EFFECTS OF VOLINANSERIN ON SLEEP AND WAKE STAGES AND THE EEG SPECTRUM DURING NICOTINE WITHDRAWAL IN RATS

by

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Dedication

To my family, whose unflinching support means the world to me and I hope one day I can return in kind.

Acknowledgements

To everyone else that believed, persisted, and achieved around me and gave me the strength to do the same. Special acknowledgements go out to my thesis committee Dr. Ward and Dr. Malin for having the patience to get this project off the ground, again, and make the most of our resources. Huge thanks to Joseph R. Campbell and Ping Sun Tsai for fast-tracking my motivation/sanity and digging into the science and reviewing my writing with me.

ABSTRACT

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Sleep disturbances, such as sleep onset latency and fragmentation commonly occur during nicotine intake and withdrawal. They may be one major factor in supporting relapse during smoking cessation. In a laboratory model of these disturbances, rats underwent surgical implantation of EEG and EMG electrodes. They were monitored and allowed to recover over several days, and a 24-hour baseline was recorded for sleep and wake stages, as well as the frequency spectrum of EEG activity. The rats were then implanted with an osmotic minipump infusing either saline or 9mg/kg/day nicotine bitartrate solution for one week. Seventeen hours after removal of the minipump, rats in their primary sleeping phase (lights on) were given an injection of either vehicle (20% DMSO and 20% Tween-80 in saline) or 3mg/kg volinanserin in that vehicle. The injection was repeated 12 hours later in their primary waking phase. EEG and EMG recordings were analyzed by KisseiComtec SleepSign Software and checked manually by

the researcher. Compared with the non-dependent controls, untreated nicotine withdrawal induced increased sleep-wake fragmentation in the lights-off phase, and alterations in the power of several EEG frequency bands. There were trends for volinanserin to prevent the fragmentation and several of the alterations in band power. Inhibiting the 5-HT_{2A} serotonin receptor with drugs like volinanserin might be a novel treatment approach to help nicotine users avoid sleep disturbances during tobacco cessation.

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CHAPTER I:

BACKGROUND

Health Impacts of Nicotine Use

Over 50 years of research has established nicotine use as a primary cause of premature deaths and other negative health ourcomes. This has not ended the smoking epidemic. A reported 63.4 million people in the U.S. aged 12 and older smoked in 2016 (USDHHS, 2014; NIDA, 2019). Evidence for the addictive properties of nicotine helps to explain the continued use of nicotine-containing products. Symptoms of withdrawal, including sleep disturbances, may cause relapse from quitting attempts. Nicotine replacement therapies (NRT's) and other pharmacological aids do not fully address problems caused by sleep disorders..

Around the late 1900s, 40 percent of the adult population used tobacco and other forms of nicotine, leading to the first Surgeon General's Report on Tobacco and Nicotine (USDHHS, 1964). This 1964 report made it clear that nicotine was a harmful substance in any form and that smoking could lead to a great many health outcomes such as chronic bronchitis, lung diseases and cancer. Since that report, numerous campaigns and laws have led to stricter labeling for product health information and advertising as well as restricting certain forms of tobacco use in and around public spaces. This included the Federal Cigarette Labeling and Advertising Act of 1965 and the Public Health Cigarette Smoking Act of 1969 (USDHHS, 2014). Use in adults had dropped dramatically by the end of the 20th century, but at the turn of the 21st century a new electronic nicotine delivery system, or e-cigarette, rose in popularity, utilizing decades-old tactics such as fruity flavorings and non-empirically based statements of health benefits, along with the introduction of high tech marketing aimed at younger consumers. E-cigarettes featuring electronic or battery powered vaporization of liquid nicotine were patented in the early

1960s. but became popular with modifications such as tanks, vaporizers, mods, vape pens and e-hookas in the late 1990s and early 2000s (USDHHS, 2016; Dutra, Grana & Glantz, 2017).

Vaping the aerosolized contents of an e-cigarette does not require burning tobacco. However, the addition of other chemicals meant to flavor and stabilize nicotine may still have health hazards. The vapor is an aerosolized liquid containing nicotine, which can still produce health problems in the user and through secondhand nicotine exposure (USDDHHS, 2016; Hughes et al., 2019). Though e-cigarettes are delivering a synthetic form of nicotine, and are technically a tar-less and tobacco-less product, federal regulations for e-cigarettes are lumped into those required for tobacco products (NIDA, 2018). A wide selection of more palatable products from e-cigarette companies, as well as lax apporach restrictions of use, have since generated a sharp increase in consumption by minors and young adults. This has been described as an 'epidemic' of youthful e-cigarette and vaporizer usage. Between 2017 and 2018, use of nicotine products including e-cigarettes soared over 50 and 75 percent in youth of middle to highschool ages, respectively (USDHHS, 2020). Recent studies implicate sleep disturbances and impaired next-day functioning as high impact factors for substance use disorders, with predictive qualities for alcohol and other illicit drug uses, in adolescents (Roehrs & Roth, 2015; Wong, Roberson & Dyson, 2015). Major reasons for prevalent nicotine use are the addictive effects of nicotine. These include nicotine craving and the aversive side affects of withdrawal, such as sleep disturbances. These can have have a subsequent impact on daytime cognitive and memory function, fatigue, impaired motor coordination and other long term effects.

Addictive Properties of Nicotine and Physiological and Chemical Impacts of Use and Withdrawal

The recent version of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) of the American Psychiatric Association (APA) lists withdrawal as "a syndrome that occurs when blood or tissue concentrations of a substance decline in an individual who had maintained prolonged heavy use of the substance" (2013). Tobacco, and its predominant addictive ingredient, nicotine, were underscored as having impacts on substance use disorders, withdrawal syndrome and sleep disorders during withdrawal (APA [DSM-5], 2013). These symptoms often cause significant distress or impairment in family, social or occupational aspects of life, which, in turn, contribute to relapse.

Nicotine is a natural substance endogenous to the tobacco plant and some vegetables (Siegmund et al., 1999). Nicotine is primarily metabolized in humans through enymes in the liver and brain into cotinine, the major indicator used to detect nicotine exposure gathered from blood plasma (Benowitz et al., 1983). Nicotine in the brain binds to nicotinic cholinergic receptors (nAChRs). These are made up of protein subunits (α2-α7 and β2-β4) and are primarily formed by a joining of at least two alpha and two beta subunits for a total of five proteins, forming a pentamer receptor (Collins et al., 2009). Nicotine acts on neurons, through nAChRs, which act as ligand-gated ion channels. At first, the activated receptors mimic the effect of acetylcholine on the nerve cell. They later desensitize the receptor, making it less responsice to acetylcholine or additional nicotine.

In the automic nervous system, which controls our internal physiology, nAChRs are found on all sympathetic ganglia cells, In the volunary motor control system, they are found in the neuromuscular junctions, which connect the nervous system to the muscles. In the central nervous system (CNS), they play a role in executive functioning, memory

and control of sleep and waking. In the brain, a high proportion of nAChRs are found in the thalamus, a region with highly structured connections to the cortex, amygdala and hippocampus. This region is linked to executive functioning, memory, and sleep-wake control (Fama & Sullivan, 2015; Gent, Bassetti & Adamantidis 2018).

Preparing tobacco leaves for human consumption can be done by heating and curing for snuff and pipe tobacco. Alternatively, burning is used to extract liquid particulates called tar droplets that are used with hundreds of other stabilizing and flavoring chemicals for cigars and cigarettes. Smoke from these is inhaled and absorbed by the lungs into the bloodstream (Benowitz, 2009). It enters the the blood through hundreds of small capillaires in tiny pouches of lung tissue called alveoli. Absorption through this method allows curculation to the brain and body in a matter of 10-20 seconds, rapidly activating the CNS and the PNS (peripheral nervous system). In the CNS, nicotine stimulates high affinity nAChRs containing $\alpha 4$ and $\beta 2$ subunits . These are often expressed on and near nerve terminals that regulate release of GABA, the main inhibitory neurotransmitter. They also regulate dopamine activity in areas such as the nucleus accumbens (NAc), ventral tegmental area (VTA), amygdala, thalamus and others (Collins et al., 2009; Lawrence, Ross & Stein, 2002). Release of dopamine in areas such as the NAc and VTA leads to activation of the dopaminergic reward system, ultimately leading to nicotine addiction. This, in turn, leads to drug seeking and cue reactivity, heightened responsiveness to stimuli associated with a drug. In the peripheral nervous system, the adrenal gland releases epinepherine, commonly known as adrenaline, increasing blood pressure, blood sugar and heart rate (Benowitz, Hukkanen & Jacob, 2009). Chronic nicotine administration also exerts indirect effects through altering activity of the monoamine oxidizing enzymes A and B. These enzyymes control levels of

the important neurotransmitters serotonin, dopamine and norepinephrine, thus affecting many nervous system functions (Volkow et al., 1999; Finberg & Rabey, 2016).

Higher absorption of nicotine happens when nicotine is in its basic pH form. Smoking provides the fastest route of administration to the brain, 10-20 seconds, versus oral administration to the mucous membranes with peak levels around 30 minutes after administration. Nicotine replacement therapies (NRT) provide slower and more gradual release of nicotine to the blood and brain in attempts to buffer the abuse potential of tobacco use. NRTs are delivered in products such as gum, patches, inhalers, nasal sprays, dissolving tablets and lozenges (Hukannen, Jacob & Benowitz, 2005). Of those treatments listed above, none to date are specifically targeted to alleviate sleep disturbances as a result of nicotine use and withdrawal.

During smoking cessation, there are several symptoms of nicotine withdrawal that impede daily functioning and mood level. These include slowed cognition and motor function, increased appetite accompanied by weight gain, irritability, depressive- or anxiety-like symptoms, and insomnia and sleep disturbances (Hughes 2007) .

Animal models of nicotine withdrawal

To be able to test NRT agents and other pharmacological cessation aids, such as veranicline and bupropion, rodent models of nicotine dependence and withdrawal were developed with highly observable effects analagous to the symptoms of human nicotine withdrawal syndrome. There are several methods to induce nicotine physical dependence in a rodent model, but a common model involves subcutaneous infusion of a nicotine bitartrate solution administered at a steady continuous rate by an osmotic minipump (Malin et al., 1992). Osmotic minipumps utilize a controlled pressure pump system whereby water is drawn through osmosis from the surrounding tissue through a semipermeable layer and into an osmotically concentrated layer. When the osmotic layer

swells with water, it compresses an internal impermeable drug filled reservoir to dispense the drug (Theeuwes & Yum, 1976). Nicotine bitartrate can be infused subcutaneously over one week at a rate of 3.15 mg/kg/day to result in rodent blood plasma levels similar to that of a heavy smoker (Malin & Goyarzu, 2009; Benowitz, Hukkanen & Jacob, 2009). Nicotine withdrwal can be initiated by injecting a nicotine antagonist drug (precipitated withdrawal) or by removing the nicotine-infusing osmotic minipump (spontaneous withdrawal). Peak behavioral withdrawal symptoms occur between 22-36 hrs after pump removal (Malin & Goyarzu, 2009).

There remains very little published research regarding nicotine withdrawal and sleep disturbances in the rat. This impedes understanding of the biological basis of nicotine-related sleep disturbances and on potential treatments for these disorders. Malin and Goyarzu (2009) point out that the main rat model of nicotine withdrawal resulted in counterparts to all of the human nicotine withdrawal symptoms except for sleep abnormalities.

Sleep disturbances in nicotine withdrawal

In human tobacco users, sleep disturbance is a prominently reported symptom of nicotine withdrawal (APA [DSM-IV-TR], 2000; APA [DSM-5], 2013). Abnormalites include delay in sleep onset (insomnia), frequent awakenings, a decrease in total sleep time and daytime tiredness. However, the relationship between the degree of nicotine dependence and sleep disturbance is somewhat complex (Shiffman et al., 2007). In studies of chronic nicotine dosing, nicotine was shown to be metabolized or cleared slowest at night, resulting in persistent plasma concentrations (Gries & Brooks, 1996).

Self-reported sleep disturbances that accompany nicotine withdrawal syndrome, such as sleep fragmentation, difficulties getting to and staying asleep, and daytime fatigue are a major factor in relapse rates for individuals trying to quit (Wetter, Fiore, Baker, &

Young, 1995; Grove et al., 2006). Recent studies implicate sleep disturbances and impaired next day functioning as high impact factors for substance use disorders in general. Sleep problems are predictive of alcohol abuse and other illicit drug use in adolescents (Roehrs & Roth, 2015;Wong, Roberson & Dyson, 2015).

The Neurobiology of Sleep

Defining characteristics of normal Sleep-Wake stages include transitions between wakefulness (W), non-rapid eye movement (NREM) sleep, and rapid eye movement (REM) sleep. These different stages of sleep and wake can be characterized by wave forms measured by electroencephalogram (EEG), measuring patterns of activity in the brain. These are typically recorded from areas of the skull in animal studies or the surface of the scalp in human studies. This can be supplemented by measuring muscle activity through electromyography (EMG). Wakefulness can involve gamma (γ) EEG waves that range from 30-120Hz, beta (β) waves ranging 15-30Hz, alpha (α) waves ranging 8-14 Hz, and theta (θ) waves ranging 4-8 Hz. Wakefulness can be characterized by low-voltage fast EEG activity (LVFA) and high muscle tone, active attention, and memory function (Brown et al. 2012). Wakefulness is followed by NREM or slow-wave sleep (SWS), which changes patterns from LVFA to large amplitude and slow waves with appearances of bursts of activity called spindles (Brown et al., 2012).

In humans there are 3 distinct stages of NREM (Silber et al., 2007), but these are harder to discern in rodents and other smaller mammals where only slow wave sleep (NREM) and sleep with total muscle paralysis but heightened brain activity (REM) can be discerned. N1 shows activity of theta waves near the base of the brain and alpha waves in the frontal lobes inducing a state of drowsiness. N2 is identified by spindles (7-15 Hz) and K-complexes and N3, or deep sleep, is characterized by high amplitude slow delta waves (1-4 HZ). NREM, the most restorative sleep phase, is followed by a phase of

mentally active sleep called rapid eye movement sleep (REM). REM is characterized by increased higher frequency β and γ waves and a paralysis of the muscle tone (Silber et al, 2007).

The various sleep-wake stages are initiated by the firing of large numbers of neurons in areas of the brain stem and midbrain. Their axons project signals to other areas of the brain such as the neocortex (Brown et al., 2012). The resulting electrical activity in the target regions can be measured at the scalp and in muscles by electroencephalogram and electromyogram (EMG) recorders, respectively. Activation or inhibition of electrical activity is influenced chemically by receptors on the neurons and neurotransmitters substances that briefly bind to those receptors (Brown et al., 2012).

It is important to note that there are usually multiple types of receptor for each transmitter. Thus, the same transmitter can have different effects on sleep-wake stages depending on the receptor and specific sub-type to which it binds. The neurotransmitter acetylcholine promotes wakefulness when it binds to targets for nicotine, the nAChRs. Nicotine acts on the nicotinic receptor system by binding to the receptors, allowing an influx of sodium and a depolarization of the cell membrane (Malin & Goyarzu, 2009; Collins et al., 2009). However, this often results in a subsequent desensitization of the receptor, a biphasic effect. The transmitter serotonin (5-HT) has varied effects on sleep-wake phases, mediated by different receptor subtypes. A major goal of the present research is to shed light on the role of the serotonin 2A receptor (5-HT_{2A}R) on sleep regulation.

Sleep Fragmentation

Important parameters of sleep quantity include total time spent in bed, sleep onset latency, total sleep time and number of awakenings. Also critical to sleep efficiency is sleep architecture (timing and percentage of each of the sleep and wake stages, and

number of transitions beteen them). Together, these exert additive effects on overall sleep health (Patterson et al., 2019). The restorative functions and homeostatic regulation of sleep require a high proportion of slow wave (SWS) or NREM sleep relative to the period of wake preceeding it (Borbely & Achermann, 1999). Reduced time and fragmented bouts of NREM sleep are a suspected factor in neurocognitive degeneration in the elderly and patients with insomnia (Monti et al., 2018). A specific marker for sleep deprivation is the power of the spectral band delta. Delta waves during SWS in NREM are noticably higher after sleep deprivation and delta power has been shown to co-vary with sleep duration and intensity (Davis et al., 2011). A recent rodent model of nicotine administration and withdrawal effects on sleep continuity and architecture show similar disruptions to total time spent asleep, NREM time and bout frequency, and reduced alertness in mice trained to orally self administer nicotine (Mathews & Stitzel, 2019).

Treatments for nicotine dependence

There are only three FDA approved medications for use in nicotine dependence treatment: nicotine replacement therapy (NRT), bupropion, and varenicline (Hughes et al., 2007). None of these treatments are targeted towards sleep disturbances, but several have adverse sleep consequences. Some of those adverse sleep disturbances include nightmares, insomnia, next day sleepiness and impariments in next day functioning and cognition (Jorenby et al., 2006) . The use of NRT or varenicline during withdrawal may increase sleep disturbances compared to those unaided in cessation, relapse to smoking, or continued tobacco use (Ashare et al., 2017). Research also suggests that sleep disturbances in regular smokers prior to cessation attempts can predict relapse rates during and after withdrawal (Peters et al., 2011).

Difficulty falling asleep and increased number of awakenings can be symptoms of both nicotine dependence treatment and withdrawal (Hughes et al., 2007). This leads to a

need for nicotine treatments that target alleviating sleep disturbances during withdrawal. Early screeing for drugs with potential effects on insomnia have generally utilized smaller mammalian species (Brown et al., 2012). Mammalian patterns of sleep are somewhat conserved across numerous species. The rat is a small species that provides useful models of both nicotine withdrawal (Malin & Goyarzu, 2010) and sleep disturbances (Brown et al., 2012). The basic symptoms of nicotine-related sleep disorders have been defined from human clinical studies. However, animal laboratory experiments are still needed to identify the molecular basis of these disorders and provide a basis for novel treaments (Shiffman et al., 2006).

Animal Models of Sleep

Sleep can be monitored by recording large areas of neuronal activity in the frontal and parietal regions of the brain from the scalp or skull, utilizing a Fast Fourier Transform (FFT) to identify wave properties. Wave types can be analyzed by their voltage amplitude and frequencies into classes of waves (α,β, γ, δ, θ), that are further characterized into the sleep stages W, NREM, REM (Brown et al., 2012). To account for activity produced by bodily rhythms (heart beat and breathing) and motor outputs (movements, grooming, eating and blinking), EMG recordings are also taken in the nuchal muscles, occular muscles, hypoglossal and upper spinal muscles (Rukhadze, Kamani & Kubin, 2014; Lu et al., 2006).

Serotonin as a Modulator of Sleep

The endogenous subtance serotonin, 5-hydroxytryptamine (5-HT), has varied receptors falling into seven family types or classes (5-HT_{1-7}). Serotonin functions as both a neurotransmitter and a hormone in the CNS and PNS and is broadly distrubuted throughout the brain and body. Serotonin is involved in the regulation of physiological responses such as arousal, neuroendocrine function, appetite, cognition, mood, memory

and sleep. It has been implicated in a vast number of neuropsychiatric disorders (Meltzer and Roth, 2013). Serotonin receptors are primarily metabotropic and are in the G protein-coupled receptor (GPCR) family. This family mediates of a great majority of cellular responses to hormones, neurotransmitters, ions, photons and other stimuli. GPCRs are recognized by seven α-helical structures spanning the cell membrane. They have have a great spectrum of biophysical and biocehmical properties within each class of receptors. (Rosenbaum, Rasmussen & Kobilka, 2014). A great deal of serotonin is produced as a hormone in the intestines. However, a large amount of what is produced in the brain originates in the dorsal raphe' nucleus of the brain stem that projects to higher areas including the cortex, hippocampus and hypothalamus (Szymusiak & McGinty, 2011). Serotonin has been implicated in regulation of sleep by modulating arousal and the amount of slow wave sleep. This regulation is accomplished through a number of functions including receptor-mediated pre- and postsynaptic inhibition, and slow rhythm firing in opposition to REM-promoting cholinergic systems (Brown et al., 2012; Szymusiak & McGinty, 2011; Zhang et al., 2018; Monti et al., 2018). Specifically, the 5-HT_{1A}, 5-$HT_{1B,}$ 5-$HT_{2A,}$ 5-$HT_{2C,}$ 5-HT_6 and 5-HT_7 subtypes are implicated directly in sleep function (Brown et al., 2012; Szymusiak & McGinty, 2011; Zhang et al., 2018; Monti et al., 2018).

Selective 5-HT_{2A}R, Receptor Antagonists/Inverse Agonists

The focus of the present research is on the serotonin receptor family (5-HT2R), specifically the sub-receptor 5-HT_{2A}, in relation to sleep abnormalites during nicotne withdrawal. The 5-HT_2 receptor has three subtypes (5-HT_{2A-C}) that are represented in major portions of the brain as well as the heart. They influence heart valve rhythms, feeding, sleep, anxiety, and numerous behavioral actions. Selective 5-HT_{2A}R, antagonists have been tested as a potential treatment for insomnia in preclinical and

clinical studies (Monti et al., 2018). As of 2008, there were 9 separate 5-HT_{2A}R antagonists or inverse agonists in clinical trials for insomnia including eplivanserin, volinanserin, quetiapine, esmirtazapine, pruvanserin, APD125, AVE8488, HY-10275 and pimavanserin (Teegarden et al., 2008). Many of those inverse agonists/antagonists of the 5-HT_{2A}R, have been shown to influence sleep cycles by increasing slow wave sleep (SWS) categorized by a spectral power density of NREM between 0.5 – 3.5 Hz, and decreasing rapid eye movement sleep (REM) in mice, rats and in humans. These drugs includ M100907 (volinanserin), pimavanserin, ritanserin, and eplivanserin (Rohers & Roth, 2015; Moriarty et al., 2008; Ancoli-Israel et al., 2011). Volinanserin, or MDL100907 ((+/-) 2,3dimethoxyphenyl-1-[2-(4-piperidine)-methanol, as well as pimavanserin, are both highly selective inverse agonist/antagonists of the 5-HT_{2A}R. Pimavanserin, in particular, has been shown to influence the SWS of healthy humans and can be hypothesized to be a marker for translational effects of sleep if administered to rats, based on currently unpublished observations (Ancoli-Isreal et al., 2011). Ancoli-Isreal et al. were able to test the effects of pimavanserin in a clinical trial of healthy humans that were still allowed to consume alcohol and smoke less than 10 cigarettes a day. The results of that sutdy showed that pimavanserin significantly affected sleep architecture, sleep profile and the spectral power density starting with a single dose and then extending to daily intake over a 14 day period. In that study Pimavanserin did not have an effect on total sleep time or the parameters surrounding REM sleep except showing a significant decrease in beta towards the end of the study. The effects of volinanerin to increase the NREM sleep in mice was further validated by utilizing wild type and 5-HT_{2A}R knock out mice. The volinanserin effects were almost completely abolished in the knock out mice, demonstrating dependence on the 5-HT_{2A} receptor.

The question remains, how effective are 5-HT_{2A}R antagonists at reversing sleep distrbances caused by abnormal conditions, such as nicotine withdrawal?

The present study tested the hypothesis that the highly selective 5-HT_{2A}R antagonist volinanserin would reverse the effects of nicotine withdrawal on sleep. Previous research in our UHCL laboratory showed that, when volinanserin was used during nicotine withdrawal, a larger percentage of sleep time was spent in NREM sleep, there was less sleep fragmentation, and greater beta and gamma power was observed on the following day, suggesting better alertness (Shahin et al., 2016). The current study will focus on replicating the basic finding of that study, as well as adding a necessary sham control condition of rats treated only with saline-filled minipumps. We hypothesized that volinanserin would help alleviate effects of nicotine withdrawal by increasing the amount of time spent asleep, particularly in NREM, reducing sleep fragmentation and increasing sleep efficiency based on alterations of various EEG specral bands.

CHAPTER II:

METHODS

Experimental Methods

Animals

The subjects were male Sprague Dawley rats (n = 16), approximately 250-300g divided into three treatment groups of saline pump-vehicle injection, nicotine pump-vehicle injection and nicotine pump-volinanserin injection. Several animals (n=4) were removed from the study due to high levels of artifact in the recordings. Rats were individually housed in a light/dark cycle (1000-2000) with food and water available ad libitum. Animals were maintained on a cycle of 12 hours with lights-on, followed by 12 hours with lights-off. It should be noted that the lights-off phase is normally their more active peiod. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Houston-Clear Lake.

EEG/EMG Implant Surgeries

Animals arriving at the UHCL Animal Research Facility were habituated for seven days to the laboratory before experimental procedures occurred. The first surgical procedure that the animals underwent was implantation of electroencephalogram and electromyogram (EEG/EMG) electrodes under isoflurane anesthesia. Animals were first placed in an isoflurane chamber until slowed breathing and atonia were observed. The animal was then placed in a nose cone extension of the isoflurane system and shaved from the base of the neck up to the eyes. The inner ears were prepped with 0.5% lidocaine cream and the surgical area cleaned with betadine. The rat was then fitted into an isoflurane nose cone extension affixed to a stereotaxic device and anchored by ear bars as well as a tooth bar and a nose restraint. Checking the depth of the anesthesia was performed by manually pinching the distal portion of the toe to assess pain reaction. The

rat was then given a subcutaneous (s.c.) block line injection of 0.25% mg/kg bupivicaine to numb the site for incision. This procedure required a single 4 cm incision starting 1 mm posterior to the eyes along the midline of the skull to the base of the neck to allow the experimenter to affix EEG electrodes to the top of the skull and EMG electrodes to the superior nuchal muscles near the base of the skull (Mathews and Stitsel, 2019; Bautista, 2014; Shahin, 2016). Experimenters utilized a scalpel blade and cotton-tipped applicators to clear blood and tissue away from the skull. Hydrogen peroxide and a cauterizing pen were used to stop any bleeding from the skull and several landmarks including bregma, lambda, the coronal and midsaggital sutures were exposed by clamping the skin back with bulldog clips. Four holes were drilled with a Dremmel electric drill with a 3/64 drill bit, each placed 2 mm caudal or rostral to bregma and 4 mm lateral to the midsaggital suture. This allowed recording electric activity from the left hemispheric and right hemispheric pareietal regions as well as the right frontal region (see Figure 1). Three 3.2mm flexible stainless steel electrodes attached to 80x0.125 stainless steel screws (36320XXXE, PlasticsOne Roanoke, VA) and one 80x0.125 stainless steel anchor screw were screwed into the skull. Three 38 mm flexible stainless steel electrodes (3632SPCXE, PlasticsOne Roanoke, VA) were stripped of the gold plating up to 1mm and bent in a less than 1mm

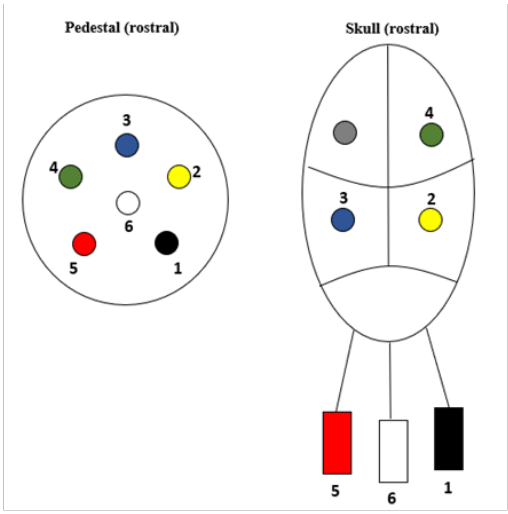

Figure 1
EEG and EMG electrode placement. EEG lectrodes are first affixed to the skull in the order listed above along with a grounding screw. EMG electrodes are then implanted into the superior nuchal muscles. All 6 electrode leads are then strung into the pedestal and fixed to the skull with dental cement.

hook shape. They were guided by hard plastic tubing into the superior nuchal muscles. Placements of the electrodes in the fronto-parietal regions as well as the superior nuchal muscles allowed the experimenters to record the electrical field activity of large groups of cortical neurons and muscle fibers, respectively. The six electrodes were threaded into a 6-channel nylon plastic electrode pedestal (MS363, PlasticsOne Roanoke, VA). The pedestal was initially held in place above the head by a mounting holder (MH363, PlasticsOne Roanoke, VA) attached to the stereotaxic device.

Once each lead was connected in place using forceps and a dental mirror, the electrode placement was recorded, and the mounting holder and pedestal were lowered onto the skull and fitted in between the four screws. Under a vented hood, a mixture of dental acrylic with gentamycin antibiotic formed a quick drying putty. It was applied with dental exploration tools under and around the pedestal to insure stable binding to the skull throughout the experiment. Within 5 minutes the acrylic dried to form a hard casing, the mounting holder was lifted, and a nylon plastic dust cap (363DC, PlasticsOne Roanoke, VA) was screwed into the pedestal to keep it clean. Animals were then sutured in front of and behind the pedestal and the surgical area was covered with topical 5% lidocaine analgesic. The animal was then removed from isoflurane anesthetic and the stereotaxic device. It was placed on a heating pad in a separate recovery cage before being returned to single housing in its home cage. The entire procedure took between 45 minutes and 90 minutes to complete. The recovery period following surgery was 7 days with daily light handling and housing 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). Movement was allowed via overhead electrical swivel commutator (SL6C PlasticsOne Roanoke, VA). To record data from the animal's EEG and EMG rhythms, a Grass model 15 Physiodata

Amplification System and model 18LT amplifiers (Grass Technologies, West Warwick, RI) were used. Beginning with the lights-on phase of the fourth day, 24 hours baseline measures of EEG and EMG during sleep/wake were recorded.

Osmotic Minipump Implant Surgeries

Following baseline recording, the animals underwent osmotic mini-pump implant surgery (Malin et al., 1992). The minipump was implanted in the subcutaneous space above the scapular region under brief isoflurane anesthesia. A two inch incision was made perpendicular to the spine above the scapular region, and a spatula was inserted into the subcutaneous space to widen a pocket for the mini pump to fit. The Alzet osmotic mini pumps had a 2 ml capacity with a 10 μl/hr flow rate and could contain enough solution to last 1 week. The pump was inserted with the flow moderator first, facing away from the incision opening. A 0.25% bupivicaine solution was applied to the incision, and several staples clamped the opening closed. Lastly, 0.5% lidocaine was applied to the surgical area. In this experiment, the pumps were filled with either 0.9% saline alone or with nicotine bitartrate (Sigma Aldrich, St. Louis, MO) in saline. The concentration of nicotine bitartrate was adjusted to produce infusions at a rate of 9 mg/kg/day for 7seven days.

Spontaneous Nicotine Withdrawal

Immediately following the 7 day infusion period, the minipumps were explanted under isoflurane anesthesia. The procedure was approximately 5 min in length per animal. Experimenters made a second incision near the initial implant, removed the pump, injected subcutaneous 0.25% bupivicaine, closed the incision with wound clips and applied topical 0.5% lidocaine. To confirm pump efficiency and appropriate overall drug administered, the amount of drug solution remaining in the pumps was measured by removing liquid in the inner chamber with an Alzet filling needle attached to a syringe.

Treatment Group Injections

Seventeen hours after the time of explant, eleven animals received an intraperitoneal (i.p.) injection of 1 mg/kg volinanserin [MDL 100907] dissolved in an injection vehicle of 20% DMSO, 20% Tween-80, 60% in saline]. Alternatively, five rats were injected with the injection vehicle alone. Thus there were three treatment groups: four rats in nicotine withdrawal treated with injection veicle alone, seven rats in nicotine withdrawal treated with 1 mg/kg volinanserin, and five sham control rats, exposed to the sme surgical procedures, but never exposed to nicotine, treated with injection vehicle alone.

These procedures were timed to correspond to the onset of peak nicotine withdrawal symptoms at 17-21 hrs and the 12 hr light phase when rats do the majority of their sleeping. The injections and the measurements were repeated during the succeeding dark phase when rats are generally more active. Throughout the experiment, polysomnography was recorded from animals utilizing VitalRecorder software (Kissie Comtec Co., Nagano, Japan).

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). After recordings, .kcd data files from Vital Recorder were analyzed through SleepSign for Animals v.2.0 (Kissie Comtec Co., Nagano, Japan). Recorded data was broken into four periods in the experimental procedures, 12 hours lights-on, 12 hours lights-off, the four hours of peak withdrawal syndrome during lights-on and the corresponding period during lights-off. These will be referred to respectively as 12 hr, 24 hr, 4 hr and 16 hr. Each period was assessed by applying software auto-screening

templates for both EEG rhythms and logical recognition of the three sleep-wake phases. After completion of the auto-screening, experimenters manually checked the EEG and EMG tracings to verify the classification of sleep-wake state into non-rapid eye movement sleep (NREM), rapid eye movement sleep (REM) or wakefulness (W) during 30 sec increments or epochs. Additionally, manual screening included identification of EMG artifacts distorting the EEG signal. Continuous FFT data was extracted with artifacts removed and placed into spreadsheets for each recording timepoint for further analysis.

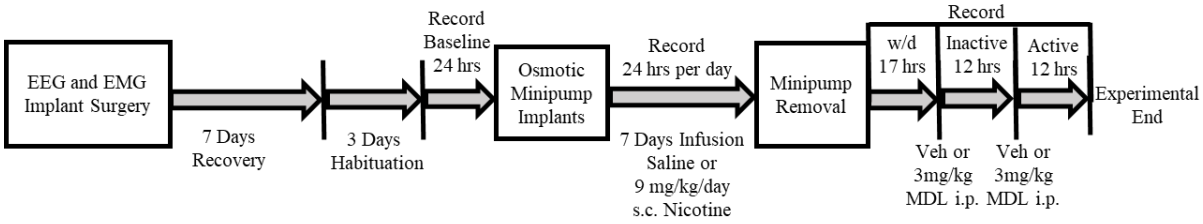

Figure 2
Experimental Timeline for EEG/EMG Polysomnography

Polysomnography data was analyzed in 30s epochs, or episodes of brain wave activity, and averaged across twelve-hour light and dark cycles. The following time delineations will observe a 24-hour clock to distinguish twelve hours of light (hours one through twelve; 12 hr) and twelve hours of dark (hours 13 through 24; 24 hr) cycles. Baseline recordings include the light cycle (12 hr) and the dark cycle (24 hr), which moving forward will be referred to as 12 hr Baseline and 24 hr Baseline periods, respectively. Similarly, the time points measured after peak withdrawal that include treatment injections of systemic 3mg/kg volinanserin (MDL) or vehicle will be referred to as 12 hr Withdrawal and 24-hour Withdrawal, respectively. Additionally, data is further analyzed into the peak withdrawal period which includes cumulative hours one through four in the light cycle of withdrawal. This peak withdrawal period (4 hr) coincide with the first four hours after the first treatment (injection) of vehicle or MDL100907. To

make comparisons with the corresponding hours of withdrawal in the dark cycle, hours 13-16 were analyzed separately following the second treatment (designated as 16 hr).

Behavior was classified into three different states by means of EEG and EMG analysis: wakefulness (W), non-REM sleep (NREMS), and rapid eye movement sleep (REM). Two separate dependent variables were measured, including fragmentation of sleep and power spectral analysis of EEG waveforms. Sleep fragmentation is defined as the transition of NREM to W stages as well as the percentage of time spent in each stage. Power spectral analysis is defined as the stage averages of each spectral band's (δ, θ, α, β) Hertz (Hz) range as a measure of voltage squared (v2) per hour over each of the four periods defined above. To normalize the data of individual animals, the power spectral analysis will be reported as the spectral band's percent of total power within a given sleep-wake stage.

Data for sleep fragmentation and power spectral analysis was analyzed using change scores comparing each animal's withdrawal data to its baseline data for corresponding 12 hr, 24 hr, 4 hr and 16 hr periods. One-way ANOVAs followed by appropriate Tukey's multiple non independent comparisons tests were used to analyze the change scores from the three treatment groups. Artifacts, defined as significant disturbance from EMG recorded movement, are removed from all analyses, which may affect the overall amount of time reported in each twelve- or four-hour period.

CHAPTER III:

RESULTS

Each spectral band of EEG recordings is defined by its frequency (Hz) on a continuum and by its amplitude (v^2) as the signal measured from area-wide scalp or skull. These variables are used to calculate the power of that band or (v^2/ Hz) and recordings are transformed into wave forms by Fourier Fast transform (FFT). Frequency of the spectral bands between 0-120 Hz are broken into 0.5 to 3.5 (delta, δ), 3.5 to 7.5 (theta, θ), 8 to 12 (alpha,α), 13 to 30 (beta,β), and 15 to 120 (gamma, γ). For the following graphs, gamma will be excluded from the analysis due to an extraction error from the data, and beta will be defined as 13 to 20 Hz.

Sleep Phases

Percent Time Spent in Sleep and Wake Stages

To identify whether the animals in this study underwent overall typical sleep patterns for the light/dark cycle (1000-2000 hours), we graphed the percentage of time in each sleep stage for both baselines and withdrawal periods in Figure 3. The figure shows that animals were typically sleeping more in the light cycle and awake more in the dark cycle throughout the study. This expected result confirms the sensitivity of the polysomnography methods employed in this study. To control for pre-existing individual differences in our animals, all data was converted into change scores from baseline to nicotine withdrawal. This was done for the four major time points in withdrawal light cycle (12 hr), dark cycle (24 hr), 4 hr peak withdrawal (4 hr) and four hours into dark cycle after peak withdrawal (16 hr).

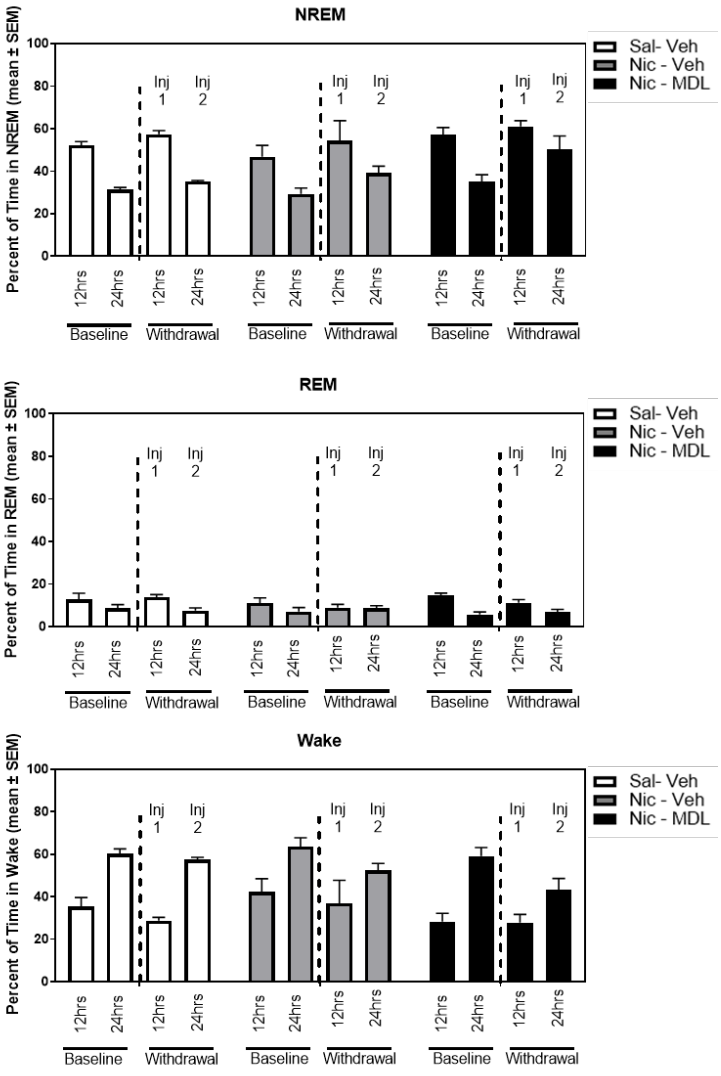

Figure 3

Percent of Time in Stages. Visual representation of the percent of time spent in NREM, REM, or Wake (mean ± SEM) for the three treatment groups. Time bins broken down into 12 hr and 24 hr Baseline and Withdrawal measures that represent light and dark phases, respectively. Treatment with either vehicle or volinanserin (3mg/kg i.p.) at 12 hr and again at 24 hr in withdrawal are denoted by injection 1 and 2, respectively. Animals experienced overall typical sleep and wake patterns at these time points.

We ran one-way ANOVAs that compared the means of the change scores from baseline of the treatment columns against each other (saline-veh, nicotine-vehicle, and nicotine-MDL) per sleep and wake stage with $\alpha = 0.05$ followed by Tukey's multiple comparisons tests. The one-way ANOVAs revealed that for NREM there were no

significant effects at 12 hr ($F(2, 13) = 0.072$, $p = 0.931$), 24 hr ($F(2, 13) = 0.813$, $p = 0.464$), 4 hr peak withdrawal ($F(2, 13) = 0.674$, $p = 0.526$) or 16 hr ($F(2, 13) = 1.094$, $p = 0.363$). There were no significant effects following Tukey's multiple comparison's test for this data set. For the percent of time spent in REM, there were significant effects of treatment for 4hr peak withdrawal ($F(2, 13) = 8.514$, $p = 0.004$), but not for any other timepoint, 12 hr ($F(2, 13) = 1.264$, $p = 0.314$), 24 hr ($F(2, 13) = 0.792$, $p = 0.473$) or 16 hr ($F(2, 13) = 0.280$, $p = 0.760$). Following Tukey's multiple comparison's test in 4 hr peak withdrawal nicotine-MDL group was significantly decreased in REM compared to saline-vehicle ($p = 0.004$). The last stage that we analyzed, change scores for percent of time spent in wake, showed no significant effects at any time point 12 hr ($F(2, 13) = 0.167$, $p = 0.847$), 24 hr ($F(2, 13) = 1.068$, $p = 0.371$), 4 hr peak withdrawal ($F(2, 13) = 0.148$, $p = 0.864$) or 16 hr ($F(2, 13) = 0.503$, $p = 0.616$). Refer to Figure 4 for graphical representation of the data.

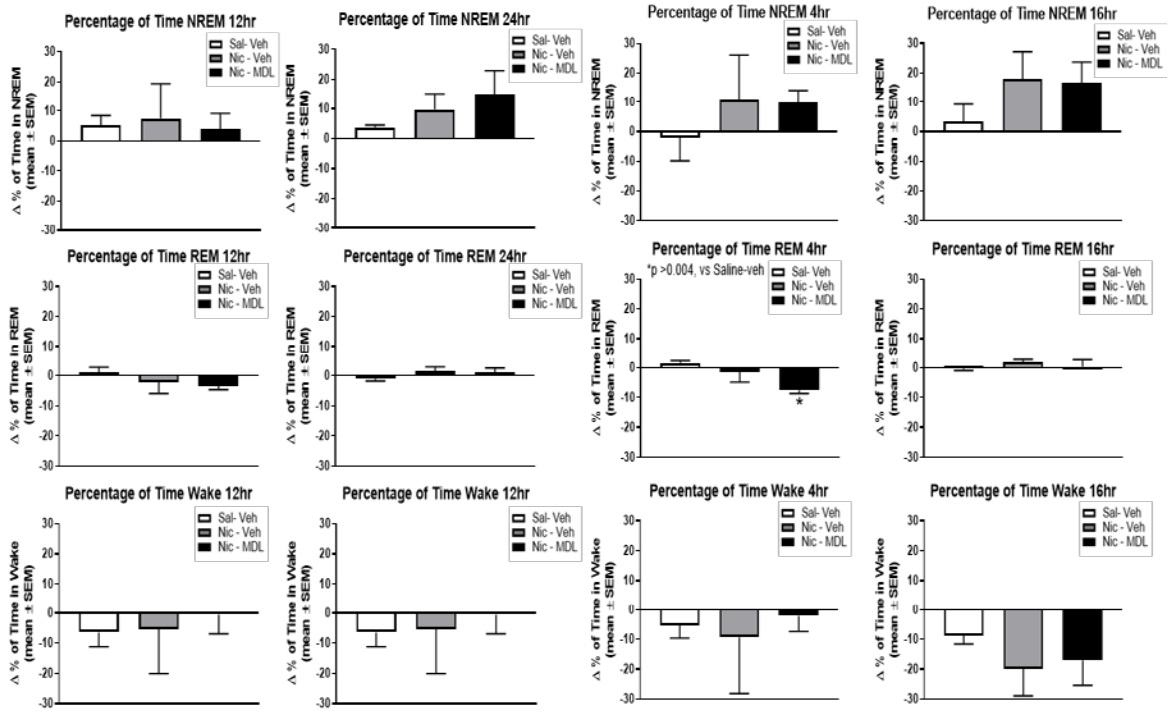

Figure 4

Percent of Time Spent in Stages, Change Scores. Change scores from baseline of percent of time spent in NREM, REM and Wake (mean ± SEM) for the three treatment groups. Time bins broken down into 12 hr and 24 hr Baseline and Withdrawal measures that represent light and dark phases, respectively. Additional time bins include 4 hr peak withdrawal (light cycle) and 16 hr post peak withdrawal (dark cycle) that correspond with treatment of vehicle or volinanserin (3mg/kg i.p.) at 12 hr and again at 24 hr in withdrawal. Animals experienced overall typical sleep and wake patterns at these time points. Significant changes from baseline are described in the text.

Sleep to Wake Transitions

For sleep to wake transitions, the one-way ANOVAs revealed no significant effects at 12 hr ($F(2, 13) = 0.250$, $p = 0.783$), 24 hr ($F(2, 13) = 1.745$, $p = 0.213$), 4 hr peak withdrawal ($F(2, 13) = 0.078$, $p = 0.925$) or 16 hr ($F(2, 13) = 2.600$, $p = 0.119$). There were no significant effects following Tukey's multiple comparison's test for this data set, refer to Figure 5 for graphical representation of the data.

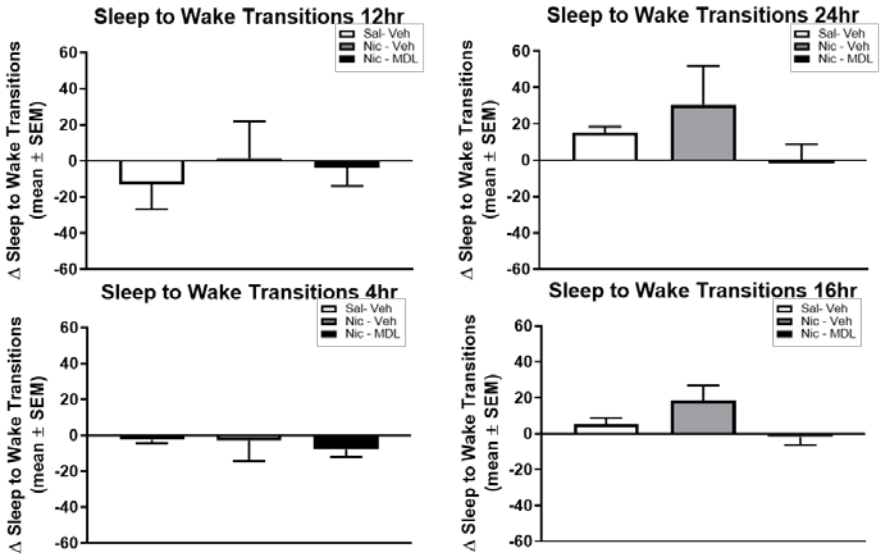

Figure 5
Sleep to Wake Transitions, Change Scores. Change scores from baseline of transitions from NREM to Wake (mean ± SEM) for the three treatment groups. Time bins broken down into 12 hr and 24 hr Baseline and Withdrawal measures that represent light and dark phases, respectively. Additional time bins include 4 hr peak withdrawal (light cycle) and 16 hr post peak withdrawal (dark cycle) that correspond with treatment of vehicle or volinanserin (3mg/kg i.p.) at 12 hr and again at 24 hr in withdrawal.

EEG Power Spectral Analysis

Percent of Each Spectral Band per Sleep and Wake Stage

We ran one-way ANOVAs for withdrawal-induced changes in the percent of total EEG power of each spectral band (δ, θ, α, β) during each sleep and wake stages. For the percent of total delta (δ) in NREM there were no significant effects at any timepoint 12 hr ($F(2, 13) = 1.465$, $p = 0.267$), 24 hr ($F(2, 13) = 2.037$, $p = 0.170$), 4 hr peak withdrawal ($F(2, 13) = 0.564$, $p = 0.583$) or 16 hr ($F(2, 13) = 2.314$, $p = 0.138$). Refer to Figure 6 for graphical representation of the data and Table 1 for all ANOVAs that did not meet significance and are not otherwise mentioned in the text. The one-way ANOVAs for percent of total theta (θ) spectral band in NREM revealed no significant effects of treatment. There were no significant comparisons following Tukey's multiple comparisons for percent of total theta in NREM, but there was a trend during the 4 hr

peak withdrawal of less decrease in theta in nicotine-MDL compared to the nicotine-vehicle group (p = 0.09). For the percent of total spectral band alpha (α) in NREM, there was a significant effect of treatment in the 16hr post peak withdrawal period (F(2, 13) = 3.974, p = 0.045). Following Tukey's multiple comparison's test there was a significant decrease following the second treatment of MDL100907 compared to the sham treatment group during 16 hr post peak withdrawal (p =0.040). For the percent of spectral band beta (β) in NREM there were no significant effects or comparisons following Tukey's.

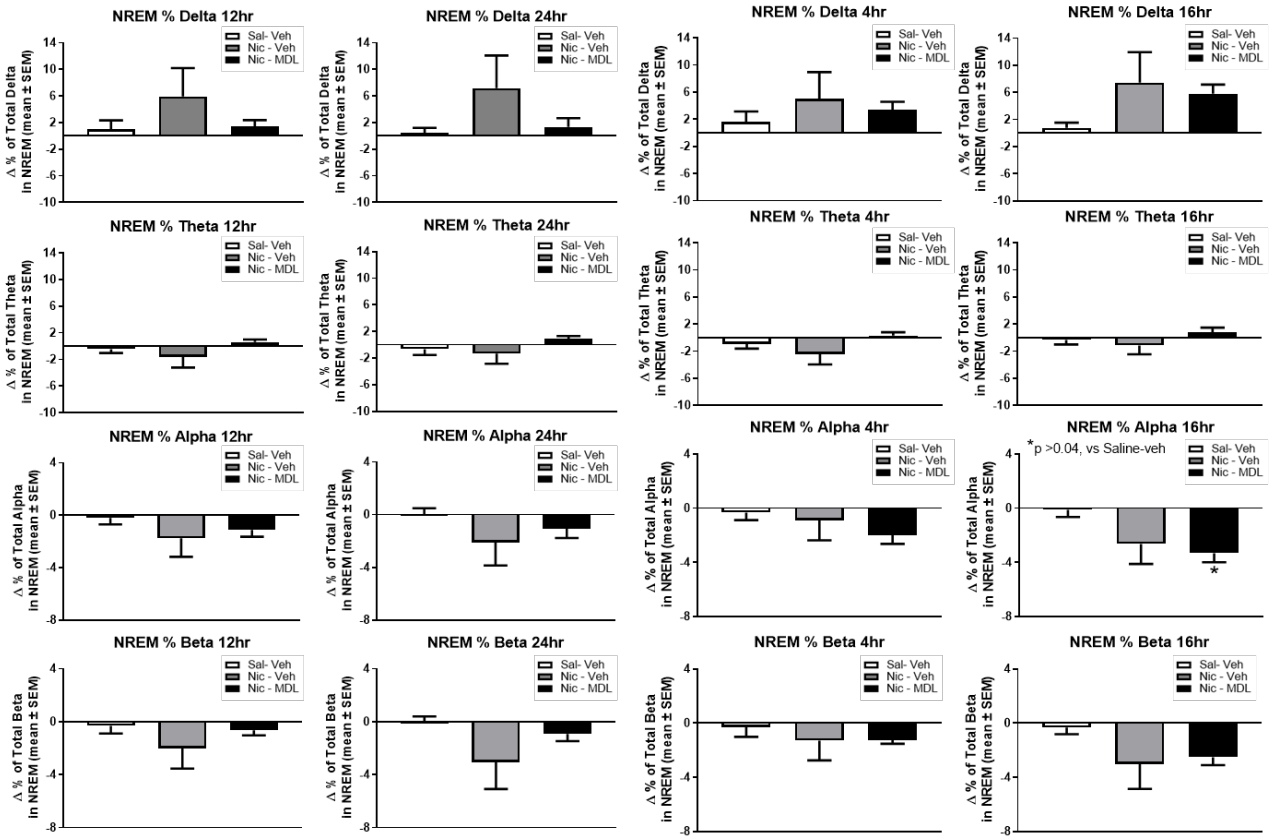

Figure 6
Percent of Total Spectral Bands in NREM, Change Scores. Change scores from baseline of percent of total delta, theta, alpha and beta power in NREM (mean ± SEM) for the three treatment groups. Time bins broken down into 12 hr and 24 hr Baseline and Withdrawal measures that represent light and dark phases, respectively. Additional time bins include 4 hr peak withdrawal (light cycle) and 16 hr post peak withdrawal (dark cycle) that correspond with treatment of vehicle or volinanserin (3mg/kg i.p.) at 12 hr and again at 24 hr in withdrawal.

The one-way ANOVAs for percent of total delta (δ) spectral band or percent of total theta (θ) in REM revealed no significant effects. There were also no significant comparisons following Tukey's multiple comparisons tests. The one-way ANOVAs for percent of total alpha (α) spectral band in REM revealed a significant effect of treatment during the sleep-intensive 12 hr period ($F(2, 13) = 7.217$, $p = 0.008$), the 4 hr peak withdrawal period ($F(2, 13) = 6.463$, $p = 0.011$) and the later 16 hr wake-intensive period ($F(2, 13) = 6.104$, $p = 0.014$). Tukey's multiple comparisons test revealed a significant decrease in alpha power during the 12 hour period compared to saline-vehicle at 12 hr ($p = 0.007$). This significant decrease of the nicotine-MDL group compared to saline-vehicle also occured in the 4 hr peak withdrawal period ($p = 0.017$), as well as a significant decrease compared to nicotine-vehicle group ($p = 0.045$). The 16 hr period after peak withdrawal also displayed the same significant decrease of the nicotine-MDL group compared to saline-vehicle ($p = 0.013$). The one-way ANOVAs for percent of total beta (β) in REM showed a significant effect of treatment at the 24 hr. period ($F(2, 13) = 3.839$, $p = 0.049$). There was also a trend for treatment in the 16 hr post withdrawal period ($F(2, 13) = 3.334$, $p = 0.068$). There were no significant comparisons following Tukey's tests for any time point. However, there was a trend toward less reduction of beta power in the nicotine-MDL group compared to nicotine-vehicle at the 24 hr. period ($p = 0.051$) and the 16 hr. post peak withdrawal period ($p = 0.062$). Refer to Figure 7 for graphical representation of the data and Table 1 for all ANOVAs that did not meet significance and is not mentioned in the text for percent delta, theta, alpha or beta in REM sleep.

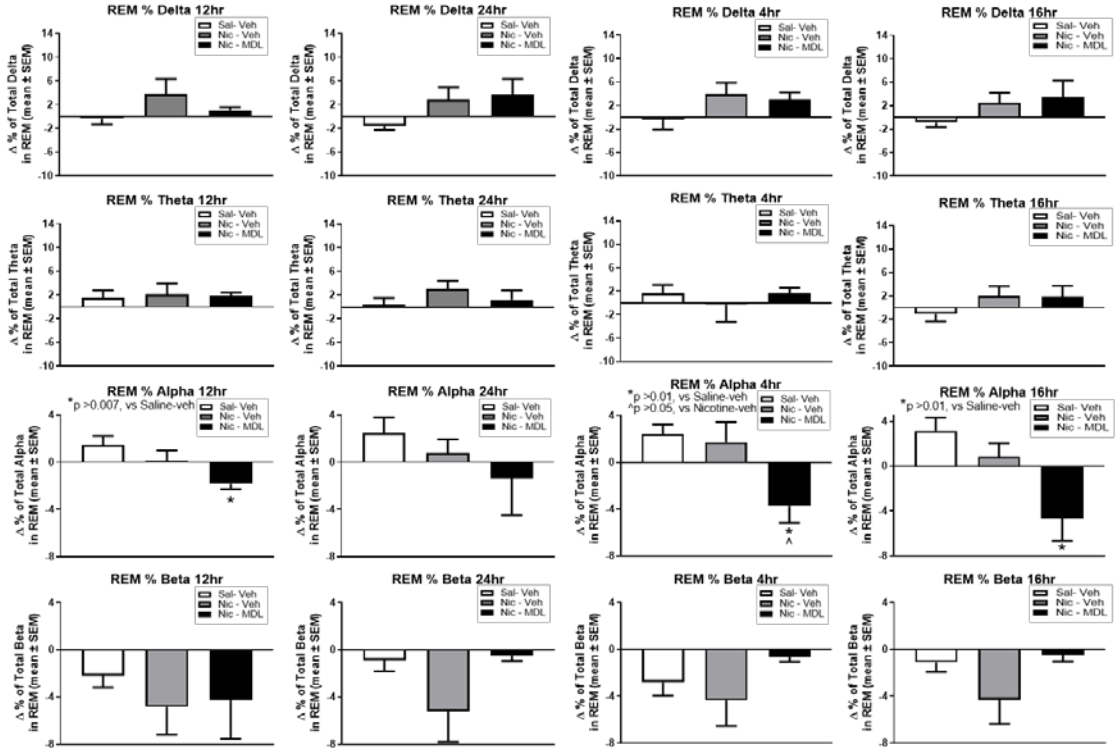

Figure 7

Percent of Total Spectral Bands in REM, Change Scores. Change scores from baseline of percent of total delta, theta, alpha and beta power in REM (mean ± SEM) for the three treatment groups. Time bins broken down into 12 hr and 24 hr Baseline and Withdrawal measures that represent light and dark phases, respectively. Additional time bins include 4 hr peak withdrawal (light cycle) and 16 hr post peak withdrawal (dark cycle) that correspond with treatment of vehicle or volinanserin (3mg/kg i.p.) at 12 hr and again at 24 hr in withdrawal.

One-way ANOVAs for percent of delta (δ) spectral band in wake revealed no significant effects of treatment in any time period. However, there was a trend approaching significance in the 12 hr. period ($F(2, 13) = 3.240$, $p = 0.072$). Tukey's comparisons revealed trends for the nicotine-vehicle group to have a bigger increase in delta power compared to the saline-vehicle ($p = 0.099$) and nicotine-MDL group ($p = 0.092$). Analysis of changes of theta (θ) power in wake revealed significant effects of treatment in both 4 hr ($F(2, 13) = 4.395$, $p = 0.035$) and 16 hr ($F(2, 13) = 4.804$, $p = 0.027$) periods. Tukey's multiple comparisons tests demonstrated a significant increase in the nicotine-MDL group compared to the saline-vehicle group for both the 4 hr peak

withdrawal period (p = 0.038) and the 16 hr period (p = 0.028). There were no significant effects for either percent of alpha (α) power or beta (β) power in wake. There was a trend for treatment in the 12 hr period of percent of total beta (F(2, 13) = 2.867, p = 0.093), but no significant comparisons following either spectral band in any time point. See Figure 8 for a graphical representation of the data in wake and Table 1 for a summary of the ANOVA analyses.

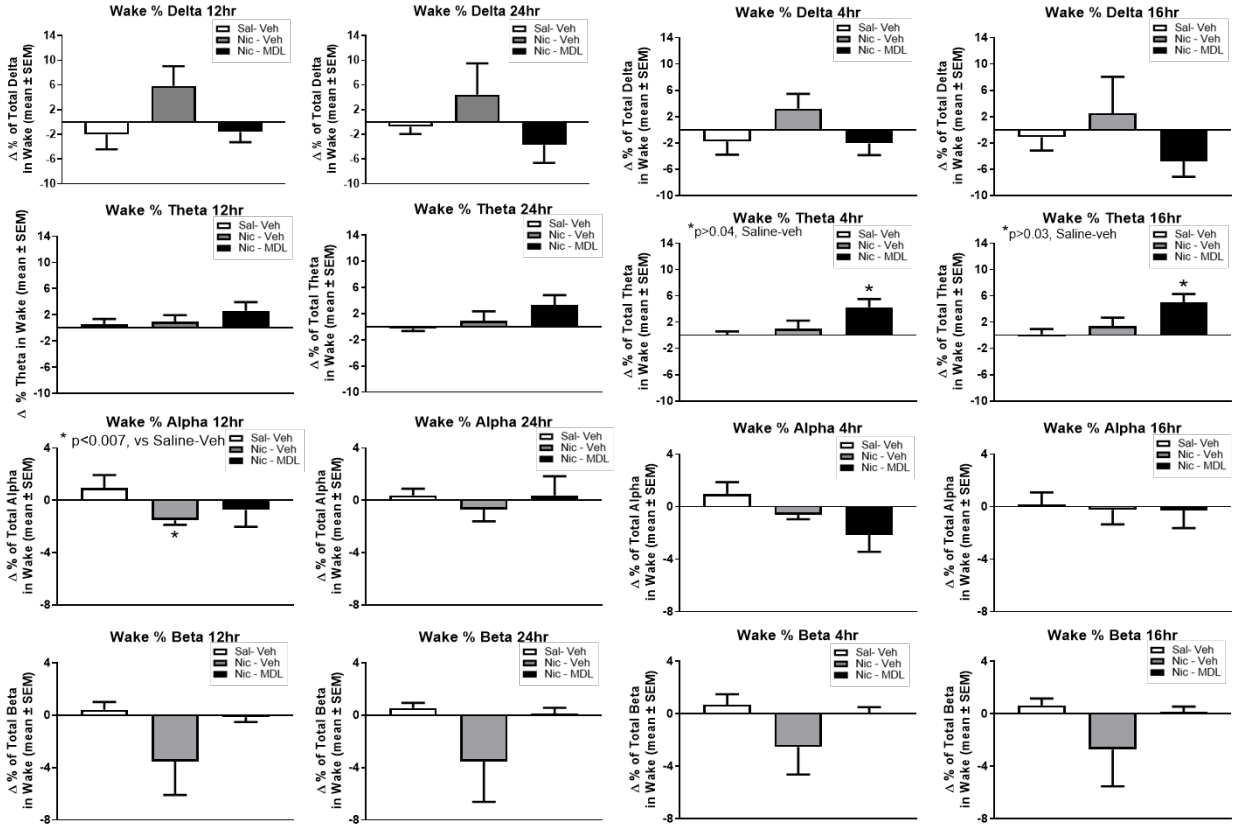

Figure 8

Percent of Total Spectral Bands in Wake, Change Scores. Change scores from baseline of percent of total delta, theta, alpha and beta power in Wake (mean ± SEM) for the three treatment groups. Time bins broken down into 12 hr and 24 hr Baseline and Withdrawal measures that represent light and dark phases, respectively. Additional time bins include 4 hr peak withdrawal (light cycle) and 16 hr post peak withdrawal (dark cycle) that correspond with treatment of vehicle or volinanserin (3mg/kg i.p.) at 12 hr and again at 24 hr in withdrawal.

Table 1

Percentage of Total Power per Spectral Band in Sleep and Wake Stages, Change Scores

Analysis Stage	Spectral Band	Time	Source of Variation	DF	F(DFn, DFd)	*P value*
NREM	**δ**	**12**	Treatment	2	F (2, 13) = 1.465	P=0.267
		24	Treatment	2	F (2, 13) = 2.037	P=0.170
		4	Treatment	2	F (2, 13) = 0.564	P=0.583
		16	Treatment	2	F (2, 13) = 2.314	P=0.138
	θ	**12**	Treatment	2	F (2, 13) = 2.058	P=0.167
		24	Treatment	2	F (2, 13) = 1.816	P=0.202
		4	Treatment	2	F (2, 13) = 2.712	P=0.104
		16	Treatment	2	F (2, 13) = 1.223	P=0.326
	α	**12**	Treatment	2	F (2, 13) = 0.873	P=0.441
		24	Treatment	2	F (2, 13) = 1.126	P=0.354
		4	Treatment	2	F (2, 13) = 1.165	P=0.343
		16	Treatment	2	F (2, 13) = 3.974	P=0.045
	β	**12**	Treatment	2	F (2, 13) = 1.114	P=0.358
		24	Treatment	2	F (2, 13) = 2.223	P=0.148
		4	Treatment	2	F (2, 13) = 0.537	P=0.597
		16	Treatment	2	F (2, 13) = 2.214	P=0.149
REM	**δ**	**12**	Treatment	2	F (2, 13) = 2.295	P=0.140
		24	Treatment	2	F (2, 13) = 1.657	P=0.229
		4	Treatment	2	F (2, 13) = 1.981	P=0.177
		16	Treatment	2	F (2, 13) = 0.941	P=0.415
	θ	**12**	Treatment	2	F (2, 13) = 0.048	P=0.954
		24	Treatment	2	F (2, 13) = 0.733	P=0.500
		4	Treatment	2	F (2, 13) = 0.413	P=0.670
		16	Treatment	2	F (2, 13) = 0.904	P=0.429
	α	**12**	Treatment	2	F (2, 13) = 7.217	P=0.008
		24	Treatment	2	F (2, 13) = 0.632	P=0.547
		4	Treatment	2	F (2, 13) = 6.463	P=0.011
		16	Treatment	2	F (2, 13) = 6.104	P=0.014
	β	**12**	Treatment	2	F (2, 13) = 0.218	P=0.807
		24	Treatment	2	F (2, 13) = 3.839	P=0.049
		4	Treatment	2	F (2, 13) = 2.589	P=0.113
		16	Treatment	2	F (2, 13) = 3.334	P=0.068
Wake	**δ**	**12**	Treatment	2	F (2, 13) = 3.240	P=0.072
		24	Treatment	2	F (2, 13) = 1.605	P=0.238
		4	Treatment	2	F (2, 13) = 1.915	P=0.187
		16	Treatment	2	F (2, 13) = 1.322	P=0.300
	θ	**12**	Treatment	2	F (2, 13) = 0.991	P=0.398
		24	Treatment	2	F (2, 13) = 2.024	P=0.172
		4	Treatment	2	F (2, 13) = 4.395	P=0.035
		16	Treatment	2	F (2, 13) = 4.804	P=0.027
	α	**12**	Treatment	2	F (2, 13) = 1.079	P=0.369
		24	Treatment	2	F (2, 13) = 0.216	P=0.809
		4	Treatment	2	F (2, 13) = 2.089	P=0.163
		16	Treatment	2	F (2, 13) = 0.044	P=0.957
	β	**12**	Treatment	2	F (2, 13) = 2.867	P=0.093
		24	Treatment	2	F (2, 13) = 2.333	P=0.136
		4	Treatment	2	F (2, 13) = 2.335	P=0.136
		16	Treatment	2	F (2, 13) = 1.754	P=0.212

CHAPTER IV:

DISCUSSION

This study evaluated the effects of nicotine withdrawal and MDL100907 on sleep quality in rodents. This included measures total sleep , sleep-wake transitions, and a power spectral analysis of EEG waveforms. During peak withdrawal, percentage of REM significantly decreased in nicotine MDL group compared to sham control. Conversely, the percentage of time in NREM tended to increase during untreated nicotine withdrawal (nicotine-vehicle). Also, in nicotine withdrawal, NREM delta power as a percentage of total EEG power tended to increase and alpha power decreased. In REM, the percentage of total alpha power during nicotine withdrawal showed signs of decrease, but it was significantly decreased in the nicotine-dependent group that received MDL100907. In wake, the, percentage of total theta power during nicotine withdrawal tended toward increase, but was significantly increased in the group that received MDL100907. Also in wake, the the percentage of total delta power tended to increase in nicotine withdrawal compared to the sham controls, but shifted toward normalization in the group that received MDL100907. A similar trend was seen in the percentage of total beta decreasing in nicotine withdrawal but seemingly normalizing in the MDL100907 group.

Together, these results indicate that in nicotine withdrawal, animals are more sleepy throughout the light and dark cycles. MDL100907 may alter this effect by causing smaller increases in delta rhythm (tyical of deep sleep), but increasd theta rhythm possibly assocaited with the drive to fall asleep during wake. These results hint at an imbalance of brain mechanisms underling wake and sleep cycles due to nicotine action and subsequent withdrawal, as seen in other studies (Logan, Williams & McClung, 2014; Pietila, Laakso & Ahtee, 1995; Matthews, 2013; Matthews & Stitzel, 2019). The results

also point to some possible counter-effects of 5-HT_{2A}R inverse agonists/antagonists on sleep disturbances caused by withdrawal from stimulant drugs of abuse (Logan, Williams & McClung, 2014; Diaz et al., 2017).

Total Sleep Parameters and Transitions

Total sleep parameters, measured by the percent of time in each sleep stage, showed that, as expected for this species, overall animals were sleeping more in the light cycle and were more awake during the dark cycle. This is indicative of normal sleep patterns for the rat. The only significant effect of treatment on total sleep parameters was a significant decrease in the percentage of time in REM during the 4hr peak withdrawal period in the nicotine-MDL group, undegoing untreated withdrawal, as compared to the sham control group. This effect of decreased REM quantity was also observed in nicotine dependent mice after 24hrs of abstinence (Matthews & Stitzel, 2019). Non-significant trends include withdrawal-induced increases in percentage of time in NREM, particulalty in the typically active dark periods (24 hr and 16 hr). Nicotine withdrawal as well as 5-HT have been found to increase slow wave sleep in both humans and rats, possibly as a mechanism to balance REM-promoting cholinergic systems.

The second sleep parameter analyzed was transitions from sleep to wake, specifically the transitions from NREM to wake identified by the inactive phase (12 hr and 4 hr) and the active phase (24 hr and 16 hr). There were no significant effects or trends for this parameter. Upon visual inspection of the graphs, however, the nicotine-vehicle group showed considerably increased transitions compared with the sham controls in the 16 hr and 24 hr period, suggesting fragmentation of sleep-wake states.. These increases were reversed in the nicotine-MDL group. This result, together with increased NREM in the typically active period, could imply an underlynig disregulation

of homeostatic and circadian processes. It might imply a rebound in slow wave sleep, following a period of poor quality sleep (Borbely & Achermann, 1999).

Power Spectral Analysis of EEG Waveforms

The EEG power (v^2/Hz) analysis for each spectral band showed significant decrease of alpha during NREM in the dark period after peak withdrawal (16 hr) for the nicotine-volinanserin group. A similar significant decrease in percent alpha power in the nicotine-MDL group was observed in both the light cycle 12 hr and peak withdrawal (4 hr) as well as the 16 hr dark cycle. Not significant, but still worth mentioning, was the increase in percentage delta power during untreated nicotine withdrawal and its seeming normalization in the nicotine-MDL group in both light and dark periods. Likewise there was a consistent trend for untreated nicotine withdrawal to reduce beta rhythm across different phases. This too was conistently reversed by MD100907L. Since MDL100907 is a highly selective inverse agonist of the 5-HT_{2A} recptor, these last two results suggst that activation of the 5HT2A receptor may contribute to imbalance of sleep-wake structure in nicotine withdrawal.

MDL100907 may have some interesting effects besides reversing effects of untreated nicotine withrawal. There were compelling and significant increases in theta rhythm for the nicotinee-MDL group during peak withdrawal and the following 16 hr period.. Additionally, trends for increases in delta power and decreases in beta power were noticably different from the sham control and the nicotine-MDL group. This could signify a delta and theta intrusion in quiet waking indicative of sleep propensity following sleep deprivation (Borbely, Tobler & Hanagasioglu, 1984).

Limitations and Future Directions

The current study is severely underpowered due to time and resource constraints. The obsered effects of nicotine withdrawal and the two treatments of volinanserin on

total sleep parameters are mostly based on visual trends. Their possible statistical confirmation that could be examined in future studies with the inclusion of more animals in each treatment group. Raw polysomnogrphy data could be analyzed to provide further information on sleep fragmentation. This could involve counting average bouts per stage or transitions from REM to Wake.

Several animals also had to be removed from this study due to high levels of artifact throughout their recordings. This could have potentially been due to one of several steps in the process, including the surgical placement of the EEG and EMG electrodes, incorrect signal sampling or amplification or misaligned recording cables in the initial recordings. To remedy any potential artifacts in future studies, one could alter the EEG electrode placement to include a reference electrode placed in a typically quiet area of the skull. Alternatively, one could use of a bipolar montague procedure, allowing recording from both hemispheres in multiple brain areas (Kadam et al., 2017).

In the current study we were unable to retrieve the complete data for the gamma power band and were forced to truncate the beta power band. Examining an expanded EEG spectrum might provide even further evidence of the cognitive effects of nicotine withdrawal as well as impacts of repeated volinanserin treatment. Future studies should include an additional control group of non-dependent rats with a saline pump and treated with volinanserin. This would complete a factorial design, so that the present data and previous data collected in our laboratory can be combined and analyzed together. Future studies might emphaize circadian rhythms and homeostatic processes to better undwesrand how perturbed sleep-wake mechanisms evolve seqquentially over time.

Potential Implications

The trends indicated by the analysis of change scores in the current study are promising for establishment of an animal model of sleep disurbances in drug withdrawal.

Also they suggest a possible alternative to current pharmacological options for nicotine cessation. Currently, the most common pharmaceuticals used for the treatment of nicotine cessation are veranicline, bupropion and benzodiazepines, which are known to have a risk of sleep disturbances. These include strange dreams, insomnia, next day sleepiness and impariments in next day functioning and cognition (Jorenby et al., 2006). The use of $5HT_{2A}R$ antagonists/inverse agonists pimavanserin and volinanserin has been shown to increase slow wave sleep after periods of sleep restriction. They consitute a potential treatment for insomnia in rats as well as humans (Monti et al., 2018; Monti & Jantos, 2006; Ancoli-Israel et al., 2011).

Further studies should to be conducted on $5HT_{2A}R$ antagonists/inverse agonists to alter the sleep disturbances following use of commonly abused stimulants (Logan et al., 2014; Shahin, 2016; Pietila, Laakso & Ahtee, 1995). Pimavanserin is already FDA-approved for Parkinson's Disease psychosis, a disease that is associated with sleep pathology including REM without atonia, increased sleep fragmentation and excessive daytime sleepiness (Comella, 2006). Pimavanserin is a good candidtate for future studies, due to it's readily soluble nature, long half-life and its non-interference with cognitive tasks even after repeated dosing. Finally, the current findings suggest that clinical patients presenting signs of substance use disorders or wanting to abstain from nicotine should be screened for sleep disturbances as well as monitored for sleep disturbances during attempts at tobacco cessation.

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