Nicotine Administration and Withdrawal and its Effect on Sleep Latency, Relevant Sleep Variables, and Endogenous Corticosterone Levels in Mice

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Abstract

NREM and REM sleep have shown an exceptional importance in controlling various cognitive functions (Brown et al, 2012), and disruption of such sleep states can cause mental and physical impairments (Christie et al, 2008). Disruptions in sleep stages (Wakefulness, REM, NREM) can be identified via EEG recordings in mice without much interference (Mckenna et al, 2008). Circadian Rhythm, hormonal markers, and stress all affect the duration, intensity and timing of these sleep stages (Brown et al, 2004; Veasey et al 2004; Christie et al, 2008). One cohort of mice (n=7) underwent a baseline condition (BL), a nicotine administration condition (Nicotine day 8 or N8), and a withdrawal condition (Withdrawal day one, or WD1) to see nicotine/withdrawals effect on sleep. Data was collected through EEG recordings of both muscle tone and brain activity. A Corticosterone immunoassay with a separate cohort was used to assess levels of corticosterone stress response to nicotine/withdrawal. **Results**: Nicotine withdrawal increased sleep latency related to baseline. Wake percentage as well as Wake Bout Duration (average length of Wake stages) were increased in withdrawal condition, and overall sleep percentage went down in withdrawal, driven by a decrease in NREM percentage. Total Stage Shifts as well as Sleep Stage Shifts decreased in withdrawal condition. REM Bout Duration also decreased during withdrawal condition. We also found that corticosterone levels did not alter significantly in either the withdrawal or nicotine condition. Mice showed no difference in any measured sleep variables in the nicotine condition compared to baseline.

Introduction

Smoking is the most substantial avoidable single cause of disease and premature death in the United States (George 2007). Around 70% of smokers express a desire to give up tobacco (CDC, 2011); however, the actual quitting prevalence is only 6.2% (CDC, 2011). Although there are many factors contributing to this disparity, consistent negative physical and mental symptoms related to smoking withdrawal is one of the leading reasons. Smokers undergo rational calculations, examining how one cigarette won't contribute much to overall health decrease, but will certainly curb withdrawal symptoms and provide pleasure (Baumeister 2017).

Nicotine produces a sense of alertness and arousal through specific physiological pathways. After smoke has been inhaled, nicotine enters arterial circulation, where nicotine travels to the brain. Nicotine binds nicotinic cholinergic receptors, which then allow the rapid influx of sodium or calcium, which leads to neuronal excitation and vesicular neurotransmitter release, respectively (Dajas-Bailador & Wonnacott, 2004). These nicotinic cholinergic receptors bind endogenous acetylcholine, which is an essential neurotransmitter for propagating excitatory or inhibitory signals in our nervous system (Belousov et al, 2001). Nicotine acts as an agonist for the receptor, allowing for exogenous intervention of signal conduction. The entry of Na+ cations into the axon cause depolarization, allowing for the activation of voltage gated calcium channels in the presynaptic terminal and subsequent vesicular release of neurotransmitters into the synaptic cleft (Dajas-Bailador & Wonnacott, 2004). One of the neurotransmitters released by stimulation of postsynaptic membrane nicotinic cholinergic receptors in the brain is dopamine. The dopaminergic neurons in

the ventral tegmental area (VTA) of the midbrain that project to the nucleus accumbens create a pleasure response when stimulated, and are integral to the drug-induced reward circuit (Nestler, 2005). Nicotine augments glutamate and γ-aminobutyric acid (GABA) release, which enhance and inhibit dopamine release, respectively (Mansvelder et al, 2000; Mansvelder & McGehee, 2002). With long-term exposure to nicotine, some nicotinic cholinergic receptors on the dopaminergic neurons become desensitized to nicotine binding, affecting release of GABA but not glutamate; it is not well understood why this selective desensitization occurs. This creates an imbalance in the regulation of nicotinic pathways (Benowitz, 2010). Typical cigarette consumption maintains near complete saturation (and thus desensitization) of nicotinic cholinergic receptors (Brody et al 2006). Smokers are using nicotine to do one of two things: to activate the drug-reward circuit, or avoid withdrawal symptoms.

The neurobiology of nicotine withdrawal has been thought to be dependent on dopaminergic neurons in the mesolimbic system. Decreased dopamine in the central amygdala as well as the VTA has been hypothesized to modulate the depressed mood and dysphoria associated with nicotine withdrawal (Watkins et al, 2000). Long term drug exposure causes a change in hedonic set point, which may increase the positive reinforcing efficacy of a drug (Koob and LeMoal, 1997). Neuroadaptations associated with long term nicotine exposure may play a role in extended abstinence by creating a heightened sensitivity to the positive reinforcing effects of nicotine use; however, adaptive mechanisms of dopamine in the mesolimbic system are still unidentified (Watkins et al, 2000). Dopamine has also been shown to competitively bind to heteromers that usually bind norepinephrine, a hormone that is integral to the

production of melatonin in the pineal gland. When Dopamine binds these receptors, melatonin production decreases, causing more arousal and wakefulness (Gonzalez et al, 2012). This new research shows the role of dopamine in sleep, and with dopamine release being amplified during nicotine use, it might be another physiological explanation for nicotine's arousing effect.

Difficulty falling asleep (increased sleep latency) is a commonly reported issue associated with smoking and withdrawal. Smokers commonly report increased sleep latency during cigarette use (Soldatos et al, 1980); This was further confirmed by a longitudinal study, which showed smokers having extended sleep latency, more complaints of insomnia, decreased total sleep time, and reduced sleep efficiency (Zhang et. al, 2006). A review of these studies, however, pointed out that smokers make other unhealthy lifestyle choices as well, such as more frequent use of alcohol and caffeine, exacerbating sleep problems initially due to nicotine use (Jaehne, 2009). Thus, a human model in which solely nicotine and sleep are examined is still something that could help elucidate nicotine's primary effects on sleep and sleep latency.

Nicotine withdrawal also seems to have an adverse effect on sleep wellness. During nicotine cessation, subjects reported decreases in sleep quality, and frequent /extended awakenings, which directly translates to increased sleep latency (Hatsukami et al 1984, Hatsukami et al, 1985; Hatsukami et al,1988; Shiffman et al, 1995). This disturbed sleep/insomnia occurs in up to 39% of cases of nicotine withdrawal, and this disturbance might increase the risk of nicotine relapse (Jaehne 2009). However, reduced sleep latency during nicotine cessation is also reported, despite discomfort

associated with withdrawal (Soldatos et al, 1980). This contradiction in results shows issues with the correlation between nicotine and sleep physiology.

The underlying physiology of why nicotine dependence and withdrawal causes sleep disturbances is still very unclear; there seems to be confusion regarding the correlation between sleep and the pharma-physiological effect of nicotine (Jaehne 2009). Many of the experiments measuring sleep during cessation attempts lacked powerful sample size, follow up data, and produced some contradictory results (Soldatos et al, 1980; Zhang et al, 2006). On top of this, many of these experiments contained confounding variables, such as alcohol and caffeine intake, which made it difficult to correlate a direct effect of nicotine on sleep wellness. One thing is clear, however; self-report studies have consistently shown results of difficulty falling asleep, increased sleep latency, and extended durations of awakenings (Hatsukami et al, 1985; Hatsukami et al,1988; Shiffman et al, 1995), strongly suggesting an effect of nicotine on sleep wellness. To assess nicotine alone and its relationship to sleep latency, rodent models should be used, due to ethical issues and environmental control issues regarding human subjects. A previous study under review for publication from our lab identified a negative impact of nicotine administration and withdrawal on sleep architecture and sleep quantity (Mathews, publication pending), but did not assess sleep latency during the inactive phase. Rodent models have proven to be reliable predictors of relevant behavior during nicotine withdrawal (Malin & Goyarzu, 2009), and using a controlled environment with no confounding variables can help further the understanding of nicotine/withdrawals various impacts on sleep physiology.

Cigarette cessation causes anxiety and stress, which cause people to relapse and begin smoking again (Le Moal 2007). This increase in stress is thought to be caused by increased levels of extrahypothalamic corticotropin releasing factor (CRF) (George et al, 2007). This increase in this neuropeptide causes overstimulation of the pituitary gland, causing excess production of ACTH. Excess ACTH overstimulates the adrenal glands, causing excess production of cortisol/corticosterone. Cortisol/corticosterone and CRF could also be potentially linked to extended nicotine withdrawal, contributing to increased stress and anxiety during nicotine cessation (Kreek & Koob, 1988). It has been speculated that nicotine use decreases endogenous production of CRF, and withdrawal contributes to increased function and production of CRF (Watkins et al, 2000). With increased levels of CRF during withdrawal, sleep may be disturbed, and sleep latency would be directly impacted by elevated circulating cortisol/corticosterone levels. However, there are contradicting studies that show an increase in plasma corticosterone in mice during nicotine administration as well (Balfour). Thus, measuring cortisol/corticosterone in relation to nicotine use and nicotine withdrawal is relevant to investigating nicotine's effect on sleep physiology.

Due to previous research showing an arousing/ reduced stress effect of nicotine and the opposite effect during withdrawal, we hypothesize that nicotine will increase sleep latency as well as reduce corticosterone levels, while nicotine withdrawal will reduce sleep latency as well as increase corticosterone levels compared to baseline.

Methods

Animals and Experimental Design

All procedures were approved by the University of Colorado's Institutional Animal Care and Use Committee and followed the National Institute of Health guide for the care and use of laboratory animals. A total of 7 individually housed, 9-week-old male C57BL/6J mice were used. One animal was excluded from statistical analyses due to signal interference and decay. The mice were kept on a 12-hour light-dark cycle, with lights on 0700, and had ad libitum access to food and water solution containing 0.2% saccharin, to help mask the sour taste of nicotine as well as reinforce the addictive properties of nicotine. Mice were weighed before the day of EEG surgery and after introduction of nicotine. To induce nicotine dependence, mice were orally administered a solution of 200 µg/ml of free-base nicotine+ 0.2% saccharin/water vehicle immediately following a one-week saccharin-only baseline. The nicotine solution was changed every 3-4 days. The volume of the remaining fluid was measured at each solution change. After 14 days of nicotine administration, withdrawal was started by replacing the nicotine solution with the 0.2% saccharin solution at ZT1.

To acquire electroencephalography (EEG) and electromyography (EMG) signals, animals were implanted with cortical EEG and intramuscular EMG electrodes. The mice were anesthetized with isoflurane and placed in a stereotaxic apparatus. Two frontal and two parietal screw electrodes (Pinnacle Technology Inc., Lawrence, KS) were implanted to obtain the EEG signal. Two flexible stainless-steel electrodes were implanted into the nuchal muscles to obtain EMG signal. All electrodes were connected

to a head mount (Pinnacle Technology Inc., Lawrence, KS) and secured with dental acrylic. Upon successful implantation, 0.1 mg/kg of Buprenorphine was intraperitoneally injected for pain management. Following surgery, mice were individually housed in recording chambers. Following a seven-day habituation and recovery period, mice were attached to recording cables (Pinnacle Technology Inc., Lawrence, KS) via an overhead swivel commutator system (Pinnacle Technology Inc., Lawrence, KS). Mice were habituated to the new cable setup for a five-day period before EEG/EMG recording began. The EEG/EMG recording paradigm was as follows: one week of baseline, two weeks 200 µg/ml base-free nicotine drinking solution and one week of withdrawal. Baseline recordings were taken on day one (BL) after the five-day habituation period, nicotine recordings were taken on day eight (N8) of the two-week nicotine period, and withdrawal recordings (WD1) were taken one day after mice had been taken off nicotine.

Mice underwent a Multiple Sleep Latency Test (MSLT) procedure in each of the three conditions (BL, N8, and WD1), as outlined in the McKenna et al 2008 study. The MSLT procedures were started at ZT 2 (n=4) and ZT 2.5 (n=3) of their light inactive period. The MSLT included three separate sleep latency trials for each mouse. For each of these three trials, the mice were kept awake for 5 minutes by means of gentle handling, and then left undisturbed for 55 minutes while EEG and EMG data were collected. This pattern was repeated 2 more times, for a total of three awakenings per mouse in three consecutive hours. Four mice were stimulated on the hour (ZT 2, 3, and 4), and three mice were stimulated on the half hour (ZT 2.5, 3.5, and 4.5). The surgery and experiment schedule are outlined in Figure one.

To assess the level of corticosterone induced by a series of three sleep latency trials, a separate cohort of animals (n=4) underwent no EEG surgery/implantation, but underwent the same nicotine treatment regimen and MSLT procedure. At the end of the third awakening, A submandibular blood draw was performed, with blood stored on ice. The blood was spun down @7000 g. To perform the immunoassay, we used DetectX® Corticosterone Enzyme Immunoassay kit (ArborAssays), and followed the instructions provided in the manual.

Data Acquisition and Analysis

All MSLT recordings were acquired using the Sirenia Acquisition system (Pinnacle Technology Inc., Lawrence, KS) at a sampling frequency of 500 Hz. The EEG was then band-pass filtered from 0.5 to 25 Hz and EMG from 0-100 Hz. The EEG/EMG signals were recorded throughout the entire experiment; however, only the three-hour MSLT period was scored and further analyzed. The 5-minute gentle handling period was also deducted from scoring and analysis to make sure only the experimental condition (55 minutes following MSLT procedure) was analyzed and reported.

Sleep scoring consisted of an initial auto-scoring by Sirenia Sleep Pro (Pinnacle Technology Inc., Lawrence, KS) software, followed by a manual assessment to confirm each epoch's accuracy. Epochs with varied theta-band amplitude and high EMG activity were scored as wake. Epochs with high frequency theta band activity accompanied with very low EMG activity were classified as rapid eye movement (REM) sleep. Epochs with high amplitude, low frequency delta band activity accompanied with low EMG activity were classified as non-rapid eye movement (NREM) sleep. Observance was broken up

into 4 second epochs, each epoch being individually classified as wake, NREM, or REM sleep.

To calculate latency to first NREM sleep, the length of the first wake bout was obtained from automated outputs. To measure sleep quantity, percent time in both NREM and REM (plus an overall combined sleep percentage) was obtained from each manually verified three-hour period. To assess sleep architecture, bout number (total number of wake bout, NREM bouts, etc.) and duration, as well as total sleep shifts and sleep stage shifts were measured from automated outputs for each three-hour period. Sleep latency is defined as the time span between the end of the five-minute gentle handling period and the first instance of NREM sleep. Since the same mice were used for each condition, a One Way Repeated Measures ANOVA test was used for statistical analysis. When a main effect was discovered, a Tukey Post-hoc analysis was run to investigate differences between groups.

After the ArborAssays Corticosterone Enzyme ImmunoAssay was performed, a colormetric test was ran against blanks (water containing no corticosterone) and a positive control (Corticosterone stock) to determine corticosterone concentration in the BL, N8, and WD1 conditions.

Results

Nicotine dependence was induced by giving mice a 0.2% saccharin solution with 200 µg/ml nicotine as their sole fluid. The solution was changed 3 times during the time in between baseline and day of EEG measurement (N8), and remaining volume was measured to confirm intake. Mice initially consumed an average of 72.95 mg/kg/day.

Consumption decreased to 38.9 mg/kg/day, and increased during the last week of exposure (63.32 mg/kg/day and 44.09 mg/kg/day, respectively. Overall, average daily consumption was 54.815 mg/kg/day. Average daily fluid consumption across the two-week period was 6.4375 ml/day.

Effect of Nicotine Treatment and Withdrawal on MSLT Sleep Quantity and Architecture

Sleep Latency

The effects of nicotine administration and withdrawal on sleep quantity and architecture were measured using EEG and EMG recordings (BL, N8, and WD1). Sleep latency was measured three times at one-hour intervals as discussed in the methods. Analyses revealed a main effect of treatment on Latency 1 ($F_{2,10}$ =13.38, p=0.0015) (Fig. 1a). No significant difference was reported in Latency 1 (L1) during nicotine exposure (p= 0.9730). During WD1, Latency 1 increased (p=0.0037) when compared to BL. No main effect of treatment was found for Latency 2 (L2) ($F_{2,10}$=0.1409, p= 8703) or Latency 3 (L3) ($F_{2,10}$ = 0.5269, p= 0.6060). A main effect of treatment was reported for Average Sleep Latency ($F_{2,10}$ = 9.87, p=0.0043), driven by the main effect found for L1. No difference was found between BL and N8 (p=0.9988); however, Average Sleep Latency increased in WD1 compared to BL (p= 0.0079) (Figure 2).

Wake/Sleep Percentages and Total Stage Shifts/Sleep Stage Shifts

When comparing sleep states, a main effect of treatment was found in the percentage of overall sleep ($F_{2,10}$=12.23, p=0.0021). No difference was found between BL and N8 groups (p=0.9790). Overall sleep percentage decreased in WD1 compared

to BL (p= 0.0045). A main effect of treatment was reported in NREM state percentage ($F_{2,10}$=16.24, p=0.007). No difference was reported between BL and N8 groups (p=0.9890), but a significant decrease in NREM state percentage was found in WD1 compared to BL (p=0.0014). No main effect of treatment was found for REM state percentage ($F_{2,10}$= 2.808, p=0.1077). A main effect of treatment was seen in Wake state percentage as well ($F_{2,10}$= 12.27, p=0.0020). No difference was found between BL and N8 (p=0.8481), but wake percentage increased in WD1 compared to BL (p=0.0043) (Figure 3).

Analysis of sleep architecture measures revealed a main effect of treatment on Total Stage Shift Bout (TSS), which is the average number of total shifts the mice underwent ($F_{2,10}$ =5.472, p= 0.0248). No differences were found between the BL and N8 group (p= 0.6820), but a decrease in TSS was seen in WD1 compared to BL (p=0.0238). A main effect of treatment was found for Sleep Stage Shift Bout (SSS), which is the average number of shifts from NREM to REM in the mice ($F_{2,10}$ = 4.573, p= 0.0389). No difference was seen for BL vs N8 (p=0.6448), but a decrease in SSS was seen in WD1 compared to BL (p=0.0353) (Figure 4).

Bout Number (Frequency) and Duration

Bout Frequency is defined as the total number of times a certain vigilance state (Wake, NREM, REM) is identified during the scored three-hour MSLT period. No main effect of treatment was seen in Wake Bout Frequency ($F_{2,10}$=1.744, p=0.2240). No main effect of treatment was seen in NREM Bout Frequency ($F_{2,10}$= 1.598, p=0.2498). No main effect of treatment was seen for REM Bout Frequency ($F_{2,10}$=3.608, p=0.0661). No

main effect of treatment was seen for Sleep Bout Frequency ($F_{2,10}$=0.9068, p=0.4346) (Figure 5).

Bout Duration is defined as the average length of the combined wake periods (Wake Bout Duration), NREM periods (NREM Bout Duration), REM periods (REM Bout duration), and sleep periods (Sleep Bout Duration). A main effect of treatment was seen for Wake Bout Duration ($F_{2,10}$=8.157, p=0.0079). No difference was seen between BL vs N8 (p=0.9962), but an increase in Wake Bout Duration was seen for WD1 compared to BL (p=0.0155). No main effect of treatment was seen for NREM Bout Duration ($F_{2,10}$=0.01088, p=0.9892). A main effect of treatment was seen for REM Bout Duration ($F_{2,10}$=5.353, p=0.0263). No differences were seen between BL vs N8 (p=0.9554), but a decrease in REM Bout Duration was seen for WD1 compared to BL (p=0.0345). No main effect of treatment was seen for Sleep Bout Duration ($F_{2,10}$=1.808, p=0.2137) (Figure 6).

Effect of Nicotine administration on Corticosterone levels

No main effect of treatment was seen for average corticosterone concentration ($F_{2,6}$=2.789, p= 0.1391) (Figure 7).

Discussion

Overall, the data seems to agree with literature that suggests a negative effect of withdrawal on overall sleep quantity and sleep latency, but disagrees with literature that shows a stimulating effect of nicotine. This study presents novel data on withdrawal causing an increase in sleep latency, which seems to correlate with the trend of nicotine

withdrawal impairing subjective sleep variables in all the studies which investigate withdrawal (Jaehne et al, 2009; Hatsukami et al, 1985; Hatsukami et al,1988; Shiffman et al, 1995).

The present study shows that nicotine withdrawal increased overall average sleep latency by affecting a variety of sleep variables. The increase in sleep latency during WD1 condition was primarily driven by an increase in Latency 1 (L1), which suggests the mice had difficulty achieving their first NREM episode once woken up and gently handled. There were no significant differences for L2 or L3 in either the N8 or WD1 condition, suggesting that mice had similar efficiency returning to sleep after the second and third handling, regardless of condition. However, no change in L2 or L3 could also be attributed to order effect; that is, the mice became habituated to the gentle handling after the first five-minute handling phase. Thus, the response to the first gentle handling (increase in latency) could be attributed to a non-specific stress response, more so than a withdrawal-mediated effect. To avoid mice becoming used to a stressor, perhaps an exploration-based stressor could be used, to minimize interference and to elucidate a non-attenuated response. N8 showed no difference in overall sleep latency or L1 compared to baseline. This seems to go against current literature that shows increased sleep latency in human subjects that are smokers versus non-smokers (Zhang et al., 2006). This difference could be accounted for by the differences in nicotine metabolism in mice versus humans, as well as the more consolidated sleep period in humans versus mice. However, the connection between withdrawal and sleep latency in the Zhang study had not been properly controlled and investigated, and this study provides novel data in relation to sleep architecture.

Due to the connection between cortisol levels and sleep disturbance (Leproult et al,1997), the mouse equivalent of cortisol (corticosterone) was also measured in this experiment. However, no significant differences between WD1 and BL were found, potentially suggesting another mediator of nicotine withdrawal affecting sleep latency. This lack of connection could also be due to the low subject size that was investigated; perhaps a larger sample size will elucidate a CRF/corticosterone mediated effect on sleep. Future studies could address this issue. Another limitation with the corticosterone measurement was that it was taken after L3, where L1 seemed to be the main stressor. Unfortunately, blood could not be drawn after L1 due to the stress associated with a blood draw potentially confounding results. Future studies could take blood after L1, assuming it would not confound other results.

The increase in sleep latency was characterized by a lower percentage of NREM sleep in WD1 compared to baseline, which correlates to the increase in Wake percentage in WD1 as well. Report of decreased active phase NREM sleep have been characterized in the with nicotine consumption in rodents (Lena et al.,2004 (injection); Salin-Pascual et al., 1999 (injection)); however, no such nicotine-mediated decrease occurred during the inactive phase which was investigated. This may be due to differences in administration (injection vs oral administration) or differences in nicotine metabolism rates in the inactive vs. active phase. The decrease in NREM sleep percentage is consistent with the idea of mice having difficulty falling asleep initally due to nicotine withdrawal. However, the decrease in Total Stage Shifts and Sleep Stage Shifts in WD1 compared to baseline suggests that mice experienced a more intense sleep pressure during withdrawal, and thus had less fragmented sleep (less stage

shifts) once they were able to fall asleep. Although sleep latency in relation to nicotine hasn't been well characterized, it does seem to be consistent with studies that show a decrease in sleep quality and efficiency as well as a decrease in sleep quantity and architecture (Jaehne 2009, Shiffman et al., 1995). This is further confirmed with the increase in average Wake Bout Duration and decrease in REM Bout Duration in WD1 compared to baseline, which suggest less sleep quantity and worse sleep quality, respectively.

The first animal model conducted by Hunter Mathews and the Stitzel lab (publication pending) showed behavioral and physiological variables of sleep and wakefulness during nicotine administration and withdrawal. Importantly, this study provides fresh data on correlation between nicotine withdrawal and sleep latency in an animal model during the inactive phase. However, some questions still exist; how does nicotine withdrawal specifically mediate the increase in sleep latency during the inactive phase, and with the current lack in literature regarding sleep and chronic nicotine exposure and withdrawal, what other physiological variables should be assessed? The current study can be applied to improve the understanding of nicotine withdrawal syndrome, and future studies should evaluate current variables with a larger subject size, as well as other variables (such as dopamine) that could potentially play a role in disturbed sleep.

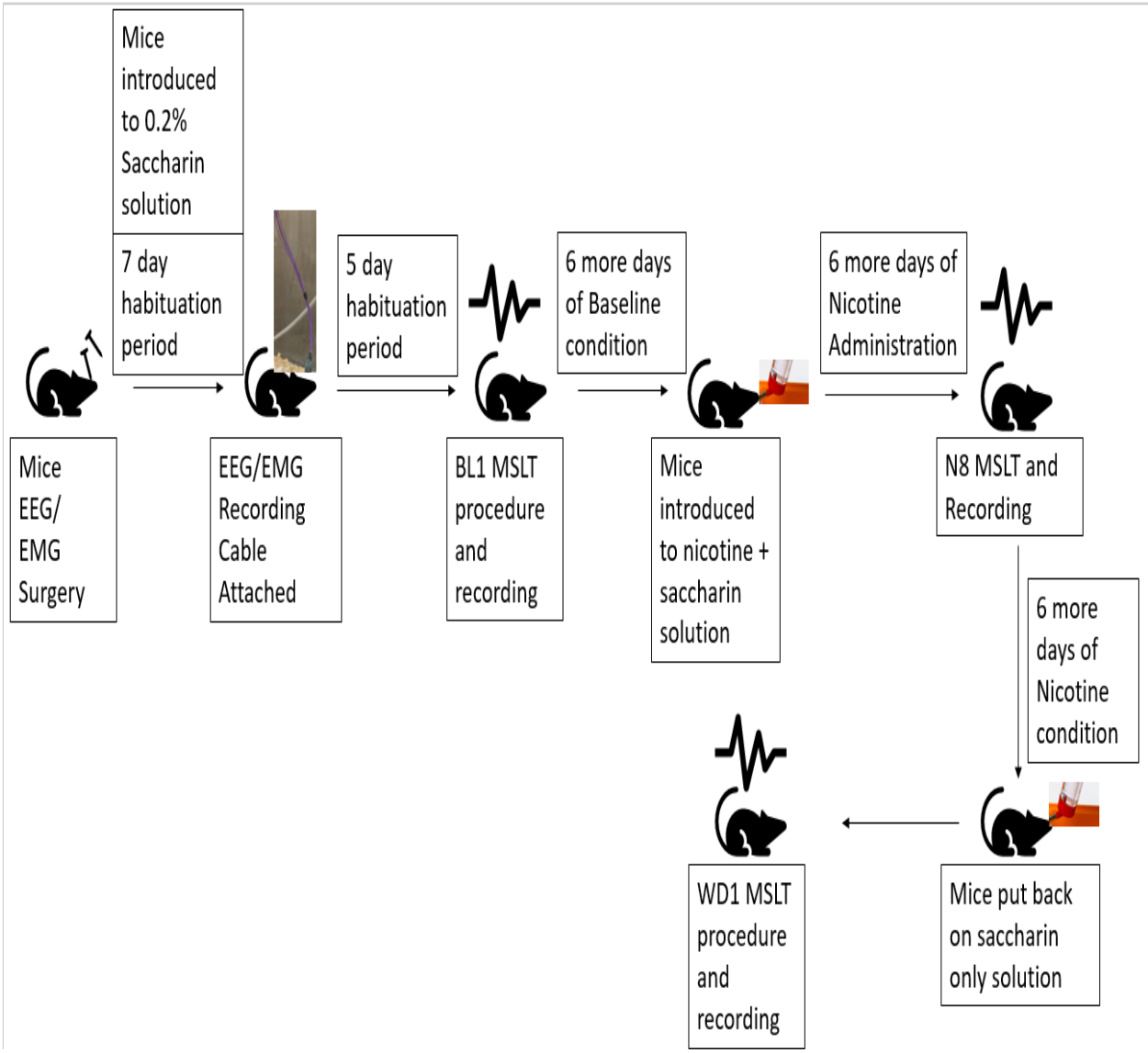

Figure 1. Paradigm of Experimental Procedure/Recording. Details When Recordings were taken, as well as habituation periods.

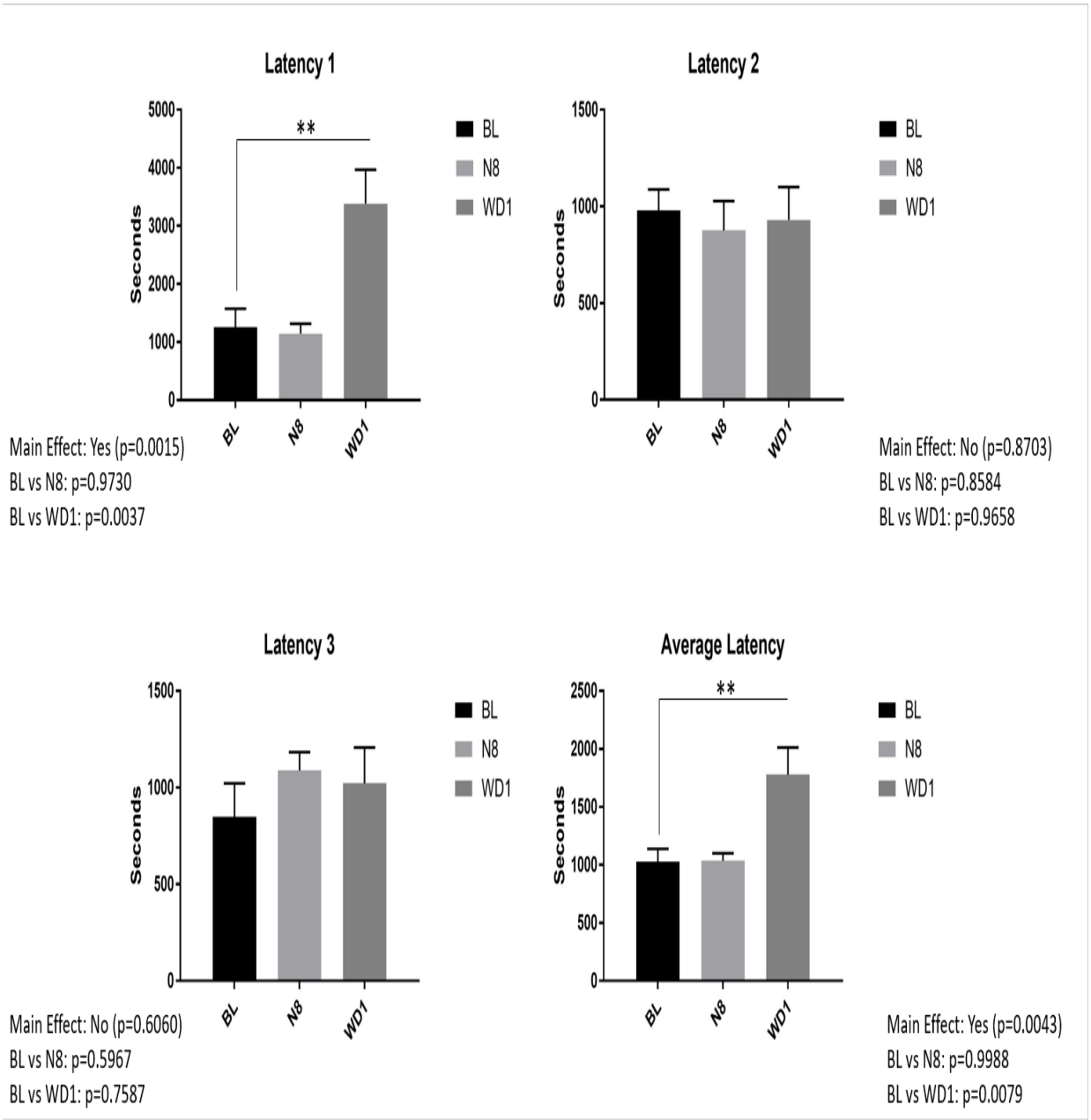

Figure 2. Effect of nicotine administration and withdrawal on Latency 1, Latency 2, and Latency 3 during the MSLT procedure. Nicotine withdrawal increased Latency 1, which drove the increase in average latency. Data are represented as mean +/- SEM. * indicates $p<0.05$, ** indicates $p<0.01$.

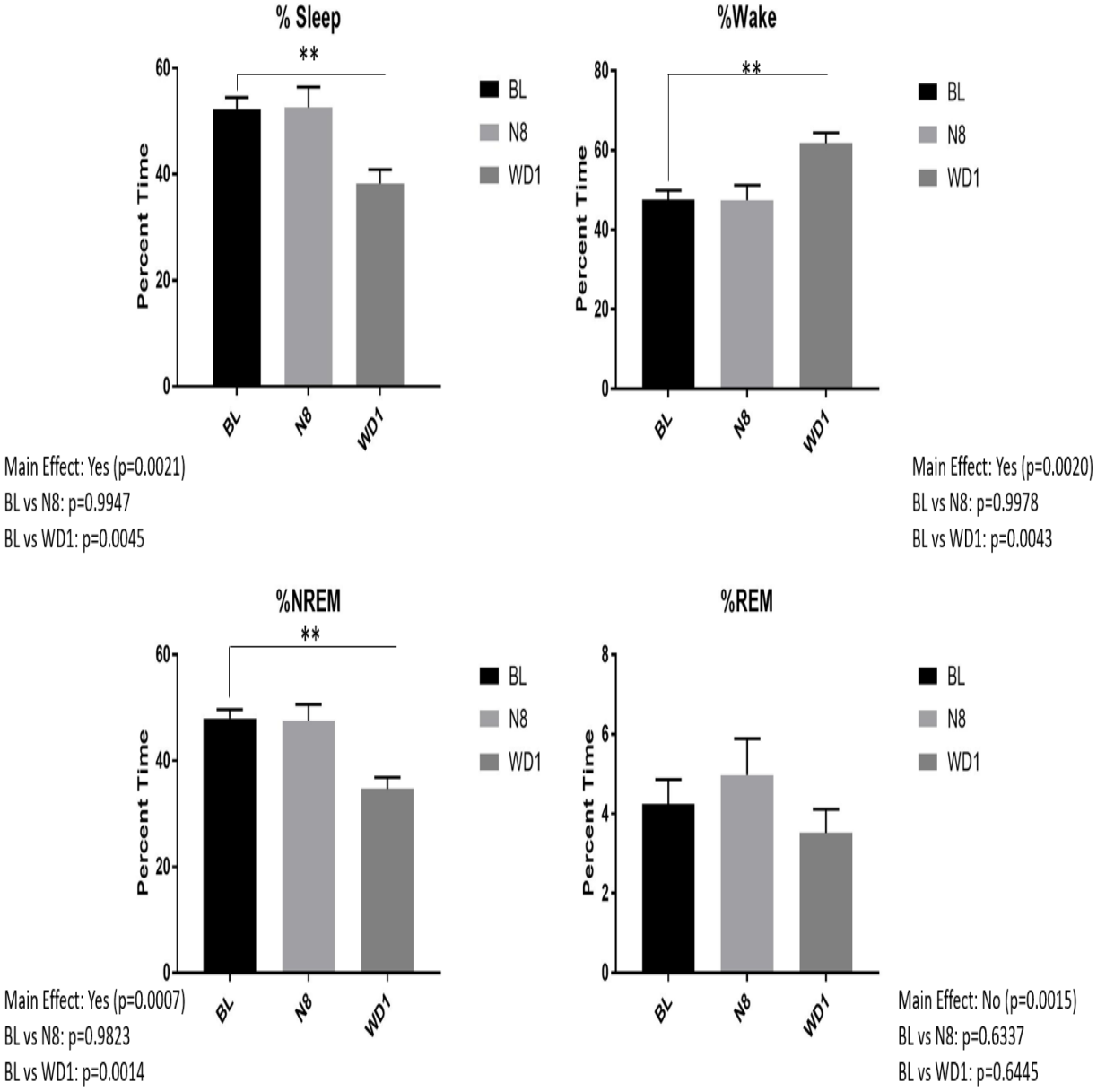

Figure 3. Effects of nicotine and withdrawal on wake/sleep state percentages in inactive phase. Nicotine had no effect on any state percentages, while withdrawal increased Wake percentage and decreased sleep percentage, via a decrease in NREM percentage. Data are represented as mean +/- SEM. * indicates $p<0.05$, ** indicates $p<0.01$.

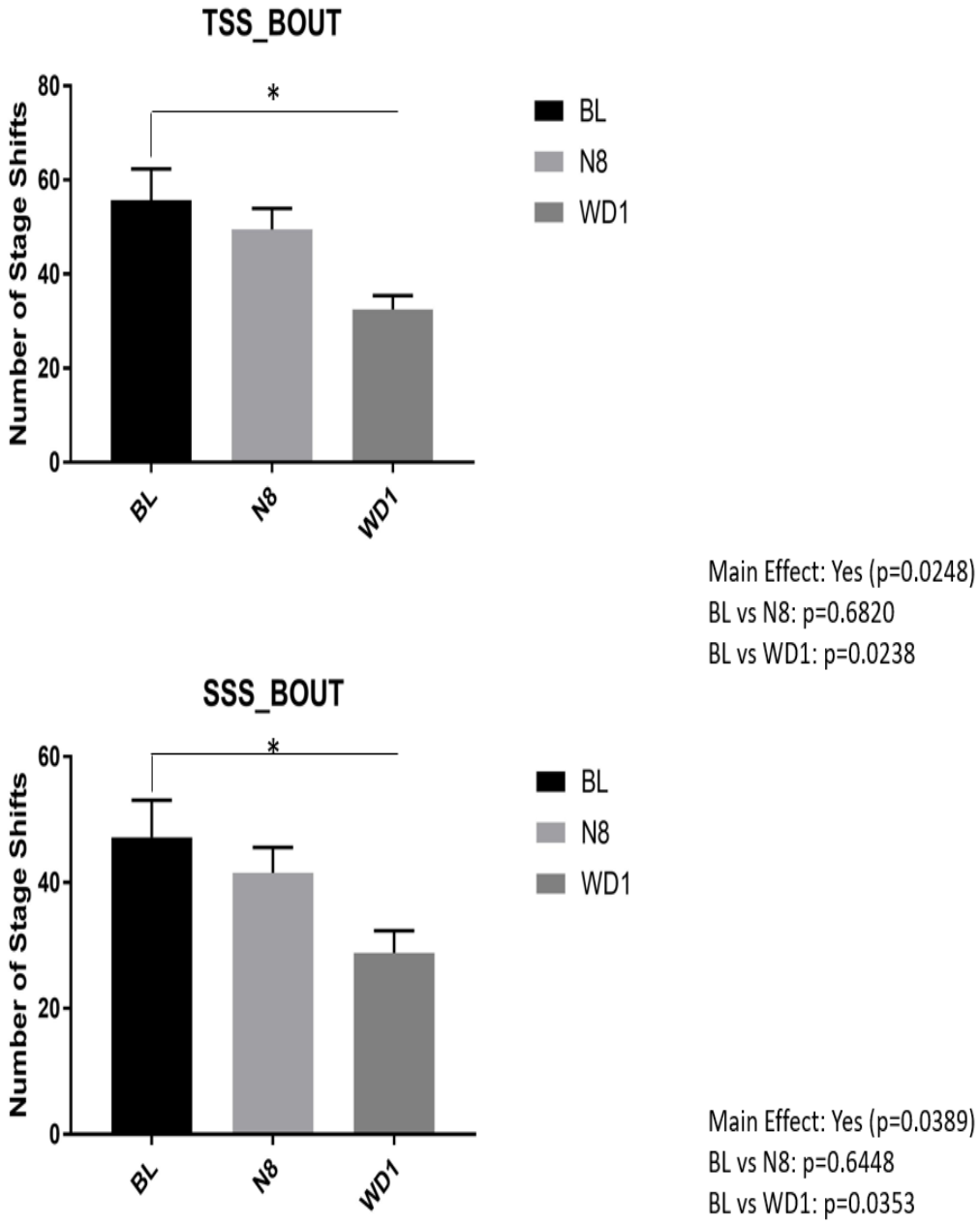

Figure 4. Effects of nicotine administration and withdrawal on Total Stage Shift Bouts (TSS) and Sleep Stage Shift Bouts. Withdrawal decreased both TSS and SSS compared to baseline. Data are represented as mean +/- SEM. * indicates $p<0.05$.

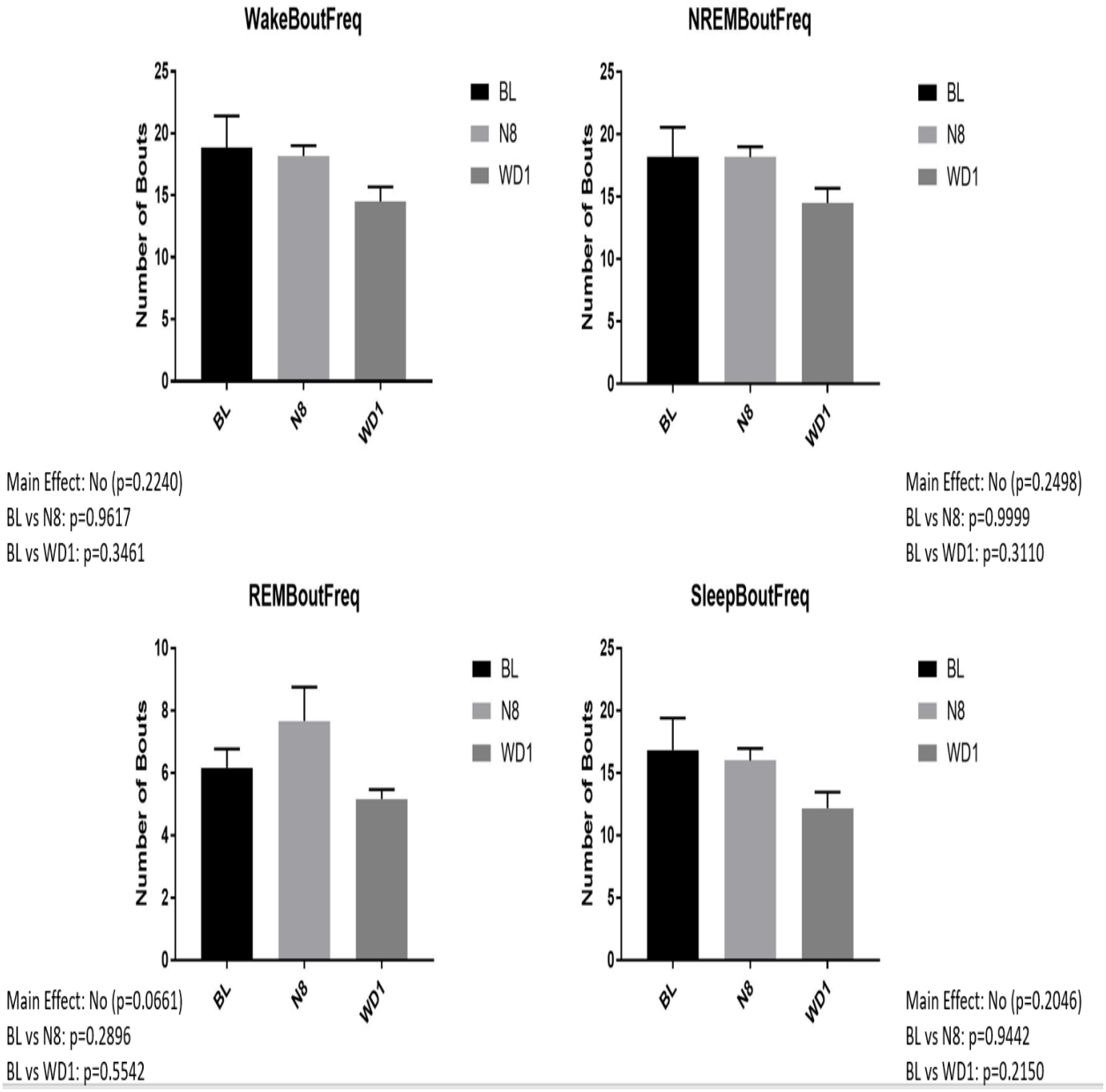

Figure 5. Effects of nicotine administration and withdrawal on Sleep/Wake stage frequencies. No main effect of treatment was seen, and no differences between N8 and BL or WD1 and BL were seen. Data are represented as mean +/- SEM.

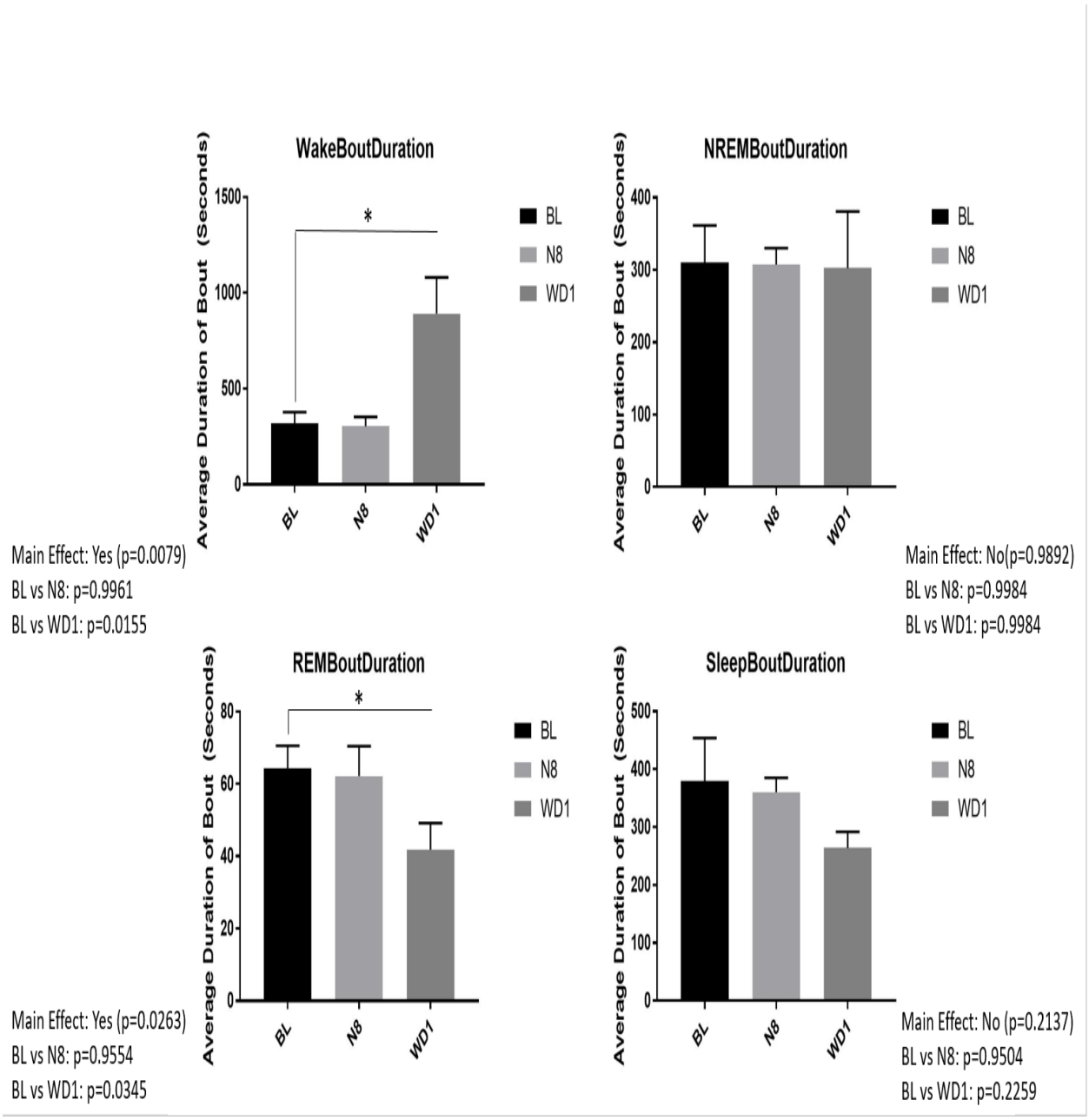

Figure 6. Effects of nicotine administration and withdrawal on Sleep/wake state duration. Withdrawal increased Wake Bout duration, and decreased REM Bout Duration. Data are represented as mean +/- SEM. * indicates $p<0.05$.

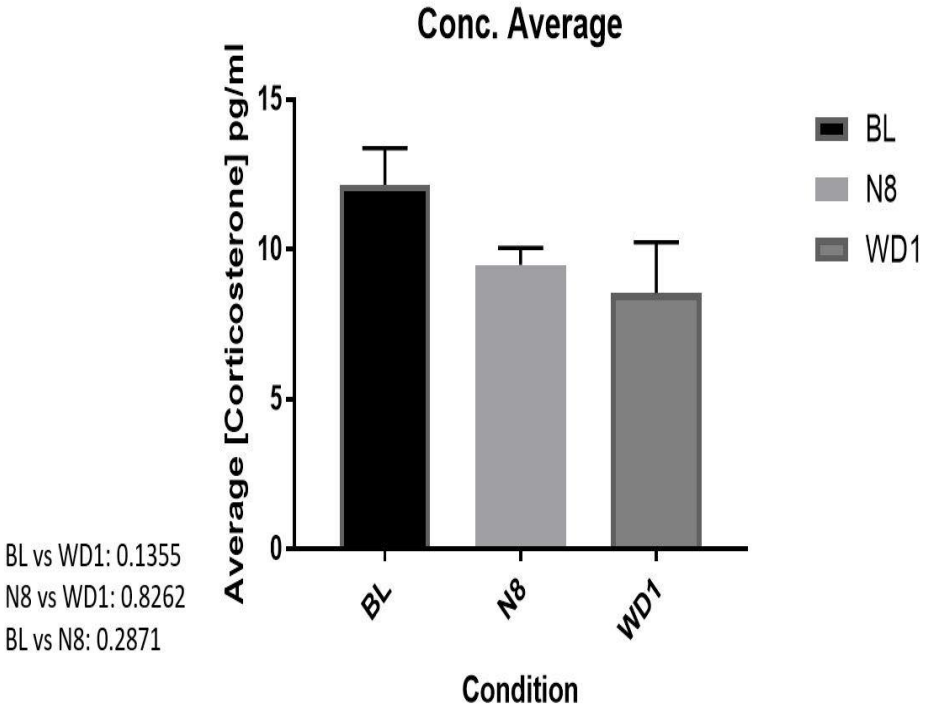

Figure 7. Effect of nicotine administration and withdrawal on corticosterone levels in submandibular blood (pg/ml). No main effect of treatment was seen, and no differences between groups was seen. Data are represented as mean +/- SEM.

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