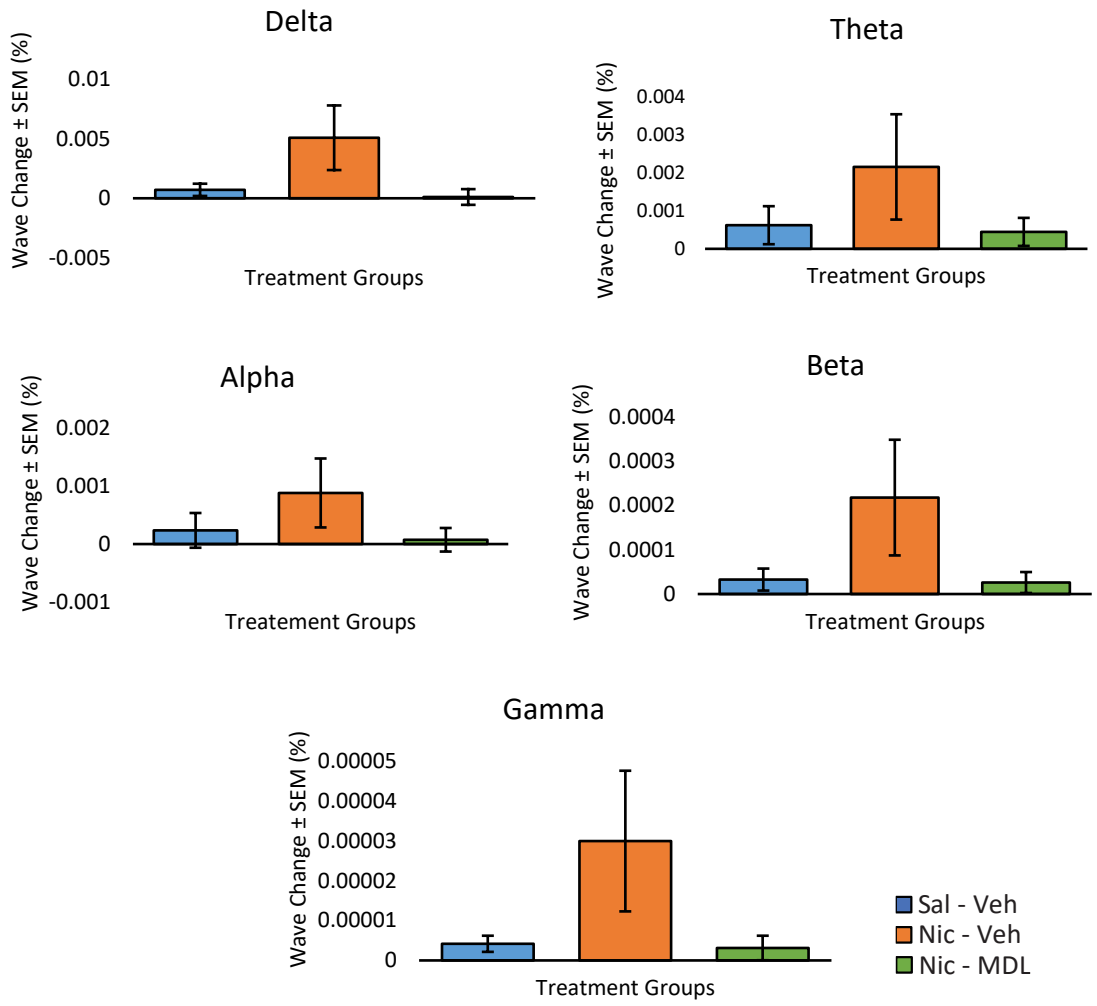

Figure 3:
Average Percent Change ± SEM in EEG Spectral Band Power During Wake from Pre-Infusion to Peak Nicotine Withdrawal (18 -22 hours post-pump removal).

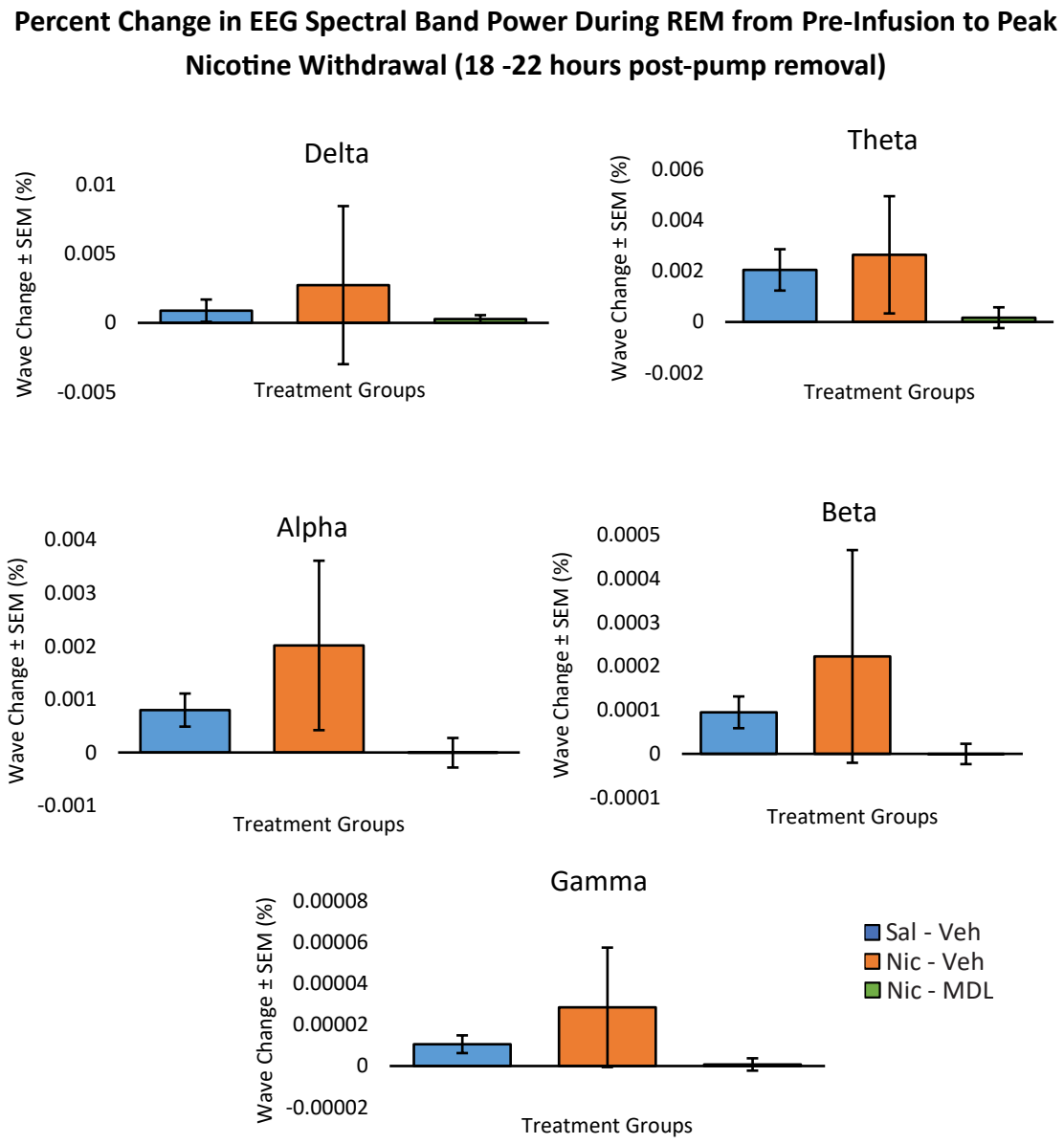

Figure 4:
Percent Change ± SEM in EEG Spectral Band Power During REM from Pre-Infusion to Peak Nicotine Withdrawal (18 -22 hours post-pump removal).

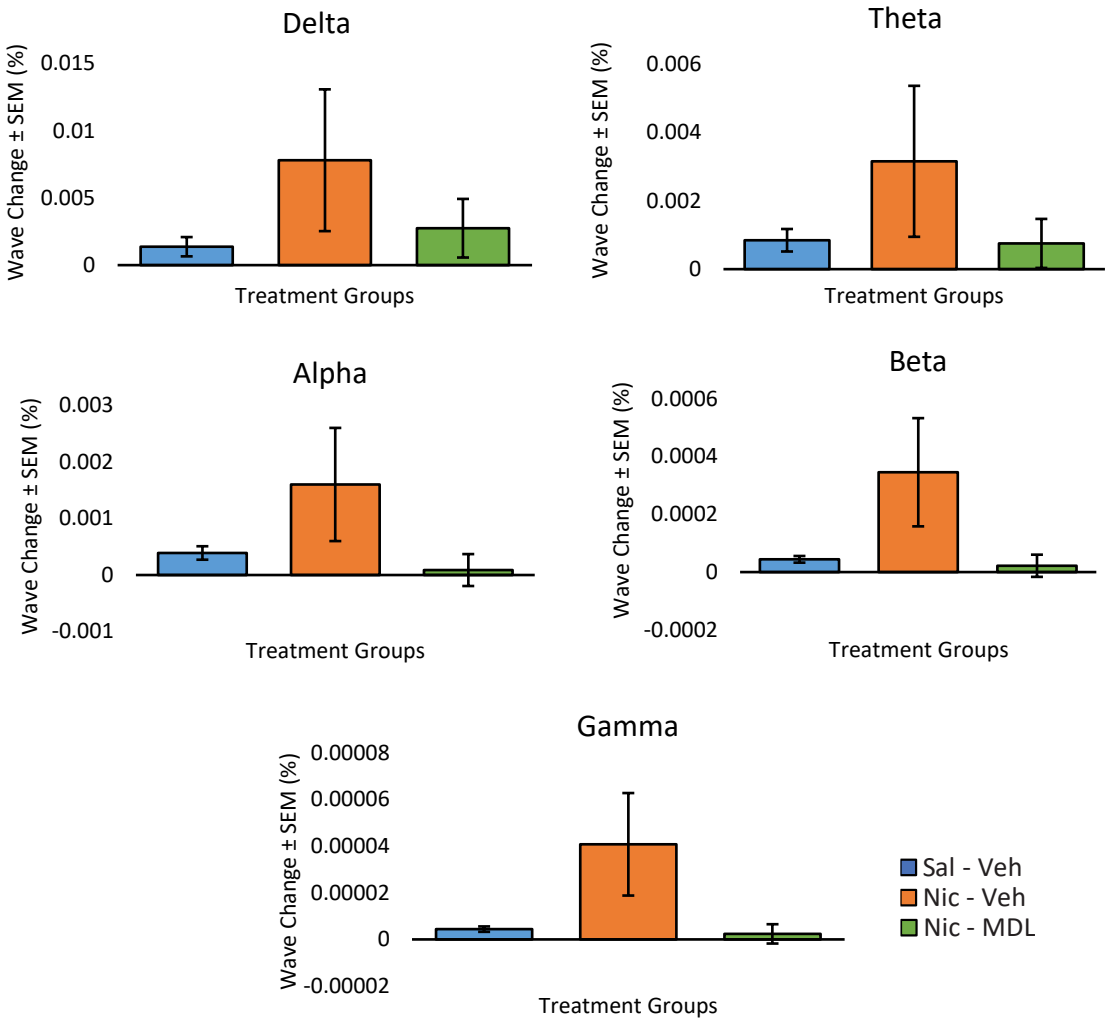

Figure 5:
Percent Change ± SEM in EEG Spectral Band Power During NREM from Pre-Infusion to Peak Nicotine Withdrawal (18 -22 hours post-pump removal).

CHAPTER IV:

DISCUSSION

The findings of this study are consistent with previous research suggesting a negative impact nicotine withdrawal has on sleep quality, and the ability for 5HT inverse agonists to ameliorate at least some negative effects of nicotine withdrawal. In humans and rodents, it has been reported that nicotine cessation leads to sleep disturbances. In smokers, these might potentially trigger a relapse to smoking. In animal models, inverse agonists were shown to prevent both constitutive and stimulated receptor activity. This study explored whether a 5HT2A inverse agonist, MDL100907, could alleviate sleep disturbances caused by nicotine cessation after chronic exposure. This study expands on previous research by specifically targeting deficient sleep, an established nicotine withdrawal sign, and examining the effectiveness of an inverse receptor agonist in treating sleep disturbances induced by withdrawal.

It was hypothesized that MDL100907 can decrease the number of bouts in each sleep stage, increase the proportion of NREM sleep, and decrease the power EEG rhythms inconsistent with their coinciding sleep stage. The results of the present study show that there was a trend for MDL100907 to have a normalizing effect across most of these measures. Consistent with previous research, withdrawal from chronic nicotine exposure tended to reduce time spent in NREM sleep (Mathews & Stitzel, 2019) and increase sleep fragmentation (Patterson et al., 2019). Conversely, rats injected with MDL100907 after chronic nicotine exposure spent more time in NREM sleep and had less fragmented sleep. These results indicate that 5HT2A receptor activity contributes to sleep disorders caused by nicotine withdrawal, suggesting it as a target for sleep disorders in withdrawal.

Nicotine withdrawal increased all EEG power across all bands in the spectrum regardless of sleep stage. Whether the rats were in wake, REM or NREM, all five frequencies increased in power. The alterations in polysomnography profile across sleep stages further solidify the role that 5HT2A plays in sleep disruption during withdrawal. In rats infused with nicotine bitartrate and injected with MDL100907, was a decrease in delta power during wake and a decrease in beta and gamma power during NREM, although these results were not significant. These trends show that 5HT2A inverse agonists have the potential to bring altered spectral power in withdrawal back to a range that is more consistent with the appropriate sleep stage. This possibility could be tested by an experiment with different MDL100907 doses and higher sample sizes.

One major limitation to this study is the presence of too many EEG artifacts during the 12-hou\r and 24-hour periods. Any rats with over 30% artifacts in EEG and EMG data were considered outliers and not included in the analyses. Since there were more artifacts present in the 12-hour and 24-hour periods than in the 4-hour peak withdrawal period, the sample size dropped significantly. Because of incongruous and low sample sizes, the 12-hour and 24-hour data that was scored and analyzed was not reported. A future direction for this study would be to focus on the 12-hour and 24-hour periods with a decent and consistent sample sized to examine the effect MDL100907 would have on bouts, time spent in each sleep stage, and EEG power. Expanding beyond the 4-hour peak withdrawal period would give further insight into whether the trends seen for the peak period could be sustained over longer periods of time.

It has been self-reported by smokers that sleep disturbances increased in cessation. These disturbances worsened with common smoking cessation treatments such as transdermal nicotine replacement and varenicline (Ashare et al., 2017). The side effects of current smoking cessation treatments, reveal a need for novel adjunctive

treatments to improve sleep during smoking cessation. To our knowledge, whether sleep disorders during actual smoking cessation can be treated by reducing 5HT2A receptor activity has yet to be investigated. Pimavanserin is another 5HT2A selective inverse agonist drug in current medical use for treatment of psychotic symptoms of Parkinson's Disease. It might potentially be used to examine the role of 5HT2A receptor activity on sleep in human disorders.

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