

**Pre- and Post-Nicotine Circadian Activity Episodes are Differentially Affected by  
Pharmacological Treatments for Drug Addiction**

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This work is dedicated to the memory of Gwen Pearson, who is probably bragging about this dissertation on the back nine in heaven, even though I'd rather she didn't.

I miss you, Grandma.

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Andrea G. Gillman

**Pre- and Post-Nicotine Circadian Activity Episodes are Differentially Affected by  
Pharmacological Treatments for Drug Addiction**

Nicotine and other drugs of abuse can act as zeitgebers and entrain persisting circadian activity episodes when administered on a 24-hour schedule. There are two types of drug-induced circadian activity episodes: a *pre-drug anticipatory episode* characterized by a rise in activity beginning 1-2 hours prior to the drug administration time that is not linked to any predictive environmental cue, and a *post-drug evoked episode* that lasts for approximately the duration of the drug's physiological half-life. The present research examined how pharmacological treatments prescribed for nicotine and other substance addictions affected pre- and post-nicotine activity episodes in adult female Sprague-Dawley rats housed in wheel boxes under constant light and rate-limited feeding. For 16 consecutive days, the rats were administered a subcutaneous "zeitgeber" injection of either nicotine or saline on a 24-hour schedule to establish pre- and post-administration activity episodes. The rats were then administered one of nine treatment conditions in place of the zeitgeber injection for two consecutive days. The treatment conditions were No Treatment, Saline Treatment, Varenicline, Mecamylamine, Acamprosate, Topiramate, Naltrexone, SB-334867, and Bupropion. The treatment phase was followed by a 4-day baseline in which no injections were administered and the rats were not disturbed. The treatment conditions had different effects on pre- and post-drug activity episodes as well as nicotine- and saline-induced episodes. All treatments

reduced post-nicotine episodes, whereas post-saline episodes were increased by some treatments and decreased by others. All treatments increased pre-saline activity levels except the No Treatment condition and Mecamylamine (a nicotinic acetylcholine receptor antagonist), which reduced pre-saline activity. In contrast, pre-nicotine episodes were significantly reduced only by the No Treatment condition and by treatment with either the  $\mu$ - and  $\kappa$ -opioid antagonist naltrexone or the orexin-1 antagonist SB-334867. These results indicate that distinct neural mechanisms mediate both pre- and post-drug circadian activity episodes as well as nicotine- and saline-induced circadian effects. These results also argue that a number of pharmacological treatments currently prescribed for nicotine addiction may exacerbate pre-nicotine anticipatory episodes, while treatment with naltrexone or SB-334867 may help to alleviate the occurrence of these episodes.

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## **Pre- and Post-Nicotine Circadian Activity Episodes are Differentially Affected by Pharmacological Treatments for Drug Addiction**

### **Introduction**

Substance dependence, often known as drug addiction, is a psychiatric disorder characterized by repeated compulsive drug use over extended periods of time that usually leads to detrimental effects on the user's social, occupational, and financial well-being (American Psychiatric Association, 2000). One of the characteristic signs of drug addiction is an inability to stop using the drug despite the fact that many drug addicts express a desire to quit. Most substance-dependent individuals require some form of treatment intervention to successfully quit, as recovery without intervention is relatively rare (Sobell, Ellingstad, & Sobell, 2000). There is a wide spectrum of pharmacological and psychosocial treatments available for drug addiction that aim to alleviate withdrawal symptoms, initiate drug abstinence, and/or prevent relapse (Dutra, et al., 2008; Nides, 2008; Soyka, et al., 2008). While some treatments are more efficacious than others, there does not appear to be a "magic bullet" that will treat all kinds of drug addictions in all types of drug users.

One of the reasons a "magic bullet" treatment is unrealistic is that long-term use of addictive drugs leads to numerous changes in several different regions of the brain, and many of these changes appear permanent. Drugs of abuse are known to promote associative learning (Hyman, Malenka, & Nestler, 2006), corrupt the mesolimbic pathways that mediate the motivational properties of reward (Di Chiara, et al., 2004), and interfere with executive decision-making (Kalivas, Volkow, & Seamans, 2005). It is



unlikely that a single treatment could address the breadth of these issues. Most drugs of abuse also have a characteristic withdrawal syndrome that begins in the early stages of the quitting process (West & Gossop, 1994) and further complicates treatment regimes.

The solution to the lack of a “magic bullet” in drug addiction treatment appears to be the individualization of drug treatment programs to account for the individual circumstances of each drug addict. Ideally, such individualized programs would account for an addict’s history of drug use, the amount of substances used, the environment(s) in which the drugs were used, individual drug-taking behaviors, and the level of motivation to quit. It is clear that no two drug users are alike, and therefore, treatment programs must have a broad scope and a great deal of flexibility to account for this fact.

One way in which individual drug addicts differ that could potentially be relevant to their treatment is their daily pattern of drug use. For example, smokers can be classified into different chronotypes based on their daily smoking patterns (Chandra, Shiffman, Scharf, Dang, & Shadel, 2007), daily fluctuations in nicotine craving, or which of the cigarettes they smoke in the course of a day is their favorite (Jarvik, Killen, Varady, & Fortmann, 1993). Heavy alcohol users can also be classified into different chronotypes based on their daily drinking schedules. Many alcohol-dependent individuals have rigid daily drinking schedules; most report that they start drinking during the same hour each day, usually before noon (Danel, Jeanson, & Touitou, 2003). There is also evidence that illicit drug users take drugs on a 24-hour schedule, as emergency room admission times for drug overdoses and drug-related medical issues show a circadian rhythm with a peak in the early evening (Erickson, Lee, Zautcke, & Morris, 1998; Morris, 1987; Raymond, Warren, Morris, & Leikin, 1992).

These regular daily consumption patterns shown by drug users provide evidence that drugs of abuse affect the timing of internally-driven rhythms that cycle on a schedule of approximately 24 hours and are known as circadian rhythms. Endogenous circadian rhythms such as the daily sleep-wake cycle are governed by neural “clocks” that receive timing signals from the external environment and send outputs to synchronize the activity of specialized cells that generate daily physiological and behavioral events (Rosenwasser, 2009). Each individual cell has a molecular clock mechanism that consists of two sets of paired genes that are expressed at opposite times of the day (Bell-Pedersen, et al., 2005), and these individual molecular clocks respond to the outputs of the neural clocks.

A great deal of evidence has emerged in recent years showing that neural and cellular circadian timing systems are corrupted by drugs of abuse. Daily nicotine administration can alter daily meal patterns (Bellinger, Cepeda-Benito, & Wellman, 2003; Bellinger, et al., 2005) and phase shift circadian rhythms of heart rate, body temperature, and locomotor activity (Jacobson, Hasenfratz, & Battig, 1994; Pelissier, Gantenbein, & Bruguerolle, 1998). Nicotine can also phase advance waking times and decrease REM sleep (Gillin, Lardon, Ruiz, Golshan, & Salin-Pascual, 1994). Daily ethanol administration can phase shift circadian activity and body temperature rhythms (Baird, et al., 1998; Eastman, Stewart, & Weed, 1994). Methamphetamine administered in the drinking water can restore circadian locomotor, body temperature, water drinking, and feeding rhythms to arrhythmic SCN-lesioned rats (S. Honma, Honma, Shirakawa, & Hiroshige, 1988). Finally, daily administrations of nicotine (Gillman, Kosobud, & Timberlake, 2008), cocaine (White, Feldon, Heidbreder, & White, 2000), methamphetamine (Kosobud, Pecoraro, Rebec, & Timberlake, 1998; Pecoraro, Kosobud,

Rebec, & Timberlake, 2000), fentanyl (Gillman, Leffel, Kosobud, & Timberlake, 2009), and ethanol (Kosobud, et al., 2009) have all been shown to entrain circadian episodes of locomotor activity that are separate from the light-entrained activity rhythm.

Studies of human drug addicts have confirmed that these individuals show extensive disruptions in endogenous circadian rhythms. In the absence of pharmacological treatment interventions, newly abstinent heroin users fail to show circadian rhythmicity in the expression of adrenocorticotrophic hormone (ACTH),  $\beta$ -endorphin, interleukin-2 (IL-2), and the mRNA of the clock genes *hPer1* and *hPer2* in peripheral blood cells for up to 30 days after the initiation of abstinence (Li, et al., 2009). These individuals also show elevated levels of cortisol and elevated expression of the clock genes *hPer1* and *hCLOCK* in addition to reduced levels of leptin, neuropeptide Y (NPY), IL-2, and tumor necrosis factor (TNF) during the first month of abstinence. These persisting drug-induced disruptions in physiological circadian rhythms may contribute to withdrawal symptoms, hinder the initiation of abstinence, and/or increase vulnerability to relapse.

Despite extensive evidence showing the involvement of circadian timing systems in the development, expression, and maintenance of drug addiction, the potential role of circadian processes in the treatment of drug addiction is largely unexplored. The present study sought to expand this area of research by examining how pharmaceutical substances prescribed to treat nicotine and other drug addictions affect circadian activity episodes in rats that are entrained to a daily nicotine administration and that appear to reflect circadian aspects of drug craving, anticipation, and/or seeking behaviors.

A major goal of this work is develop procedures, results, and hypotheses that can lead to improvements in the individualization of drug treatment programs. Specifically, the present study will help to clarify the predictability of how drug users with strong circadian patterns of drug use (and presumably strong circadian-based drug cravings) will respond to pharmacological treatments for drug addiction. Additionally, the results of this study will help to isolate the neurotransmitter systems that mediate nicotine-induced circadian episodes, as several different treatments with a variety of pharmacological profiles will be tested.

### **Circadian Timing Mechanisms**

The Earth rotates on its axis approximately once every 24 hours. This rotation organizes each 24-hour day into one long period of light and one long period of dark across most of the planet's surface. This basic day/night structure has existed throughout the evolution of most of the organisms on Earth (Hunt, 1979). Consequently, most organisms evolved some kind of endogenous timing or "clock" mechanism to synchronize physiological and behavioral processes to the 24-hour day. Most physiological events and behaviors that are critical to the survival of an animal show circadian or 24-hour rhythmicity, including the sleep-wake cycle (Aschoff, 1965), the consumption of large daily meals (Mistlberger, 1994), fluctuations in body temperature (Aschoff, 1983), and the release of several hormones, including corticosteroids that regulate physiological responses to stress (Moore & Eichler, 1972).

Endogenous or internally-driven circadian rhythms have two important characteristics. First, as these rhythms are generated by cells within the body, they have an intrinsic rhythm that is approximately, but often not equal to 24 hours (Aschoff, 1965;

Aschoff, Hoffmann, Pohl, & Wever, 1975). Second, the period lengths of these intrinsic rhythms can be shortened or lengthened to match up with special time cues known as zeitgebers that signal the passage of a day in the external environment. Most endogenous circadian rhythms respond to the zeitgeber of light; specifically, the transition from light to dark throughout the day that occurs as the sun rises and sets. However, other external stimuli are able to act as zeitgebers, including large meals (Mistlberger & Rusak, 1987), social stimuli (Mrosovsky, Reeb, Honrado, & Salmon, 1989), and administration of drugs of abuse (Kosobud, et al., 2007). If no zeitgebers are present in an organism's environment, these internally-driven rhythms will continue to oscillate on a schedule of approximately, but usually not exactly 24 hours. Such rhythms are known as free-running rhythms and are essentially the expression of biological errors in time-keeping. These characteristics are critical to the identification of both endogenous circadian rhythms and unknown zeitgebers and to distinguish them from Pavlovian stimulus-response associations which do not show free-running rhythmicity.

At the cellular level, circadian rhythms are regulated by two sets of paired elements known as “clock” genes that are expressed at opposite times during the 24-hour day in a transcriptional-translational autoregulatory feedback loop (Bell-Pedersen, et al., 2005). The active part of the rhythm (such as the release of a hormone) is generated by the expression of the positive or “ON” genes. This gene expression then encodes the activation of the negative or “OFF” genes in the feedback loop. These negative genes prevent the expression of the positive genes until they are degraded by metabolic processes in the cell. Once the negative genes are fully degraded, the positive genes can be expressed, and the feedback loop begins again. This entire process takes

approximately 24 hours to complete, and the length of this feedback loop dictates the free-running periodicity of endogenous circadian rhythms.

This basic molecular feedback loop occurs in some iteration in every organism in which circadian rhythms have been studied to date. In mammals, the positive genes in the genetic clock are known as CLOCK (Circadian Locomotor Output Cycles Kaput) and BMAL1 (Brain-mediated ARNT-like Protein 1), two genes in the nucleus of the cell that form a heterodimer (Ko & Takahashi, 2006). A paralog of CLOCK known as NPAS2 can also dimerize with BMAL1 and appears to function in place of CLOCK in some regions of the brain (DeBruyne, Weaver, & Reppert, 2007). The formation of a CLOCK:BMAL1 (or NPAS2:BMAL1) heterodimeric protein chain in the nucleus of a cell activates the transcription of the negative genes in the mammalian molecular clock. These negative genes are known as *Period* (*Per*), which has three subtypes, *Per1* – *Per3*, and *Cryptochrome* (*Cry*), which has two subtypes, *Cry1* and *Cry2* (Ko & Takahashi, 2006). *Per* and *Cry* form a second heterodimer that acts in a negative feedback loop to suppress their own transcription in the nucleus by inhibiting the expression of the CLOCK:BMAL1 heterodimer. As the *Per:Cry* complex degrades, CLOCK and BMAL1 are re-expressed, and the feedback loop starts over.

Each individual cell that expresses this molecular feedback loop can operate independently of other cells. However, the cacophony of individually oscillating cells can be synchronized by the outputs of a group of cells that collectively act as a master clock system or oscillator (Morin & Allen, 2006; Verwey & Amir, 2009). The master clock sends electrical and/or chemical outputs to dictate the activity of downstream cells, and, when a zeitgeber is present in the environment, receives inputs that allow it to match

its own oscillation with the presentation of the zeitgeber. To date, there are three basic types of oscillator systems that have been identified by their zeitgebers: the light-entrainable oscillator system, the food-entrainable oscillator, and the drug-entrainable oscillator.

**The light-entrainable circadian system.** As stated above, most endogenous circadian rhythms respond to the zeitgeber of the daily light/dark cycle. When light photons enter the eye, they activate three basic types of photoreceptors on the most posterior layer of the retina: rods, cones, and melanopsin-containing cells (Morin & Allen, 2006). The activation of these retinal photoreceptors stimulates neurons that project to numerous regions of the brain that mediate vision and control the muscles of the eye. Light-induced activation of retinal photoreceptors also activates a circadian visual pathway that is independent of the primary visual and oculomotor pathways.

The circadian visual pathways stretch from the retina to the suprachiasmatic nuclei (SCN) of the hypothalamus both directly via the retinohypothalamic tract (RHT) (Moore & Lenn, 1972) and indirectly via the geniculohypothalamic tract (GHT) that passes through the intergeniculate leaflet (Swanson, Cowan, & Jones, 1974). The SCN are regions of approximately 11,000 neurons (Guldner, 1983) that act as a master clock to synchronize the activity of cells in the brain and periphery to produce light-entrainable behavioral and physiological rhythms (Ralph, Foster, Davis, & Menaker, 1990; Silver & Schwartz, 2005; Stephan & Zucker, 1972). In mammals, the SCN acts as the master clock for the majority of the known endogenous circadian rhythms, including the sleep-wake cycle (Ibuka & Kawamura, 1975; Mistlberger, 2005), locomotor activity (Schwartz & Zimmerman, 1991; Stephan & Zucker, 1972), the release of hormones such as adrenal

corticosterone (Moore & Eichler, 1972), and metabolic processes such as dopamine metabolism (Perlow, Gordon, Ebert, Hoffman, & Chase, 1977). When the SCN is lesioned, these processes lose their periodicity and become arrhythmic.

The SCN has four major output pathways that have been identified (LeSauter & Silver, 1998; Morin & Allen, 2006). The rostral or anterior pathway projects to the medial preoptic area (which receives vasopressin), the ventrolateral septum, the bed nucleus of the stria terminalis, and the anterior paraventricular nucleus of the thalamus, which receives both vasopressin and vasoactive intestinal polypeptide (VIP). The lateral pathway projects to the intergeniculate leaflet and appears to interact with the primary visual pathways. The posterior pathway projects to the posterior paraventricular thalamus, the precommissural nucleus, and the olivary pretectal nucleus. Finally, the periventricular pathway projects to several other hypothalamic nuclei, and many of the peptides transmitted to these nuclei by the SCN neurons have been described. VIP and gastrin-related peptide (GRP) are transmitted to the subparaventricular zone, vasopressin and VIP are transmitted to the paraventricular hypothalamic nucleus, and vasopressin, VIP, and GRP are all transmitted to the dorsomedial hypothalamic nucleus. The SCN also projects to the ventromedial hypothalamic nucleus, the arcuate nucleus, and the premammillary area in the periventricular pathway. There is also evidence that the SCN can transmit chemical outputs without axonal projections via diffusible chemical pathways, as SCN-driven locomotor rhythms can still persist when all efferent projections of the SCN are severed (Hakim, Debernardo, & Silver, 1991).

The RHT and GHT transmit several different types of neurotransmitters that affect the firing of SCN neurons. The primary neurotransmitters of the RHT are



glutamate (Ebling, 1996) and pituitary adenylate cyclase-activating peptide (PACAP) (Morin & Allen, 2006), although this pathway also transmits aspartate, nitric oxide, and substance P. The transmission of glutamate in the RHT and the sensitivity of SCN neurons to the effects of glutamate correspond to the effects of light on the SCN (Gillette & Mitchell, 2002). Both light pulses and glutamate transmission can induce phase shifts in SCN-driven circadian rhythms during the subjective night (the dark phase of the light/dark cycle), but not during the subjective day. In contrast, PACAP appears to act as the functional opposite of glutamate and light; PACAP transmission can induce phase shifts during the subjective day, but not at night.

The primary neurotransmitter of the GHT appears to be GABA (Morin & Blanchard, 2001), although this pathway also transmits neuropeptide Y, neurotensin, and enkephalin (Morin & Allen, 2006). Although GABA is generally considered a universally inhibitory neurotransmitter, SCN neurons show differential responses to GABA during the day and at night. During the subjective day, GABA transmission elicits an excitatory response from SCN neurons, whereas during the subjective night, GABA has an inhibitory effect on these cells. GABA also appears to serve as the major synchronizing mechanism among the neurons within the SCN and is transmitted widely throughout the region (Morin & Allen, 2006).

Although photic information from the RHT and GHT provide the major zeitgeber input into the SCN, there are several other afferent pathways that can affect the activity of SCN neurons. Serotonin is transmitted to the SCN from projections that originate in the median raphe nucleus (Glass, DiNardo, & Ehlen, 2000). Acetylcholine is transmitted from the basal forebrain and brainstem (Bina, Rusak, & Semba, 1993), and SCN neurons

respond to these cholinergic inputs during the subjective night, but not the subjective day (Morin & Allen, 2006). The SCN also receives inputs from several other hypothalamic nuclei, including the dorsomedial and ventromedial hypothalamic nuclei, and from the regions of the brain that mediate interoception, including the insular cortex, the infralimbic cortex, the parabrachial nucleus, the olfactory cortex, and the autonomic nervous system via the vagus nerve (Krout, Kawano, Mettenleiter, & Loewy, 2002; Moga & Moore, 1997).

Although light is undoubtedly the most powerful zeitgeber of SCN-driven rhythms, several non-photic stimuli have been shown to affect the firing of SCN neurons and induce phase shifts in SCN-driven rhythms during the subjective day when the SCN does not respond to light pulses and shows maximal *Period* gene expression (Morin & Allen, 2006). In most cases, the phase shifts induced by these non-photic stimuli are associated with decreased *Per* expression. Non-photic stimuli that are able to phase shift SCN-driven rhythms include novel wheel-running activity (Maywood & Mrosovsky, 2001) and treatment with addictive drugs including the benzodiazepine analog brotizolam (Yokota, et al., 2000), fentanyl (Vansteensel, et al., 2005), and nicotine (Ferguson, Kennaway, & Moyer, 1999; Trachsel, Heller, & Miller, 1995).

**The food-entrainable circadian system.** Although the majority of endogenous circadian rhythms have been shown to be controlled by the SCN, there is a small subset of circadian rhythms that clearly are neither entrained by light nor controlled solely by the outputs of the SCN. Animals with complete bilateral SCN lesions do not lose circadian periodicity in locomotor activity, body temperature, or adrenal corticosterone rhythms that have been entrained to the regular daily presentation of a large meal

(Krieger, Hauser, & Krey, 1977; Phillips & Mikulka, 1979; Stephan, Swann, & Sisk, 1979a, 1979b). Like the zeitgeber of light, a large daily meal can only entrain such activity when presented at an interval greater than 23 hours or less than 29 hours (Mistlberger, 1994; Stephan, 1981), a time window known as the range of entrainment. Further, when animals are kept in constant light or constant dark and on a restricted daily feeding schedule, the light-entrainable, SCN-driven locomotor rhythm will free-run independently of the food-driven anticipatory locomotor rhythm (Stephan, et al., 1979a). In contrast to light-entrained rhythms, peak levels of food-entrained rhythms emerge several hours *before* the zeitgeber is presented and drop off shortly after the meal is consumed (Mistlberger, 1994). As with all endogenous circadian rhythms, food-anticipatory rhythms will persist on an approximately circadian schedule for several days after the meal is withheld.

At the molecular level, the pre-feeding episodes of food-anticipatory rhythms appear to be associated with the expression of the *Period2* and *Cryptochrome* clock genes. Although *Per1* mutant mice show regular food-anticipatory activity, *Per2* mutants do not show significant food-anticipatory rhythms (Feillet, et al., 2006). Likewise, *Cry1/Cry2* double knockout mice show greatly reduced food-anticipatory activity compared to wild type mice (Iijima, et al., 2005). CLOCK mutant mice are arrhythmic for light-entrainable circadian rhythms, but do show normal food-anticipatory activity to a daily meal (Pitts, Perone, & Silver, 2003). However, mice deficient for NPAS2 (a paralog of CLOCK that is not expressed in the SCN but is expressed in other forebrain areas) take a long time to acquire food-anticipatory activity (Dudley, et al., 2003), so the expression of pre-feeding activity bouts may not be solely derived from the expression of

*Per2* and *Chryptochrome*. It has been reported that BMAL-1 deficient mice do not show food-anticipatory activity rhythms and that restoration of BMAL1 expression is sufficient to restore these rhythms (Fuller, Lu, & Saper, 2008a), but these results have been strongly contested (Fuller, Lu, & Saper, 2008b; Fuller, Lu, & Saper, 2009; Mistlberger, et al., 2009a; Mistlberger, et al., 2009b; Mistlberger, et al., 2008; Pendergast, et al., 2009). It has also been suggested that food-anticipatory circadian rhythms may operate completely independently of the known mammalian molecular clock (Storch & Weitz, 2009).

The food-entrainable oscillator that governs food-anticipatory rhythms was originally believed to be a discrete region that operated much like the SCN with a discrete zeitgeber input pathway and widespread output pathways to synchronize cells throughout the brain and periphery. This hypothesis appears to be false, as several decades of studies have shown that food-anticipatory rhythms persist after lesions of the vast majority of the brain (Davidson, 2009; Mistlberger, 1994, 2009). The location and circuitry of the food-entrainable oscillator is currently the subject of considerable controversy among researchers.

In the past decade, a great deal of research on the food-entrainable oscillator system has focused on the dorsomedial hypothalamic nucleus (DMH). Activity of DMH neurons has been linked to several circadian rhythms, including feeding, the sleep-wake cycle, and the transmission of corticosteroids (Chou, et al., 2003; Elmquist, Ahima, Elias, Flier, & Saper, 1998; Verwey & Amir, 2009). As stated above, the DMH is a major target of SCN efferent signals and receives vasopressin, VIP, and GRP from this region (Morin & Allen, 2006). In addition to the SCN, the DMH also receives projections from most of the other hypothalamic nuclei, particularly the preoptic area (Thompson &

Swanson, 1998). The DMH also receives projections from the brainstem and several areas of the telencephalon, including the ventral subiculum, the infralimbic cortex, the lateral septal nucleus, and the bed nucleus of the stria terminalis. The DMH receives inputs of both the satiety-associated neuropeptide leptin and the hunger-associated neuropeptide ghrelin (Elmquist, et al., 1998; C. B. Lawrence, Snape, Baudoin, & Luckman, 2002).

The DMH itself has three major efferent projection pathways to other hypothalamic nuclei. First, the DMH projects to the paraventricular hypothalamic nucleus (PVH), a region associated with the transmission of corticosteroids and the autonomic responses to stress (Elmquist, et al., 1998). Second, the DMH transmits GABA to the ventrolateral preoptic nucleus (VLPO), a region of neurons whose activity promotes sleep (Chou, et al., 2003). Finally, the DMH transmits glutamate to the lateral hypothalamus (LH), the origin of most of the neurons that transmit the neuropeptide orexin, which is sometimes known as hypocretin (De Lecea, et al., 1998; Preti, 2002). In contrast to the VLPO, activity of LH neurons is linked to wakefulness, and the transmission of orexin from these neurons promotes wakefulness and decreases REM sleep (Piper, Upton, Smith, & Hunter, 2000) and induces feeding in rats (Sakurai, et al., 1998). In sum, the activation of DMH neurons is directly influenced by the SCN and peripheral feeding-related inputs and is associated with arousal, stress responses, and feeding.

Studies that have used lesions to examine the function of the DMH in food-anticipatory rhythms have reported widely different results depending on the techniques and measurements utilized. Excitotoxic (ibotenic acid) lesions of the DMH have been

shown to reduce the circadian rhythm of *ad libitum* feeding (Chou, et al., 2003) and to greatly reduce food-anticipatory (general) locomotor activity and increases in body temperature as measured by implanted telemetry transmitters (Gooley, Schomer, & Saper, 2006). However, radiofrequency lesions of the DMH do not abolish food-anticipatory locomotor activity as measured by sensors both in the cage and in the food cup (Landry, Simon, Webb, & Mistlberger, 2006; Landry, Yamakawa, Webb, Mear, & Mistlberger, 2007). These apparently contradictory findings may be due to methodological differences (Davidson, 2009; Gooley & Saper, 2007; Landry & Mistlberger, 2007; Mistlberger, 2009), but it remains unclear whether the inactivation of DMH neurons has a demonstrable effect on food-anticipatory circadian rhythms.

Numerous studies have examined how restricted daily feeding affects clock gene oscillation and expression in the brain and periphery. A single daily meal elicits rhythmic *Per1* and *Per2* expression in the DMH, the nucleus of the solitary tract (NTS), and the area postrema (AP) that is synchronized to food-anticipatory activity and food availability and is not present in rats fed *ad libitum* (Mieda, Williams, Richardson, Tanaka, & Yanagisawa, 2006). Restricted daily feeding schedules will also entrain the rhythms of *Per1*, *Per2*, *Per3*, and *Cry1* expression in the liver independently of the light/dark cycle but do not alter clock gene expression in the SCN (Damiola, et al., 2000; Hara, et al., 2001). Although lesions of the DMH will phase advance the *Per2* rhythm in the liver, a large meal can still entrain this rhythm in DMH-lesioned animals (Tahara, Hirao, Moriya, Kudo, & Shibata, 2010). Therefore, although the DMH is likely an important relay for the synchronization of food-anticipatory circadian rhythms, it does not appear to act as an oscillator that is comparable to the SCN.

Food is an example of a natural reward, and like other rewarding stimuli, palatable food activates the mesocorticolimbic pathways that mediate the motivation to seek rewards (Wise, 2004). Other rewarding stimuli have also been shown to entrain anticipatory circadian rhythms independent of feeding when presented on a 24-hour schedule, including water access (Mistlberger, 1992) and highly palatable treats such as sucrose (Pecoraro, Gomez, Laugero, & Dallman, 2002) and chocolate (Ángeles-Castellanos, Salgado-Delgado, Rodríguez, Buijs, & Escobar, 2008). Both food and chocolate entrain *Per1* expression in the prefrontal cortex and both the core and shell of the nucleus accumbens. However, a large daily meal has also been shown to entrain *Per1* expression in hypothalamic structures, including the DMH and the arcuate nucleus, while daily chocolate access does not entrain *Per1* expression in these structures. Therefore, circadian anticipation of caloric or metabolic stimuli such as a palatable daily meal appears to be mediated in the hypothalamus, whereas circadian anticipation of rewards appears to be mediated in corticolimbic structures and not in the hypothalamus.

**The drug-entrainable circadian system.** Acute administration of drugs of abuse produces rewarding effects, and like the natural rewards discussed above, administration of these drugs activates the corticolimbic structures that mediate the motivation to seek rewards (Di Chiara & Imperato, 1988; Wise, 2004). Like food and other natural rewards, drugs of abuse have also been shown to entrain circadian rhythms of locomotor (wheel-running) activity episodes when presented on a 24-hour schedule (Kosobud, et al., 2007). Specifically, repeated subcutaneous or intraperitoneal injections of methamphetamine (Kosobud, et al., 1998), nicotine (Gillman, et al., 2008), cocaine (White, et al., 2000), fentanyl (Gillman, et al., 2009), and ethanol (Kosobud, et al., 2009) have been shown to

entrain circadian activity episodes in adult rats. These episodes emerge 1-2 hours before the daily drug administration time and last for 3-6 hours after the administration, depending on the drug and dosage given. Once these pre- and post-drug activity episodes are entrained, they will persist for at least two days if the drug is withheld.

Further, as with the zeitgebers of light and food, drug zeitgebers appear to have a specific and limited range of entrainment. Injections of these same drugs on 31- or 33-hour schedules do not entrain anticipatory episodes 1-2 hours before the next administration time (at hours 29-30 post-administration) (Gillman, et al., 2009; Pecoraro, et al., 2000; White, et al., 2000). Instead, an “ensuing” episode of activity occurs approximately 24 hours after each administration, even though the next injection is not administered until 7-9 hours after the ensuing activity bout.

Although daily injections of saline have been shown to entrain weak persisting circadian episodes (Timberlake, Gillman, Leffel, & Kosobud, 2009), and the act of picking up rodents once a day has been shown to entrain small bouts of activity (Goel & Lee, 1997), not all drug injections are able to act as zeitgebers. The antipsychotic drug haloperidol, a dopamine receptor antagonist with a high affinity for D2 receptors and a low affinity for D1 receptors (Irving, Adams, & Lawrie, 2006), does not entrain either anticipatory locomotor episodes on a 24-hour schedule or ensuing activity episodes on a 31-hr schedule (Gillman, et al., 2009).

As stated above, drug-entrained circadian activity episodes manifest as a continuous bout of activity that begins approximately two hours prior to the circadian administration time and lasts for 3 to 6 hours after administration, depending on the drug and dose. Similarly, the ensuing activity episodes recorded approximately 24 hours after



each administration on 31- or 33-hour schedules usually emerge 22-23 hours after the injection and last for a total of 3-6 hours. Although these drug-entrained episodes appear as a single circadian activity episode in the data, subsequent evidence suggests that there are actually two separate episodes entrained to the drug administration time that are at least partially mediated by separate mechanisms.

The first episode has been designated the pre-drug episode (Gillman, et al., 2008; Gillman, et al., 2009). For all drugs of abuse, this episode emerges 1-2 hours prior to the drug administration time and is generally considered to end at the administration time. Pre-drug activity episodes closely resemble food-anticipatory activity rhythms, which also emerge 1-2 hours prior to the zeitgeber presentation and drop off after the zeitgeber is consumed (Mistlberger, 1994). In human smokers and polydrug (cocaine and heroin) users, the hours leading up to drug administration are most closely associated with linear increases in craving (Preston, et al., 2009; Shiffman, et al., 2002). The hours leading up to a relapse to cigarette smoking in abstinent individuals are associated with increases in negative affect (Shiffman & Waters, 2004). In polydrug (cocaine and heroin) users, the hours prior to drug use and drug craving are associated with several triggers for drug use, especially the sight of the drug and both positive and negative affect (Epstein, et al., 2009). Based on these convergent data, pre-drug circadian activity episodes are assumed to reflect a circadian-based drug craving, anticipation, and/or seeking behavior.

The second episode has been designated the post-drug episode, and it begins immediately after the drug is administered and has different activity profiles and durations depending on the drug and dose administered. Post-drug episodes appear to reflect the acute drug effects in addition to a circadian-synchronized effect that may

influence the rewarding properties of the drug and set the timing for compensatory changes in physiological systems to counteract the excitotoxic effects of the drug.

In some cases, environmental conditions have been shown to differentially affect pre- and post-drug episodes. Pre-drug episodes can be suppressed if an auditory cue is paired with the effects of the drug, but post-drug episodes are not affected by presentation of the same cue (Gillman, et al., 2008). Further, when rats are kept under a fixed light/dark cycle, post-drug episodes last for an extended period of time compared to when the rats are kept under constant light or a variable light/dark cycle (Gillman, Kosobud, & Timberlake, 2007). In contrast, pre-drug episodes are not affected by different lighting regimes. Pre- and post-nicotine episodes also have different dose-response curves (Gillman, Kosobud, & Timberlake, 2010). When the nicotine dose is reduced and then restored over time, post-drug episodes show an inverse dose-response effect where the number of wheel turns per mg of nicotine administered increases as the dose is decreased and decreases as the dose is increased. In contrast, pre-drug episodes maintain a direct linear dose-response relationship as the dose is changed over time. These results suggest that post-drug episodes likely show sensitization and tolerance and that pre-drug episodes do not, but this has yet to be confirmed.

As with food-entrainable circadian rhythms, the mechanisms that produce drug-entrainable circadian rhythms have not been definitively identified. Although a great deal is known about how drugs of abuse affect the brain over time (see next section), few studies have explicitly examined the effects of drugs of abuse from a circadian perspective. Like the food-entrainable oscillator, the drug-entrainable oscillator system appears to operate at least partially independent of the light-entrainable master clock in

the SCN. Arrhythmic SCN-lesioned rodents given *ad libitum* access to methamphetamine in the drinking water show restoration of free-running circadian locomotor, feeding, drinking, body temperature, and corticosterone rhythms that do not entrain to the light-dark cycle (K.-I. Honma, Honma, & Hiroshige, 1987; S. Honma, et al., 1988; Tataroglu, Davidson, Benvenuto, & Menaker, 2006) but do entrain to a large meal given at 24- or 27-hour intervals (S. Honma, Honma, & Hiroshige, 1989; S. Honma, Kanematsu, & Honma, 1992). Methamphetamine administration can also to lengthen the free-running period of circadian activity in non-lesioned animals (K. Honma & Honma, 1986).

Although the results above suggest that the drug-entrainable oscillator system overlaps with the food-entrainable oscillator system, the SCN may also play a role in the expression of drug-induced and drug-shifted circadian rhythms. As stated earlier, the SCN receives cholinergic, glutamatergic, and GABAergic inputs (Morin & Allen, 2006), and these neurotransmitter systems are known to be affected by drugs of abuse (Benowitz, 2008; Kalivas, 2009; Koob, 2004). There is also evidence that the methamphetamine-sensitive circadian oscillator that restores free-running rhythmicity to arrhythmic rodents may interact with or be influenced by the SCN, as intact animals often show two separate (light-entrainable and methamphetamine-entrainable) rhythms with relative phase coordination (Tataroglu, et al., 2006).

To further complicate the issue, the methamphetamine-sensitive circadian oscillator does not appear to require the presence of any of the clock genes that have been identified as essential for the functioning of the light-entrainable circadian clock system. Arrhythmic *Per1/Per2* double knockouts, *Cry1/Cry2* double knockouts, and BMAL1

knockouts all show restored circadian locomotor rhythmicity when methamphetamine is administered *ad libitum* in the drinking water (S. Honma, Yasuda, Yasui, van der Horst, & Honma, 2008; Mohawk, Baer, & Menaker, 2009). CLOCK, *Tau*, and NPAS2 mutants, all of which show ultradian or infradian periodicity, experience a lengthening in their locomotor activity rhythm when administered methamphetamine (Masubuchi, Honma, Abe, Nakamura, & Honma, 2001; Mohawk, et al., 2009). Further, these effects of methamphetamine are not affected by SCN lesions.

Despite the negative results, there is considerable evidence that drugs of abuse can affect the known circadian clock genes. When methamphetamine is administered in the drinking water, the locomotor activity rhythm of intact (non-SCN-lesioned) rats becomes desynchronized from the light/dark cycle, and this desynchronized rhythm is correlated with phase shifts of *Per1*, *Per2*, and BMAL1 expression in the caudate-putamen and the parietal cortex, but not in the SCN, the nucleus accumbens, or the cingulate cortex (Masubuchi, et al., 2000; Masubuchi, Honma, Abe, Namihira, & Honma, 2007). In rats, morphine administration eliminates the daily rhythms of *Per1* and *Per2* in both the hypothalamus and in peripheral blood cells (Li, et al., 2009). Likewise, chronic ethanol administration has been shown to attenuate the expression of *Per2* in the SCN and the arcuate nucleus of the hypothalamus and to eliminate the periodicity of *Per3* in the SCN and *Per1* in the arcuate nucleus (Chen, Kuhn, Advis, & Sarkar, 2004). Finally, chronic cocaine administration has been shown to increase *Per1* and *Per2* expression and decrease NPAS2 expression in the hippocampus as well as increase *Per1* and CLOCK expression and decrease *Per2*, BMAL1, *Cry1*, and NPAS2 expression in the caudate-putamen (Uz, et al., 2005).

There is also a great deal of evidence that the known circadian clock genes have roles in drug addiction. *Per1* knockout mice do not show locomotor sensitization or conditioned place preference to cocaine (Abarca, Albrecht, & Spanagel, 2002), which indicates that the *Per1* gene may be critical for the development of both sensitization and Pavlovian associations to cocaine. Rodents deficient in N-acetylserotonin and melatonin also fail to sensitize to cocaine and also lack circadian periodicity in *Per1* expression (Akhisaroglu, Ahmed, Kurtuncu, Manev, & Uz, 2004); therefore, the role of *Per1* in sensitization and Pavlovian conditioning to cocaine may be mediated through the actions of these transmitters.

In contrast to *Per1* knockouts, *Per2* knockouts show robust conditioned place preference to cocaine and increased locomotor sensitization to cocaine compared to wild-type mice (Abarca, et al., 2002). These results have been interpreted to mean that individuals with poorly-functioning *Per2* genes may have an increased vulnerability to drug addiction. *Per2* mutants also consume greater amounts of alcohol than wild-type mice and show downregulation of the excitatory amino acid transporter 1 (EAAT1) on synaptic astrocytes, resulting in high levels of synaptic glutamate (Spanagel, et al., 2005). Elevated alcohol consumption in *Per2* mutants can be reduced with administration of the glutamate NMDA receptor antagonist acamprosate, an anti-craving drug used to treat alcoholism.

The CLOCK gene appears to be important for the firing of dopaminergic neurons in the ventral tegmental area (VTA) that mediate the motivational properties of rewarding stimuli such as food and drugs of abuse (Wise, 2004). Like *Per2* mutants, CLOCK knockout mice show increased sensitization and robust conditioned place preference to

cocaine (McClung, et al., 2005). CLOCK mutants also show increased firing and bursting in VTA dopamine neurons and have elevated levels of tyrosine hydroxylase, the rate-limiting enzyme in dopamine synthesis. As with *Per2*, these results indicate that poorly functioning CLOCK polymorphisms may increase an individual's susceptibility to drug addiction.

Studies of human population genetics have shown mixed results in attempts to link clock gene polymorphisms with drug addiction. One of the earliest studies failed to show evidence for *Per1*, *Per2*, or CLOCK polymorphisms that contribute to cocaine addiction (Malison, Kranzler, Yang, & Gelernter, 2006). However, one study has identified a variant of the *Per2* gene in humans that may contribute to alcoholism (Spanagel, et al., 2005) and another has identified a polymorphism of the CLOCK gene that may contribute to co-morbid alcohol use and depression, but not to alcoholism alone (Sjoholm, et al., 2010).

In summary, while the ability of methamphetamine to entrain locomotor activity rhythms does not appear to involve the known light-entrainable molecular clock feedback loops, these clock genes appear to play a role in drug addiction and mediate many of the effects of drugs of abuse. *Per1* appears to be critical for the development of behavioral sensitization, whereas *Per2* and CLOCK appear to mediate a protective effect to prevent sensitization to drugs of abuse as well as the formation of drug-paired Pavlovian associations.

There is still a great deal that remains to be discovered about how and where drugs of abuse affect endogenous circadian timing systems. Drugs of abuse are known to induce widespread and often permanent changes to the learning, memory, motivational,

and cognitive regions of the brain. The next section will provide an overview of the neural mechanisms of drug addiction with a special emphasis on potential connections to circadian timing systems.

### **Neural Mechanisms of Drug Addiction**

Fundamentally, drug addiction is a disorder of goal-directed behavior. The brain contains a complex circuit of motor and cognitive pathways that motivate an individual to seek stimuli that are critical to survival and reproduction and to avoid stimuli that are detrimental to these goals (Cardinal, Parkinson, Hall, & Everitt, 2002). Thus, it is no accident that survival-related stimuli such as food and water tend to have rewarding properties, whereas harmful stimuli tend to be repulsive. In the early stages of drug use, drugs of abuse show rewarding qualities, and their administration reliably elicits pleasurable acute effects (Kalivas & Volkow, 2005). Administration of these drugs activates the motivational circuits of the brain and induces the drug user to seek the rewarding drug effects again and again.

However, over time, repeated administration of drugs of abuse leads to a number of permanent alterations in the brain. The pleasurable effects of the drug decline steadily, while the motivation to seek the drug continues to escalate (Kalivas & Volkow, 2005). The drug-directed motivation of an addict is often so strong that it is not interrupted by strongly negative stimuli and states such as drug-induced medical problems (i.e. cancer, liver disease, etc.), the loss of financial security, or the disruption of important social relationships (American Psychiatric Association, 2000). In short, drugs of abuse cause permanent damaging changes to the motivational survival circuits of the brain.

**Acute drug effects.** Drugs of abuse cover a wide pharmacological spectrum, including psychostimulants, opiates, sedatives, and depressants. Each individual drug has a distinct set of acute physiological and behavioral effects. Nicotine consumption leads to increased arousal and alertness, a relaxed mood, and a reduction in appetite (Benowitz, 2008). Amphetamine administration causes hyperactivity, increased heart rate and breathing rate, and a number of psychological effects including euphoria, increased concentration, anxiety, and paranoia (Seiden, Sabol, & Ricaurte, 1993). Opioids such as heroin and morphine were first developed as analgesics but also induce euphoria and relaxation when used recreationally (Zacny, 1995). Alcohol can be stimulating at low doses, but at high doses it acts as a depressant and causes intoxication, impaired judgment, blurred vision, and many other effects (Eckardt, et al., 1998).

Each type of drug interacts with a unique subset of the dozens of neurotransmitter systems that facilitate cell-to-cell communication in the nervous system. For example, the acute psychostimulant effects of nicotine are primarily derived from the drug binding to and activating nicotinic acetylcholine receptors (Benowitz, 2008). Cocaine and amphetamine interfere with dopamine reuptake in the synapse which leads to an increase in dopamine transmission (Giros, Jaber, Jones, Wightman, & Caron, 1996). Both ethanol and opiates inhibit the firing of GABAergic neurons; ethanol binds to GABA receptors on these neurons while opioids bind to endogenous opioid receptors (Di Chiara, 1995; Koob, 2004). As the brain is a highly complex and integrated circuit, these primary drug actions have widespread and long-term consequences. Despite their different pharmacological profiles, drugs of abuse cause several common effects in brain functioning, the sum of which presumably constitutes the disorder of drug addiction.



***Dopamine.*** One of the first physiological “links” discovered among the different classes of addictive drugs was that acute administration of each of these drugs led to the transmission of dopamine in the shell of the nucleus accumbens from neurons that originate in the ventral tegmental area (VTA) of the midbrain (Di Chiara, et al., 2004; Di Chiara & Imperato, 1988). The VTA also has dopaminergic projections to the ventral pallidum, the prefrontal cortex (PFC), and the amygdala that are affected by drugs of abuse (Kalivas & Volkow, 2005). The transmission of dopamine from the VTA to the nucleus accumbens appears to mediate the motivation to seek rewarding stimuli (Cardinal, et al., 2002; Wise, 2004), and these VTA neurons also release dopamine when an individual is exposed to natural rewards such as palatable food (Hernandez & Hoebel, 1988) and stimuli associated with sex and reproduction (Aragona, et al., 2006; Fisher, Aron, & Brown, 2005). By activating VTA dopamine neurons, drugs of abuse signal to the brain that drug consumption is a desirable action and should be repeated if the drug is encountered again.

Each addictive drug enhances VTA dopamine transmission via different mechanisms, but the end result appears to be similar (Di Chiara, 1995). Nicotine binds to nicotinic acetylcholine receptors on the dopamine neurons in the VTA which cause the neurons to fire action potentials and release dopamine from their axon terminals in the nucleus accumbens. Psychostimulant drugs such as amphetamine and cocaine bind to the dopamine reuptake transporter on the axon terminals in the NAc, which increases the transmission of dopamine by preventing it from being taken back up into the presynaptic neuron. Both ethanol and opioid drugs enhance dopamine transmission through a process called disinhibition where the drugs bind to receptors on neurons that transmit GABA to

the VTA dopamine neurons and inhibit their firing. By preventing the GABA neurons from firing, these drugs allow the dopamine neurons to fire without inhibition. Ethanol disinhibits the VTA dopamine neurons by binding to GABA-A receptors on the GABA neurons, while opioids bind to  $\mu$ -opioid receptors on these neurons.

***Pavlovian conditioning.*** The formation of Pavlovian associations between a rewarding stimulus and reward-related cues in the external environment is a critical component of adaptive motivation. These associations allow an organism to predict the location and/or temporal availability of a rewarding stimulus without having to directly encounter the stimulus itself. The establishment and maintenance of Pavlovian associations involves several different brain regions, including the VTA, the nucleus accumbens, the amygdala, the prefrontal cortex, and the orexin neurons of the hypothalamus (Aston-Jones, et al., 2010; Day & Carelli, 2007; Kalivas & Volkow, 2005; See, 2005).

As noted above, the VTA also has dopaminergic projections to the amygdala (Kalivas & Volkow, 2005; See, 2005). The basolateral amygdala (BLA) is an important region for the processing of emotional information (Cardinal, et al., 2002; Day & Carelli, 2007) and is critical for the formation of Pavlovian associations between drugs of abuse and drug-associated environmental cues (See, 2005). Both the core and shell of the nucleus accumbens receive excitatory glutamatergic inputs from the basolateral amygdala, and these pathways are believed to mediate cue-induced drug seeking.

While drugs of abuse directly stimulate VTA neurons to release dopamine in the shell of the nucleus accumbens, drug-paired cues elicit dopamine release in the core of the nucleus accumbens (Ito, Dalley, Howes, Robbins, & Everitt, 2000). The development

of Pavlovian associations to drugs of abuse can be prevented if dopamine antagonist drugs are administered directly into the NAc core (Yun, Nicola, & Fields, 2004) or if this region is lesioned (Parkinson, Olmstead, Burns, Robbins, & Everitt, 1999). Drug-paired cues also appear to develop rewarding qualities in and of themselves, as rats trained to press a lever to receive cocaine or heroin will also press a lever solely to see a light that was previously paired with the administration of the drug (Di Ciano & Everitt, 2004).

The nucleus accumbens core and shell both have inhibitory GABAergic projections to the ventral pallidum, a nucleus of the basal ganglia that is an important motor region of the brain (Stratford & Kelley, 1999). Both drugs of abuse and their associated Pavlovian cues disinhibit these GABA projections and therefore activate the neurons of the ventral pallidum (Day & Carelli, 2007). The disinhibition of this motor area appears to lead to reward-related motor processes such as consumption.

***Orexin.*** The lateral hypothalamus (LH) is another important structure for the consumption of both natural and drug rewards. Unlike the motor functions of the adjacent ventral pallidum, the lateral hypothalamus drives reward consumption through the release of the neuropeptide orexin (Dube, Kalra, & Kalra, 1999; Sakurai, et al., 1998). The orexin neurons of the lateral hypothalamus project to diverse regions of the brain that are involved in the outputs of many endogenous circadian rhythms, including sleep, arousal, and reward-related behaviors (Sakurai, 2007). Most notably, the lateral hypothalamus has orexin projections to the VTA (Peyron, et al., 1998) and to the insular cortex (Date, et al., 1999), a subregion of the prefrontal cortex that has been strongly implicated in drug craving in substance-dependent individuals (Naqvi & Bechara, 2009; Naqvi, Rudrauf, Damasio, & Bechara, 2007).

The orexin neurons of the lateral hypothalamus appear to play a role in conditioned behavioral drug-seeking responses to drug-paired Pavlovian cues and environmental contexts (Aston-Jones, et al., 2010). A number of studies have shown that the transmission of orexin from these neurons is critical for triggering cue- and context-induced reinstatement of drug-seeking in rodent self-administration and conditioned place preference paradigms (Aston-Jones, et al., 2010; Boutrel, et al., 2005; Harris, Wimmer, & Aston-Jones, 2005; A. J. Lawrence, Cowen, Yang, Chen, & Oldfield, 2006). This function appears to be specifically mediated by the orexin-1 (OX1) receptor, as administration of the OX1 antagonist SB-334867 strongly attenuates cue-induced drug-seeking behaviors (A. J. Lawrence, et al., 2006; Smith, Tahsili-Fahadan, & Aston-Jones, 2010). The activity of LH orexin neurons correlates with the expression of conditioned place preference to morphine and cocaine, and this drug-induced conditioned place preference is reduced following administration of SB-334867 (Harris, et al., 2005).

The mechanisms by which drugs of abuse activate orexin neurons and subsequently contribute to the expression of conditioned behavioral responses to drug-paired cues have not been definitively isolated. The afferent projections to the lateral hypothalamus orexin neurons that are most strongly activated during conditioned place preference to cocaine originate in the lateral septum and the bed nucleus of the stria terminalis (Aston-Jones, et al., 2010), so these input pathways may be critical for drug-induced activation of LH orexin neurons. The orexin projections to the VTA appear to be critical for the expression of cue-induced drug-seeking, as microinjections of orexin into the VTA can reinstate extinguished conditioned place preference to morphine (Harris, et

al., 2005) and microinjections of SB-334867 into the VTA can suppress morphine-conditioned place preference (Narita, et al., 2006).

It has been suggested that orexin transmission in the VTA facilitates glutamate transmission to the VTA dopamine neurons from neurons that originate in the medial prefrontal cortex (Aston-Jones, et al., 2010). The transmission of glutamate in this pathway is known to be critical for the maintenance of Pavlovian associations between drugs and environmental cues (Kalivas & Volkow, 2005), and orexin inputs to these neurons appear to both enhance the glutamate-induced excitation of VTA neurons and to simultaneously inhibit excitatory inputs from other regions (Aston-Jones, et al., 2010). Through these actions, orexin transmission from the lateral hypothalamus appears to promote the firing of VTA dopamine neurons in reward-paired behavioral contexts.

***Glutamate.*** The acute effects of drugs of abuse appear to be primarily driven by their effects on dopamine transmission. However, these effects happen at the early stages of drug use, and as initial recreational drug use progresses into drug addiction, many of these dopaminergic effects are reversed (Kalivas, et al., 2005). The chronic effects of drugs of abuse are believed to result from their effects on glutamate, a universally excitatory neurotransmitter. In addition to their effects on dopamine, drugs of abuse also acutely enhance glutamate transmission, mostly via the same mechanisms by which they stimulate dopamine release (Dalia, Uretsky, & Wallace, 1998; Reid, Fox, Ho, & Berger, 2000; Reid, Hsu, & Berger, 1997). Over time, this excessive transmission of glutamate appears to be the major mechanism by which chronic drug use leads to permanent changes in neural structure and function.

**Chronic drug effects.** Although acute (short-term) administration of drugs of abuse leads to the activation of VTA dopamine neurons, these neurons decrease in size following chronic drug administration (Skolnick-Tavron, et al., 1996) and fire action potentials at a greatly reduced rate (Zhang, Hu, & White, 1998). Additionally, postsynaptic neurons in the nucleus accumbens and the prefrontal cortex express fewer dopamine receptors (Dagher, et al., 2001; Volkow, et al., 2001). The net result of these changes is reduced dopamine transmission in the mesocorticolimbic pathways of the brain. This reduction may partially explain why the acute rewarding effects of the drug diminish over time while the urge to take the drug continues to escalate (Robinson & Berridge, 1993; Solomon & Corbit, 1974).

While dopamine transmission is attenuated with chronic drug use, glutamate transmission continues to be evoked by drugs of abuse at all stages of drug use (Kalivas, 2009; Kalivas, et al., 2005). Excessive glutamate transmission is believed to underlie many of the characteristic behaviors and physiological changes that occur in drug-dependent individuals, including behavioral sensitization, compulsive drug use, and relapse.

**Tolerance.** Chronic drug users are able to consume enormous quantities of drugs that would produce toxic or fatal effects in naïve drug users (American Psychiatric Association, 2000). This phenomenon is known as tolerance, and is formally defined as a decreased effect of the drug as the dosage is increased or held constant over time (Stewart & Badiani, 1993). Tolerance usually occurs as a reduction in the physiological effects of the drug, and common examples include attenuation of nicotine-induced changes in mood

and heart rate (Perkins, et al., 1994), alcohol-induced intoxication (Bennett, Cherek, & Spiga, 1993), and opioid-induced analgesia (Collett, 1998).

The mechanisms of drug tolerance are not well understood, but tolerance is generally believed to occur as a result of reduced signal intensity at the drug's primary pharmacological targets as well as changes in the extent of gene expression and protein transcription within the target neurons (Littleton, 2001). For example, tolerance to the effects of nicotine is associated with increased expression of nicotinic acetylcholine receptors (Benowitz, 2008) and with changes in the expression patterns of the subunits that make up these receptors (McCallum, Collins, Paylor, & Marks, 2006). Tolerance to cocaine and morphine has been linked to an upregulation of the transcription factor cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) that occurs as a result of chronic drug-induced dopamine transmission (Nestler, 2004). Alcohol tolerance is linked to an upregulation of alcohol dehydrogenase enzymes in the liver that work to metabolize alcohol and remove it from the bloodstream (Eckardt, et al., 1998; Redmond & Cohen, 1971).

***Behavioral sensitization.*** Behavioral sensitization is the functional opposite of tolerance; it is defined as an increased effect of the drug as the dosage decreases or stays the same (Stewart & Badiani, 1993). Sensitization generally occurs to the behavioral effects of drugs such as locomotor activation and drug seeking and is particularly pronounced for psychostimulants such as nicotine, cocaine, and amphetamine (Booze, et al., 1999; Camp, Browman, & Robinson, 1994).

The development of behavioral sensitization has been linked to a prolonged increase in excitatory synaptic transmission known as long-term potentiation (LTP) at

glutamatergic synapses in the VTA (Kauer, 2004). These glutamate projections primarily originate in the medial prefrontal cortex and are an important pathway for the expression of reward-seeking behaviors (Capriles, Rodaros, Sorge, & Stewart, 2003; Cardinal, et al., 2002). As with LTP in the hippocampus that underlies learning and memory, LTP in the VTA is initiated by the upregulation of NMDA glutamate receptors on the post-synaptic cells at this synapse (Saal, Dong, Bonci, & Malenka, 2003). The initiation of both sensitization and LTP in the VTA can be prevented with the administration of both competitive and noncompetitive NMDA antagonist drugs (Ohmori, Abekawa, Muraki, & Koyama, 1994).

Once LTP is established, NMDA receptors at the synapse are downregulated, and the number of AMPA glutamate receptors is increased (Boudreau & Wolf, 2005). AMPA antagonist drugs can block drug-seeking behaviors in animals that are chronically administered drugs of abuse (Cornish & Kalivas, 2000), but NMDA antagonists have no effect on established sensitization (Karler, Chaudhry, Calder, & Turkanis, 1990). The increased numbers of AMPA receptors at potentiated synapses is associated with the upregulation of two transcription factors, CREB and  $\Delta$ FosB, that alter the patterns of gene expression within VTA neurons, increase the expression of AMPA receptor subunits, and alter the sensitivity of these neurons to glutamate transmission (Nestler, Barrot, & Self, 2001; Olson, et al., 2005).

Three additional factors have been found to contribute to the potentiation of VTA glutamatergic synapses and therefore enhance behavioral sensitization to chronic drug administration. First, stressful stimuli such as a foot-shock have been shown to enhance LTP at these synapses (Saal, et al., 2003). Numerous studies have linked stressful stimuli



and situations to the development of drug addiction (Koob & Kreek, 2007), and stress-induced drug-taking appears to hasten the process of sensitization. Second, chronic drug administration also blocks the development of long-term depression, a prolonged weakening of synaptic strength, at VTA glutamatergic synapses (Jones, Kornblum, & Kauer, 2000). Third, as stated earlier, orexin inputs to the VTA appear to augment cortical glutamate transmission and promote both behavioral sensitization and the expression of conditioned drug-seeking (Borgland, Taha, Sarti, Fields, & Bonci, 2006).

***Compulsive drug consumption.*** One notable difference between drug addicts and recreational drug users is that drug addicts show compulsive or irresistible drug-taking behavior, whereas recreational users are generally better able to self-regulate their drug consumption (American Psychiatric Association, 2000). Individuals who can be classified as substance-dependent show reduced neural activity in both the prefrontal cortex and the nucleus accumbens (Goldstein & Volkow, 2002), and changes in these regions are believed to underlie the compulsive aspect of chronic drug use.

In normal, non-drug-using individuals, the neurons of the prefrontal cortex and nucleus accumbens are activated by the presentation of a rewarding stimulus (Cardinal, et al., 2002). However, in drug addicts, the prefrontal cortex is only activated when presented with a drug-paired cue such as a video of a person taking drugs (Garavan, et al., 2000). These individuals also show reduced D<sub>2</sub> receptor signaling and therefore have a predominance of D<sub>1</sub> receptors that are coupled to a stimulatory G-protein that promotes inhibition of neuronal activity (Volkow, et al., 2001). The net result of these changes is that only very strong excitatory inputs, such as the excessive glutamate transmission produced by chronic drug use, can activate these motivational circuits. This serves to

focus the behavior of the drug addict towards drug-seeking and away from other rewarding stimuli (Kalivas, et al., 2005).

**Craving.** Drug craving is defined as a strong urge to consume a drug and is another characteristic of fully developed drug addictions. Drug craving appears to be one of the permanent aspects of drug addiction; unlike withdrawal, drug addicts may experience craving for several months, years, or for the rest of their life, even if they successfully quit (Friedmann, Saitz, & Samet, 1998).

Surprisingly, drug craving is not usually associated with withdrawal symptoms (Robinson & Berridge, 1993; Tiffany, Warthen, & Goedeker, 2007); instead, researchers have found that drug craving is reliably induced by certain environmental factors. When a drug addict experiences prolonged abstinence, consumption of a small amount of the drug can induce further craving – this phenomenon is known as drug-induced drug craving (Stewart, 2000). Exposure to drug-paired cues such as the sight of the drug or being in a place where the drug is normally consumed can reliably produce cue-induced drug craving, particularly in recently abstinent addicts (Brody, et al., 2007; Garavan, et al., 2000). Exposure to stressful situations can also induce drug craving in both drug-using and abstinent drug addicts, and this is known as stress-induced craving (Sinha, et al., 2005; Stewart, 2000). Finally, there is some evidence that heavy drug users may experience craving on a circadian schedule, particularly in the first few hours after waking (Chandra, et al., 2007; Danel, et al., 2003; Jarvik, et al., 1993).

The insular cortex has been strongly implicated in mediating drug craving in the brain. Activation of insular neurons correlates with reported drug cravings in drug addicts (Garavan, et al., 2000; Goldstein & Volkow, 2002). Smokers with bilateral

stroke-induced damage to the insula occasionally spontaneously quit smoking without experiencing nicotine craving (Naqvi, et al., 2007). In rats, conditioned place preference to amphetamine can be reversibly blocked by the temporary inactivation of the insula with lidocaine (Contreras, Ceric, & Torrealba, 2007).

The insular cortex integrates interoceptive cues from the autonomic and sensory nervous systems to monitor the current state of the body (Craig, 2002). It appears to facilitate drug-seeking by integrating the peripheral and visceral effects of the drugs with emotional and Pavlovian memories (Naqvi & Bechara, 2009). The insula also receives afferent signals from other brain regions, most notably glutamate projections from the amygdala and the ventral striatum (Cardinal, et al., 2002; Naqvi & Bechara, 2009) and orexin projections from the lateral hypothalamus (Date, et al., 1999; Hollander, Lu, Cameron, Kamenecka, & Kenny, 2008).

Although the role of insular mechanisms in the various types of craving have not yet been defined, activation of the VTA and the nucleus accumbens (in the ventral striatum) have been linked to drug-induced drug craving, and stress-induced drug craving has been linked to the release of corticotrophin releasing factor (CRF) by norepinephrine neurons in the bed nucleus of the stria terminalis (Stewart, 2000). Cue-induced craving has been linked to activation of the anterior cingulate cortex (Brody, et al., 2007; Garavan, et al., 2000). The orexin inputs to the insula have been linked to motivational drug-seeking, as administration of the orexin-1 antagonist SB-334867 into the insula reduces nicotine self-administration in rats and reverses the effects of nicotine on intracranial self stimulation thresholds (Garavan, et al., 2000).

***Withdrawal.*** Each drug has a distinct and characteristic withdrawal syndrome that drug addicts experience when they stop taking the drug. Withdrawal symptoms usually emerge 24-48 hours after the last drug administration and can last for several days or weeks depending on the drug and the severity of the addiction (Foy, Kay, & Taylor, 1997; McGregor, et al., 2005; Piasecki, Fiore, & Baker, 1998). Withdrawal from nearly all drugs of abuse is associated with anxiety, depression, and sleep disturbances (West & Gossop, 1994). Nicotine withdrawal also induces irritability and increased appetite. Alcohol withdrawal is characterized by tremors, nausea, excessive sweating, and occasionally seizures. Opioid withdrawal includes dysphoria, sweating, nausea, and pain. Amphetamine withdrawal induces agitation, increased appetite, and some psychotic symptoms. The withdrawal symptoms of cocaine are not as severe as for other drugs of abuse, but have been reported to include dysphoria, fatigue, and increased appetite.

***Relapse.*** Finally, when attempting to quit taking drugs, drug addicts have an extremely high chance of experiencing relapse, the resumption of drug-taking after a period of abstinence. Even in structured treatment programs, the majority of patients will relapse within a year of initial abstinence (McLellan, Lewis, O'Brien, & Kleber, 2000). Psychiatrists generally believe that relapse is an inescapable aspect of addiction. The most common explanations for relapse correspond to many of the most common triggers for drug craving; drug addicts often relapse after exposure to drug-associated stimuli and contexts or after experiencing stressful or traumatic situations (Marlatt, 1996).

### **Treatment of Drug Addiction**

Most pharmacological and behavioral treatments for drug addiction aim to prevent relapse after an addict has quit using the drug for an extended period of time.

Specifically, most treatments are administered to reduce subsequent drug craving, alleviate the symptoms of drug withdrawal, or to help a drug addict transition to a drug-free lifestyle. Some treatments are targeted toward particular drugs or drug classes, such as opiates, while other treatments can be applied across the entire spectrum of addictive drugs. Currently, most evidence-based drug treatment programs include a combination of psychosocial and pharmacological interventions in order to maximize the efficacy of the program.

**Psychosocial interventions.** Several types of psychosocial treatment interventions have been shown to be effective in the treatment of drug addiction, including contingency management, cognitive behavior therapy, motivational interviewing, and counseling (Carroll & Onken, 2005; Dutra, et al., 2008). Many treatment programs utilize a combination of these approaches. Contingency management programs offer a reward in exchange for continued good behavior. In the case of drug addiction treatment, the good behavior is usually abstinence from drug use as measured by urine samples or compliance with the prescribed treatment program, and the rewards can include money, access to methadone, career training, or vouchers for food and other necessities (Carroll & Onken, 2005; Petry, 2000). Cognitive behavior therapy is based on operant conditioning procedures and generally involves coaching a drug addict to recognize both the antecedents and the consequences of drug use and to develop a set of skills to help avoid and deal with situations that could lead to relapse. Motivational interviewing, also known as motivation enhancement therapy, is a form of counseling that is designed to increase an individual's motivation to change their behavior and remain abstinent (Carroll & Onken, 2005; Soyka, et al., 2008). This form of

psychosocial intervention is most often used to treat alcoholism. Finally, most drug treatment programs include some kind of counseling, either private, with family, or in group settings. Behavioral family and couples therapies tend to be the most effective, as they engage a drug user's social network to promote the maintenance of abstinence and the prevention of relapse (Carroll & Onken, 2005).

**Drug withdrawal treatments.** As stated earlier, drug withdrawal syndromes occur when a drug of abuse is unable to act in the body, either due to low systemic drug levels or due to the presence of an antagonist drug that prevents the drug from binding to its primary receptors (West & Gossop, 1994). Antagonist drugs that act in this manner are not effective treatments for substance dependence because they induce drug cravings that can't be immediately alleviated by taking the addictive drug. An example of this kind of antagonist is mecamylamine, a nicotinic acetylcholine receptor antagonist that induces nicotine cravings in smokers, but does not have adverse effects on non-smokers (Nemeth-Coslett, Henningfield, O'Keeffe, & Griffiths, 1986). A similar antagonist is naltrexone, a  $\mu$ - and  $\kappa$ -opioid antagonist that blocks the effects of opiate drugs such as heroin and morphine and can induce dysphoria and other withdrawal symptoms in current and recently abstinent users (Crowley, Wagner, Zerbe, & Macdonald, 1985).

Many drug withdrawal treatments involve some kind of drug replacement therapy in which a substitute compound is administered that allows addicts to wean themselves off of the drug without experiencing strong withdrawal. One of the most common examples of drug replacement is methadone, a weak opioid receptor agonist that is administered to treat addictions to heroin and morphine (Farrell, et al., 1994). The symptoms of opioid withdrawal are extremely unpleasant, and by alleviating these

symptoms, methadone administration increases the chances of successful quitting. Another common example of drug replacement therapy is nicotine replacement therapy (NRT). Nicotine is the main addictive component of tobacco (Office of the Surgeon General, 1988), and in NRT, nicotine is administered alone without the other components of tobacco, usually in the form of an oral lozenge or a dermal patch (Silagy, Lancaster, Stead, Mant, & Fowler, 2004). Varenicline, a weak nicotinic acetylcholine receptor agonist, is a pharmaceutical form of drug replacement therapy for nicotine addiction that treats nicotine withdrawal symptoms as well as nicotine craving (Rollema, et al., 2007).

**Drug craving treatments.** Drug withdrawal symptoms will eventually cease once an addict has been abstinent for a sufficient period of time, usually several days or weeks (West & Gossop, 1994). Despite the absence of the drug withdrawal syndrome, drug addicts often still experience drug cravings for many months or years, particularly when exposed to stressful situations or environmental cues that were previously paired with the effects of the drug, such as the sight of a person smoking a cigarette (Friedmann, et al., 1998). There are several pharmaceutical treatments with a variety of pharmacological profiles that are prescribed to reduce or alleviate drug cravings.

Consistent with the role of glutamate in the chronic effects of drugs of abuse, pharmacological treatments that target glutamate transmission have shown efficacy in the treatment of several different substance addictions. Acamprosate is primarily considered to be a weak glutamate NMDA receptor antagonist (Spanagel & Zieglgansberger, 1997), although it has been reported to enhance NMDA-mediated transmission in the nucleus accumbens (Berton, Francesconi, Madamba, Zieglgansberger, & Siggins, 1998). Acamprosate is primarily prescribed for the treatment of alcoholism (Kranzler & Van

Kirk, 2001; Spanagel & Zieglansberger, 1997). Topiramate is a glutamate AMPA/kainite antagonist and GABA agonist drug that was originally prescribed as an anticonvulsant but has also been used to treat cravings in alcoholics, smokers, and cocaine addicts (Flórez, et al., 2008; Kampman, et al., 2004; Reid, Palamar, Raghavan, & Flammino, 2007).

Dopamine receptor antagonists are generally prescribed to treat psychosis and do not have a great deal of efficacy for the treatment of drug addictions (Nestler, 2002). However, drugs that enhance dopamine transmission can alleviate craving in some individuals. For example, bupropion is a dopamine (DAT) and norepinephrine transporter (NET) reuptake inhibitor and weak acetylcholine receptor antagonist that enhances dopamine transmission and has been prescribed for smoking cessation in addition to its original indication as an antidepressant (Dwoskin, Rauhut, King-Pospisil, & Bardo, 2006).

Although opioid receptor antagonists are not effective in alleviating opioid withdrawal symptoms, drugs that target these receptors have shown some efficacy in alleviating drug craving. Naltrexone, a  $\mu$ - and  $\kappa$ -opoid receptor antagonist, is particularly effective in reducing reported cravings in alcoholics and in opioid addicts who are not experiencing withdrawal symptoms (Kosten, Kreek, Ragunath, & Kleber, 1986; Kranzler & Van Kirk, 2001; Spanagel & Zieglansberger, 1997). Naltrexone has also been proposed as a treatment for nicotine craving although it is seldom prescribed for this purpose (Covey & Glassman, 1999; O'Malley, et al., 2006). By blocking opioid receptors, naltrexone also indirectly suppresses dopamine transmission in the nucleus accumbens (Benjamin, Grant, & Pohorecky, 1993).



Finally, given the recent findings linking the activity of lateral hypothalamic orexin neurons to cue-induced drug seeking and other aspects of drug addiction (Aston-Jones, et al., 2010; Hollander, et al., 2008), some researchers have proposed prescribing orexin receptor antagonist drugs to alleviate drug craving (Scammell & Saper, 2007). Currently, the drug most likely to be utilized is the OX1 receptor antagonist SB-334867, but this drug has not been approved for this purpose and is not yet commercially available (Bingham, Cai, & Deehan, 2006; Rodgers, et al., 2001).

### **Purpose of Research**

Given that drug users can experience several different types of craving, and each type of craving is at least partially mediated by different neural pathways and neurotransmitter systems, the total alleviation of drug craving through pharmaceutical treatment intervention is a daunting task. For example, a single treatment drug could alleviate stress-induced drug craving while exacerbating cue-induced drug craving. The present study is based on the assumption that the anticipatory activity that emerges prior to a daily subcutaneous injection of nicotine (pre-nicotine) reflects a circadian-based drug craving that could potentially impact the treatment of drug addicts who show strong circadian patterns of drug consumption. The neural mechanisms that mediate circadian-based drug craving have not yet been determined, but it is assumed that these mechanisms are at least partially distinct from those that produce stress-induced, cue-induced, and other types of drug craving. Therefore, a treatment that reduces circadian-based craving could increase other types of craving, and vice versa.

The present study tested the effects of seven pharmacological treatments on nicotine-induced pre- and post-drug circadian activity episodes. A number of

pharmacological treatments for nicotine addiction have been shown to alleviate some form of craving in abstinent human smokers, but these treatments have not been tested for their efficacy on circadian-based drug craving. The treatment drugs used in the present study have shown efficacy either for smoking cessation or for the treatment of alcoholism, and most were selected based on the known pharmacological profile of nicotine. The acetylcholine-targeted treatments used were varenicline, a weak nicotinic acetylcholine receptor agonist that is essentially a pharmacological form of nicotine replacement therapy (Rollema, et al., 2007), and mecamylamine, a nicotinic receptor antagonist that will show how pre- and post-nicotine episodes are affected by a drug that is known to enhance rather than alleviate craving in human smokers (Nemeth-Coslett, et al., 1986). The glutamate-targeted treatments included acamprosate, an NMDA receptor antagonist that has not specifically been tested for smoking cessation, but is often prescribed for the treatment of alcoholism (Spanagel & Zieglgansberger, 1997). Acamprosate has also been shown to alleviate excessive alcohol consumption in *Period2* mutant mice (Spanagel, et al., 2005), so the pharmacological actions of this drug may include drug-entrainable molecular timing mechanisms. Topiramate, an AMPA and kainate receptor antagonist, was also used as a glutamate-targeted treatment, and this drug has shown efficacy both for alcoholism treatment and for smoking cessation (Flórez, et al., 2008; Kampman, et al., 2004; Reid, et al., 2007). Bupropion, an established pharmacological treatment for smoking cessation, was used as a treatment to specifically target dopamine transmission (Dwoskin, et al., 2006). Bupropion inhibits the dopamine reuptake transporter (DAT), so administration of this treatment will increase dopamine transmission.

Naltrexone, a  $\mu$ -opioid receptor antagonist, was also tested as a treatment in the present study. Although naltrexone is primarily prescribed for the treatment of alcoholism, it has also shown some efficacy for smoking cessation when combined with nicotine-replacement therapy (Krishnan-Sarin, Meandzija, & O'Malley, 2003; O'Malley, et al., 2006). Nicotine administration has been shown to enhance the transmission of endogenous opioids in addition to stimulating dopamine and glutamate transmission (Margioris, Markogiannakis, Makrigiannakis, & Gravanis, 1992). Stimulation of endogenous opioid pathways is believed to mediate some of the rewarding effects of nicotine (Corrigall, Herling, & Coen, 1988) and may explain a portion of the efficacy of naltrexone observed in smoking cessation programs.

Finally, the rewarding effects of nicotine have also been shown to be at least partially mediated by the neuropeptide orexin via the orexin-1 (OX1) receptor (Corrigall, 2009; Hollander, et al., 2008). For this reason, the present study also included the selective OX1 antagonist SB-334867 as a treatment. As stated earlier, this drug is not currently available or prescribed for smoking cessation or the treatment of other substance addictions, although it has been proposed for this purpose (Scammell & Saper, 2007).

In addition to the seven pharmacological treatments listed above, the present research also included two control treatments: a saline injection and a no treatment condition. The saline treatment in the present paradigm is analogous to the smoking of denicotinized cigarettes which allow a smoker to extinguish Pavlovian associations between the effects of nicotine and sensory cues associated with the route of administration (i.e., the cigarette) (Rose, 2006). The no treatment control condition is

analogous to quitting “cold turkey,” the most common method of smoking cessation (Fiore, et al., 1990; Solberg, Asche, Boyle, McCarty, & Thoele, 2007).

The nicotine dosage used in the present study, 1.0 mg/kg, has been established in previous studies of nicotine-induced circadian activity episodes to entrain robust pre- and post-drug wheel-running episodes that persist for at least two days after nicotine is withheld (Gillman, et al., 2007; Gillman, et al., 2008). These previous studies also used a dorsal subcutaneous injection route to minimize the amount of handling and restraint necessary to administer the drug. This administration route was used for all nicotine, saline, and treatment injections throughout the present study so that the only difference between the drug and treatment conditions was the substance administered. Due to temporal and financial constraints on this research, only a single dose was used for each treatment drug. Whenever possible, the treatment doses were selected based on previous studies that have shown that particular dose to have an effect on the behaviors or physiological effects produced by high doses of nicotine (see Method section). If these data were not available, the treatment doses selected were high enough to alter the effects of another drug of abuse in the absence of toxic effects.

The present study also investigated the effects of the nine treatment conditions on saline-induced circadian activity episodes in addition to nicotine-induced episodes. Previous studies have shown conflicting results on the ability of saline to act as a zeitgeber. Some studies have not shown entrainment of locomotor activity to daily saline administration (Gillman, et al., 2008; Pecoraro, et al., 2000), while others have shown small but significant persisting episodes of wheel-running activity entrained to saline injections (Timberlake, et al., 2009). At best, saline injections appear to be only a weak

zeitgeber that is easily overshadowed by more salient rewards. In the present study, saline was used as a zeitgeber control injection to examine the effects of the treatments on drug-naïve animals that have been weakly entrained to a daily administration time and to provide further insight on the neurotransmitter mechanisms that produce pre- and post-drug circadian activity episodes.

As stated earlier, daily administrations of nicotine and other drugs of abuse entrain two distinct activity episodes in which activity counts are significantly higher than acclimation activity levels at the same time of day. The pre-drug anticipatory episode consists of steadily increasing activity levels that emerge 1-2 hours prior to the daily administration time. The post-drug evoked episode begins immediately after the drug is administered and lasts for several hours, depending on both the drug and dosage given. Entrainment is considered to occur if repeated daily administration of a drug produces significant pre- and post-drug episodes and if both of these episodes persist for multiple days after the drug is withheld.

For the purposes of this research, pre-nicotine circadian activity episodes are assumed to reflect a circadian-based nicotine craving and/or nicotine-seeking behavior. Therefore, a treatment that significantly reduces pre-nicotine activity episodes and eliminates their persistence is assumed to alleviate this circadian-based craving. In contrast, a treatment that significantly enhances these episodes is assumed to exacerbate circadian-based craving. Post-nicotine circadian activity episodes are assumed to reflect the activation of an endogenous circadian timing system as an acute effect of the drug. A treatment that significantly reduces or eliminates the amplitude and persistence of post-

nicotine episodes is assumed to interrupt this timing mechanism, while a treatment that does not significantly change these episodes is assumed to not affect this timing system.

Finally, the effects of the tested treatments on pre- and post-nicotine- and saline-induced activity episodes should help to illuminate which neurotransmitter mechanisms mediate the expression of these episodes. Given that these episodes normally persist for several days following drug cessation, if administration of a treatment is followed by an immediate and significant attenuation of an activity episode, an opposite pharmacological action is assumed to at least partially mediate that activity. For example, if the nicotinic acetylcholine receptor antagonist mecamylamine significantly reduces an activity episode, then that episode is assumed to be at least partially driven by acetylcholine transmission via nicotinic receptors. Conversely, if treatment administration significantly increases activity levels in a circadian episode, the pharmacological action of the treatment is assumed to partially mediate the expression of that activity. For example, if the dopamine reuptake inhibitor bupropion significantly increases activity levels in a circadian episode, then the expression of that episode is assumed to be partially driven by dopamine transmission. Finally, if a treatment does not significantly alter a circadian activity episode, it will be tentatively concluded that the receptor and/or neurotransmitter targets of that treatment are not involved in the expression of that activity episode while reserving the possibility that higher or lower doses of the treatment may have an effect.

## **Method**

All experimental procedures were approved by the Institutional Animal Care and Use Committee at Indiana University Bloomington.

### **Subjects**

Subjects were 144 female Sprague-Dawley rats obtained from the rodent colony in the Department of Psychological and Brain Sciences at Indiana University Bloomington or from Harlan Industries. Female rats were used because previous studies of drug-entrained circadian episodes have generally used female rats (Gillman, et al., 2008; Kosobud, et al., 2007), and because male rats generally show a decline in wheel running with age (Peng, Jiang, & Hsu, 1980). At the beginning of the study, the mean age of the subjects was 103.53 days (SD = 15.78 days) and the mean body weight was 250.04 g (SD = 20.05 g). The rats were divided into a total of 18 experimental groups based on the zeitgeber injections (nicotine or saline) and the administered treatment (no treatment, saline, varenicline, mecamylamine, acamprosate, topiramate, naltrexone, SB-334867, and bupropion). Eight rats were assigned to each experimental group.

### **Apparatus & Conditions**

All rats were individually housed in cages with attached wheels for 50 days, except for four of the saline zeitgeber groups that received the acamprosate, topiramate, naltrexone, and SB-334867 treatments, which were housed for 28 days. The cages were kept in light- and sound-isolated cabinets equipped with a ventilation fan to maintain airflow and mask outside noise. Each cabinet contained 8 cages, and each experimental group was isolated within a single cabinet.

The rats were continuously monitored throughout the study for wheel running, water drinking, and feeding activities. Water was available *ad libitum*, and water bottles and cage bedding were changed once every week as per institutional guidelines. The numbers of wheel turns were recorded with microswitches, and water bottle licks, head entries in the feeder, and the numbers of food pellets consumed were recorded with photobeam sensors. Data were recorded continuously in one-minute bins using the Med PC-IV program (MedAssociates, Inc).

Throughout the study, the rats were kept under constant light that varied as a function of the location within the cage. Constant light was used to prevent entrainment to a light/dark cycle, which acts as a zeitgeber for most locomotor, behavioral, and physiological circadian rhythms (Bell-Pedersen, et al., 2005). Under constant lighting conditions, the estrous cycle is usually suspended in adult female rats (Fitzroy Hardy, 1970). Light intensity was ~45 lux in the wheels and ~275 lux in the cage where the food hopper and water bottle were located. Light intensity in the room outside the cabinet where injections were performed and body weights were recorded was ~215 lux. These light intensities are considered “bright light,” but are lower than the 300 lux intensity that reliably produces arrhythmia in adult rats (Cambras, et al., 1998). Light-entrainable physiological and behavioral rhythms will free-run under constant light, and the period of these free-running rhythms lengthens as the light intensity increases (Daan & Pittendrigh, 1976). Therefore, under the present study conditions, the free-running light-entrainable rhythms should show periods of approximately 25-26 hours and be easily distinguishable from the drug-entrained circadian episodes, which should show 24-hour periodicity.



For the duration of the study, food access was rate-limited to no more than two 97-mg pellets (NOYES pellets, Test Diets, Inc.) per 5-minute period. Food pellets were accessible from a food receptacle in the cage equipped with an infrared photodetector. The dispensation of the food pellets was controlled by the Med PC-IV program, which was programmed to dispense two pellets if five minutes had elapsed since the previous pellets were dispensed and if there were no pellets currently present in the receptacle. This rate-limited food access was used to prevent entrainment to a large daily meal which has been shown to act as a zeitgeber for circadian food-anticipatory locomotor and body temperature rhythms (Mistlberger, 1994). Under the rate-limited feeding schedule, rats readily consume their daily nutritional requirements, but they are unable to consume pellets fast enough and in sufficient quantities to constitute a meal that is large enough to entrain circadian food-anticipatory rhythms. In adult female rats, food-anticipatory activity generally emerges when meal size is 5 g or larger in a 2-hr period (Mistlberger & Rusak, 1987).

### **Drug Solutions & Administration**

Summaries of all drug solutions administered are listed in Table 1. All zeitgeber injections and treatments were administered via dorsal subcutaneous injections at a dosage volume of 1.0 ml/kg. All drug solutions were refrigerated at approximately 4°C when not in use.

**Zeitgeber injections.** Nicotine hydrogen tartrate powder (Sigma Pharmaceuticals, St. Louis, MO) was dissolved in 0.9% NaCl solution to a concentration of 1.0 mg/ml (free base weight). The pH of the solution was adjusted to approximately 7.4 using NaOH solution. Nicotine was administered at a dosage level of 1.0 mg/kg.

Table 1

*Pharmacological Profiles and Dosages of Subcutaneous Zeitgeber and Treatment Injections<sup>a</sup>*

<b>Substance</b>	<b>Pharmacological Target</b>	<b>Dosage</b>
<b>Zeitgeber Injections</b>		
Nicotine	Nicotinic acetylcholine receptor agonist; enhances dopamine, glutamate, orexin transmission	1.0 mg/kg
Saline	n/a	n/a
<b>Treatment Injections</b>		
No Treatment	n/a	n/a
Saline Treatment	n/a	n/a
Varenicline	Nicotinic acetylcholine receptor partial agonist	1.0 mg/kg
Mecamylamine	Nicotinic acetylcholine receptor antagonist	0.57 mg/kg
Acamprosate	Glutamate NMDA receptor antagonist	50 mg/kg
Topiramate	Glutamate AMPA/kainate receptor antagonist	50 mg/kg
Naltrexone	$\mu/\kappa$ -opioid receptor antagonist	10 mg/kg
SB-334867	Orexin-1 receptor antagonist	10 mg/kg
Bupropion	Dopamine reuptake transporter inhibitor	20 mg/kg

<sup>a</sup>All injections administered at a dosage volume of 1.0 ml/kg.

This dosage of nicotine has been shown to entrain robust pre- and post-drug circadian activity episodes in adult female rats under constant light and rate-limited feeding without adverse effects (Gillman, et al., 2008). This dosage has also been shown to produce conditioned place preference to nicotine injections, as do higher and lower doses (Le Foll & Goldberg, 2005).

A solution of 0.9% NaCl solution was used for all saline injections.

**Treatment injections.** Varenicline tartrate (Pfizer) was dissolved in 0.9% NaCl solution to a concentration of approximately 1.0 mg/ml. Varenicline was administered at a dosage level of 1.0 mg/kg. This dosage has been shown to produce a response rate similar to 0.4 mg/kg nicotine in a drug discrimination paradigm, and this effect of varenicline is blocked by the administration of 0.56 mg/kg mecamylamine (Rollema, et al., 2007).

Mecamylamine hydrochloride powder (Sigma) was dissolved in 0.9% NaCl solution to a concentration of approximately 0.57 mg/ml. Mecamylamine was administered at a dosage level of 0.57 mg/kg. This dosage has been shown to eliminate somatic signs of nicotine withdrawal in rats 24 hours after nicotine administration ceases (Watkins, Epping-Jordan, Koob, & Markou, 1999).

Acamprosate sodium (Toronto Research Chemicals) was dissolved in 0.9% NaCl solution to a concentration of approximately 50 mg/ml. Acamprosate was administered at a dosage level of 50 mg/kg. This dosage has been shown to reduce ethanol drinking in rats during reinstatement (Czachowski, Legg, & Samson, 2001), but is not known to specifically alter any behavioral or systemic effects of nicotine.

Topiramate powder (US Pharmacopeia) was suspended in 0.9% NaCl solution to a concentration of approximately 50 mg/ml and mixed in a warm water bath. Topiramate was administered at a dosage level of 50 mg/kg and vigorously stirred prior to each daily administration time. This dosage has been shown to attenuate the release of dopamine, norepinephrine, and serotonin in the nucleus accumbens produced by the administration of 0.4 mg/kg nicotine (Schiffer, et al., 2001).

Naltrexone hydrochloride (Tocris) was dissolved in 0.9% NaCl solution to a concentration of approximately 10 mg/ml. Naltrexone was administered at a dosage level of 10 mg/kg. This dosage has been shown to decrease responding for food under a fixed interval schedule when administered in combination with 1.0 mg/kg nicotine (Corrigall, et al., 1988).

SB-334867 (Tocris) was dissolved to a concentration of approximately 10 mg/ml in a vehicle that consisted of 88% sterile water, 2% dimethylsulphoxide (Sigma), and 10% 50 mM 2-hydroxy- $\beta$ -cyclodextrin. SB-334867 was administered at a dosage level of 10 mg/kg and stirred prior to each daily administration time. In rats, this dosage has been shown to extinguish cocaine-seeking behavior (Boutrel, et al., 2005) and to reduce the consumption of high-fat food pellets (Nair, Golden, & Shaham, 2008). A lower dose of SB-334867 (4 mg/kg) has been shown to significantly reduce both i.v. nicotine self-administration and to reduce lever-pressing for nicotine rewards in a progressive ratio schedule (Hollander, et al., 2008).

Bupropion hydrochloride (Sigma) was dissolved in 0.9% NaCl solution to a concentration of approximately 20 mg/ml. Bupropion was administered at a dosage level

of 20 mg/kg. This dosage has been shown to attenuate somatic nicotine withdrawal signs in rats (Malin, et al., 2006).

### **Study Schedules**

The basic study schedule lasted 50 days (Table 2). The study began with a 5-day acclimation phase in which the rats were only disturbed twice at approximately 1200 to record body weights. The remainder of the study consisted of two zeitgeber injection series, each followed by a 2-day treatment series and a 4-day baseline phase in which all injections were withheld and the rats were left undisturbed unless entry was required by equipment malfunctions. The first zeitgeber injection series lasted 16 days, and the second zeitgeber injection series lasted 8 days. Within each injection series, nicotine or saline was administered at the same hour each day within the first 30 minutes of the hour.

The daily administration time was shifted (earlier or later) by at least 3 hours between zeitgeber injection series 1 and 2. During the treatment series, the treatments were administered at the same time of day that the zeitgeber injection had been administered in the preceding injection series.

Four of the saline zeitgeber groups were tested in an abbreviated 28-day paradigm due to temporal and financial constraints. These groups received the acamprosate, topiramate, naltrexone, and SB-334867 treatments. In this abbreviated paradigm (Table 1), the acclimation saline injections and the second zeitgeber injection, treatment, and baseline phases were omitted so that the rats received only a single 16-day zeitgeber injection series followed by a 2-day treatment phase and a 4-day baseline phase.

Table 2

*Study Schedule and Subcutaneous Injections Administered*

	No. of	Nicotine Zeitgeber	Saline Zeitgeber
Study Phase	Days	Group	Group
Acclimation	5	None	None
Acclimation Injections <sup>a</sup>	8	Saline	Saline
Zeitgeber Injection Series 1	16	Nicotine	Saline
Treatment Series 1	2	Treatment <sup>b</sup>	Treatment <sup>b</sup>
Baseline Phase 1	4	None <sup>c</sup>	None <sup>c</sup>
Zeitgeber Injection Series 2 <sup>a</sup>	8	Nicotine	Saline
Treatment Series 2 <sup>a</sup>	2	Treatment <sup>b</sup>	Treatment <sup>b</sup>
Baseline Series 2 <sup>a</sup>	4	None <sup>c</sup>	None <sup>c</sup>

<sup>a</sup>Omitted for Saline-Acamprosate, Saline-Topiramate, Saline-Naltrexone, and Saline-SB334867 groups.

<sup>b</sup>Treatments administered were one of the following: No Treatment (rats left undisturbed on treatment days), Saline injection, Varenicline, Mecamylamine, Acamprosate, Topiramate, Naltrexone, SB-334867, or Bupropion. All treatments were administered at the same time as the zeitgeber was administered in the preceding injection series.

<sup>c</sup>Rats not disturbed on indicated days.

## Data Analysis

The two hours prior to a zeitgeber injection time were designated as the pre-injection (PRE) period, and the three hours immediately following an injection were designated as the post-injection (POST) period. The PRE period for each individual zeitgeber and treatment injection was calculated for the period 22-24 hours after that particular injection (i.e., on the following day). The remaining 19 hours of each 24-hour day were designated as the rest-of-day (ROD) period. The activity measures analyzed in the statistical analyses were the wheel turns, water licks, head entries into the feeders, and the number of pellets consumed.

Entrainment was determined by estimating the period length of the drug-related and free-running activity cycles from the actograms and by statistical analysis of the activity counts across the acclimation phase, the zeitgeber injection series, and the treatment and baseline days. Multivariate repeated measures analyses with simple planned comparisons were performed to compare the PRE, POST, and ROD periods among the acclimation and zeitgeber injection series to test for significant episodes of activity before and after the drug injection times. Activity levels in the PRE and POST periods that were significantly higher in the zeitgeber injection series than in the acclimation injection series were interpreted as significant episodes. To determine persistence of significant episodes, repeated measures analyses were also performed to compare the treatment and baseline days to the zeitgeber injection series for the No Treatment control groups. Activity levels that were not significantly different between the treatment or baseline phases and the zeitgeber injection series were interpreted as persisting episodes.

The percent change in activity from the zeitgeber injection series was used to examine the effects of the treatments on pre- and post-drug episodes. This measure was calculated by subtracting the amount of activity in either the PRE or POST period on each of the individual treatment and baseline days from the mean amount of activity in that period on the last four days of the zeitgeber injection series. This difference value was then divided by the injection series amount to obtain the percent different in activity. As the PRE period used for each injection was calculated on the following day, there was one fewer baseline day of data available for the PRE period than for the POST period. Multivariate repeated measures analyses with planned comparisons were then performed to compare the percent difference in activity on the individual treatment and baseline days to the percent difference in activity during the zeitgeber injection series, which was equal to zero in all cases. Therefore, percent differences on the treatment and baseline days that were significantly different from zero (no change in activity) were interpreted as a significant effect of the treatments on the activity levels.

Finally, to examine the effect of the treatments on the reacquisition of nicotine-induced entrainment, multivariate repeated measures comparisons were performed between the first and second zeitgeber injection series for an average of activity in the PRE and POST periods in both the first 4 days of each series and the last 4 days of each series.

Two rats were excluded from the analyses of the treatment effects because their activity counts and percent difference values for all of the four activity measures were extreme outliers compared to the other seven rats in their treatment group (more than 3



interquartile ranges from the group means). The excluded rats were rat #3 from the Nicotine-Naltrexone group and rat #9 from the Saline-Bupropion group.

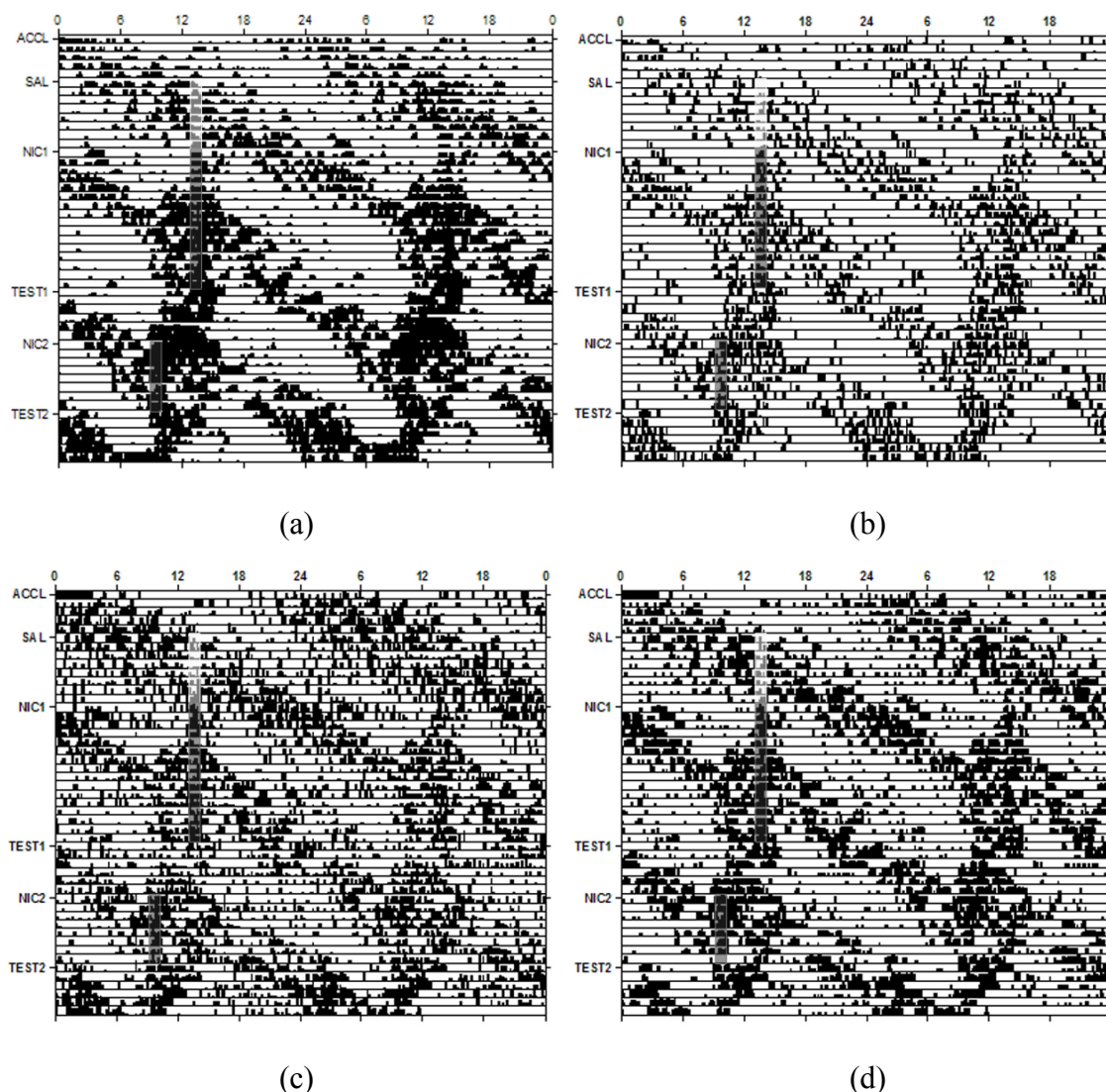
## Results

### Episodic Entrainment to 24-hour Injection Schedules

**Entrainment to nicotine zeitgeber.** Actograms of wheel running, water drinking, head entries into the feeders, and food pellet consumption for the Nicotine-No Treatment control group are depicted in Figure 1 for rats that received the nicotine zeitgeber. As in previous studies, nicotine-induced circadian activity episodes were independent of the free-running activity rhythms for all four activity measures. Period lengths of the activity cycles ( $\tau$ ) were approximately 24 hours for nicotine-induced episodes, whereas the free-running rhythms were approximately 25-26 hours in length.

As established in previous studies, drug-induced episodic entrainment requires that four major criteria be met. Repeated administration of a zeitgeber drug on a 24-hour schedule must induce (1) a pre-administration activity episode and (2) a post-administration episode, both of which are significantly higher than acclimation activity levels at the corresponding daily time periods. These significant pre- and post-drug episodes (3 & 4) must also persist for multiple days after the drug is withheld. As no treatment drug was administered to the No Treatment control groups, these rats received a total of six test days following each injection series in which the drug was withheld and the rats were not disturbed.

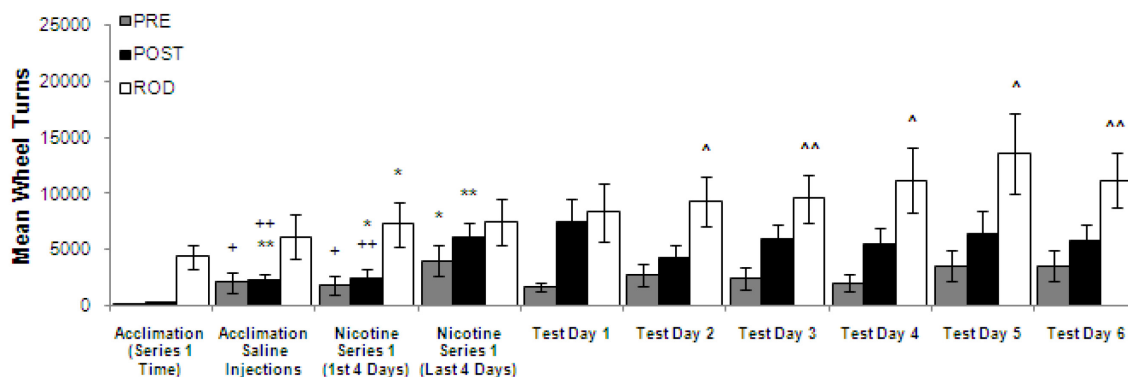
Repeated nicotine administration induced post-drug activity episodes that were higher than activity levels at the corresponding time of day during the acclimation phase. In Injection Series 1, wheel counts in the POST period (3 hours following the nicotine injection) were significantly higher than acclimation wheel counts,  $F(1, 7) = 18.769, p <$



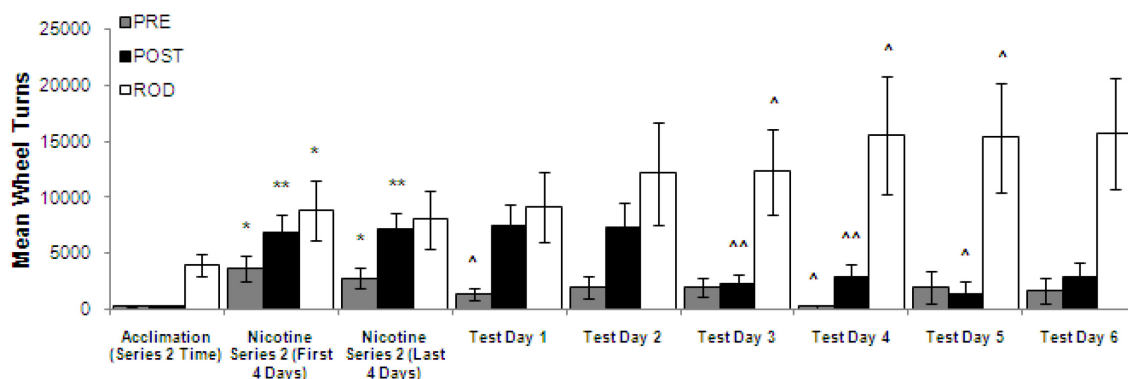
*Figure 1.* Double-plotted actograms of wheel turns (a), water bottle licks (b), head entries in the feeder (c), and food pellet consumption (d) depicted as black tick-marks for a representative rat (#2) from the Nicotine-No Treatment control group kept under constant dim light and rate-limited feeding. The y-axes are the days of the study with the first study day at the top of the graph, and the x-axes are the hours of the day starting at midnight (hour 0). Each horizontal line depicts two consecutive days, and the second day is repeated at the beginning of the next line. Rats in the Nicotine-No Treatment group received 5 days of acclimation (ACCL), 8 days of saline injections (SAL), and 2 separate nicotine injection series (NIC1 and NIC2), each followed by a 6-day test phase (TEST1 & TEST2) in which the rats were not disturbed. Subcutaneous nicotine injections are marked with transparent gray boxes on the left side of the actograms, and saline injections are marked with white boxes. For this group, the daily administration time was 1300 during Series 1 and 0900 during Series 2. The free-running (light-entrainable) activity rhythms are visible as diagonal bouts approximately 26 hours in length. The pre- and post-nicotine episodes are visible as vertical bouts before and after the nicotine administration times and persisting into the test phases.

.01 (Figure 2), as were water licks,  $F(1, 7) = 8.028, p < .05$  (Figure 3), head entries in the feeder,  $F(1, 7) = 9.685, p < .05$  (Figure 4), and pellet consumption,  $F(1, 7) = 68.136, p < .001$  (Figure 5). Post-nicotine episodes of wheel-running, water drinking, head entries, and pellet consumption were also significantly higher than post-saline episodes following the acclimation saline injections,  $F(1, 7) = 10.797 - 20.847, p < .05$ . On the test days when nicotine was withheld (Test 1), both the POST period wheel counts,  $F(1, 7) = .063 - 4.774, p = .065 - .810$ , and the head entries,  $F(1, 7) = .011 - 4.740, p = .066 - .918$ , were not significantly different from the POST period during Injection Series 1, and therefore these activity episodes are considered to have persisted for all six of the test days. In contrast, the water licks and pellet consumption persisted for only one test day,  $F(1, 7) = 2.010 - 4.502, p = .072 - .199$ , and were significantly lower than the injection series levels on the second and third test days,  $F(1, 7) = 7.431 - 37.370, p < .05$ . During Injection Series 2, all of the activity measures in the POST period were significantly higher than acclimation wheel counts,  $F(1, 7) = 19.855, p < .01$ , water licks,  $F(1, 7) = 32.286, p < .01$ , head entries,  $F(1, 7) = 6.802, p < .05$ , and pellet consumption,  $F(1, 7) = 26.985, p < .01$ . POST period wheel running, water licks, and pellet consumption each persisted for two test days,  $F(1, 7) = .018 - 2.001, p = .200 - .897$ , and head entries persisted for five test days,  $F(1, 7) = .199 - 3.453, p = .106 - .669$ .

Pre-nicotine episodes were not as consistent as the post-nicotine episodes. In Injection Series 1, PRE period wheel counts were significantly higher than both acclimation wheel counts,  $F(1, 7) = 7.902, p < .05$ , and acclimation saline injection wheel counts,  $F(1, 7) = 10.388, p < .05$ , at the corresponding time of day (Figure 2). These PRE period wheel counts also persisted for all six days of Test 1,  $F(1, 7) = .080 - 2.877, p =$

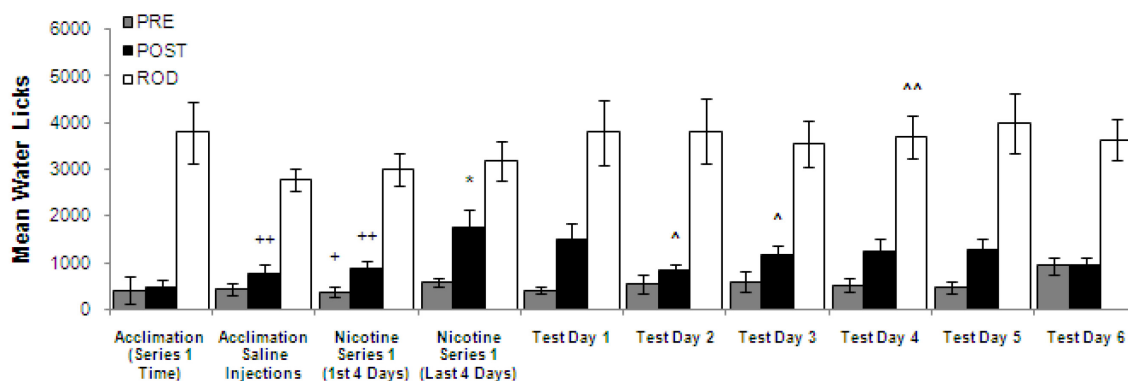


(a)

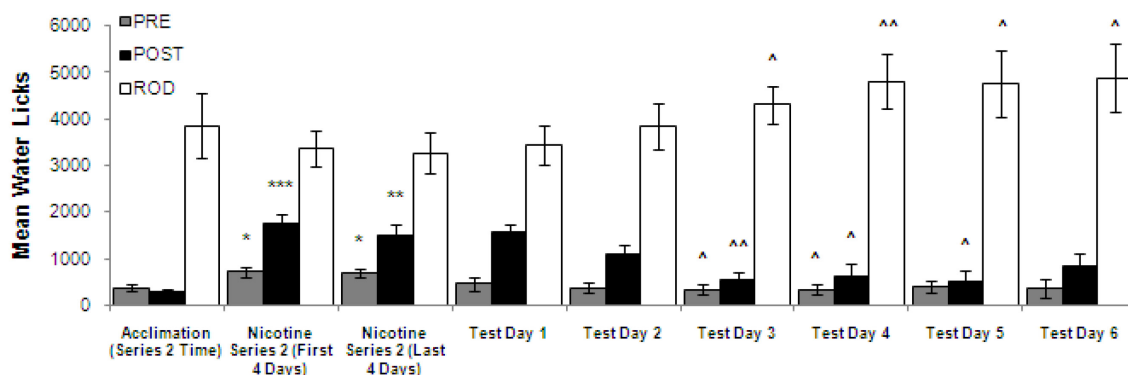


(b)

*Figure 2.* Mean wheel turns for Nicotine-No Treatment control group rats throughout the 50-day study. Rats received 5 days of acclimation followed by (a) 8 days of acclimation saline injections at 1300, 16 days of nicotine injections at 1300, 6 test days in which the rats were not disturbed, and (b) 8 days of nicotine injections at 0900 followed by 6 additional test days. The pre-drug period (PRE) is 22-24 hours following each nicotine injection. The post-drug (POST) period is 0-3 hours following each nicotine injection. The rest-of-day (ROD) period is 3-22 hours following each nicotine injection. Mean wheel turns that are significantly higher than acclimation wheel turns: \* $p < .05$ , \*\* $p < .01$ . Mean wheel turns that are significantly lower than the last 4 days of Nicotine Series 1: + $p < .05$ , ++ $p < .01$ . Mean wheel turns that are significantly different from the last 4 days of Nicotine Series 1: ^ $p < .05$ , ^^ $p < .01$ .

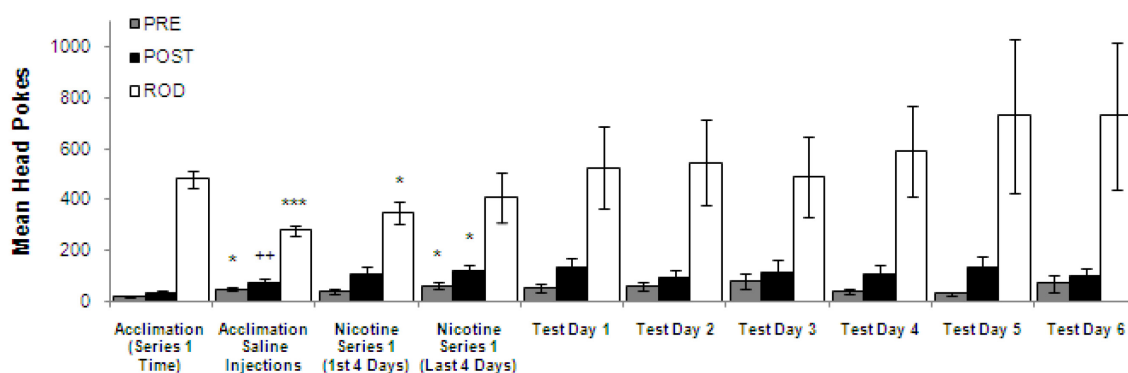


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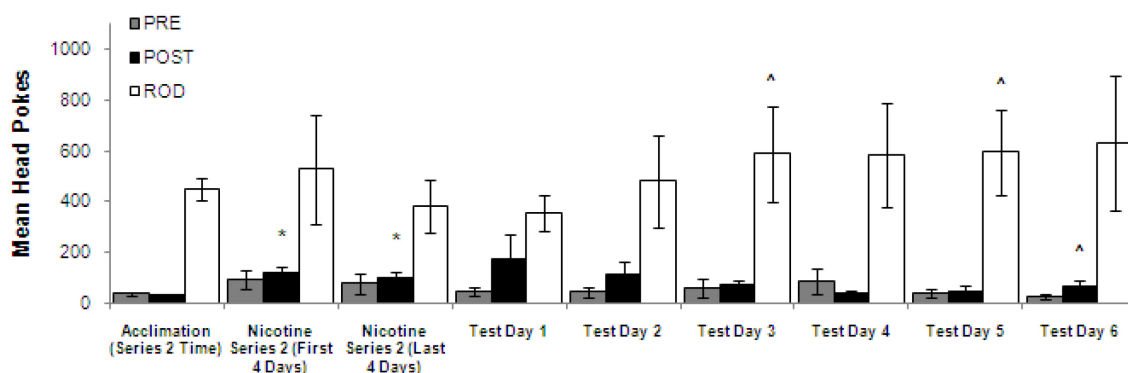


(b)

**Figure 3.** Mean water licks for Nicotine-No Treatment control group rats throughout the 50-day study. Rats received 5 days of acclimation followed by (a) 8 days of acclimation saline injections at 1300, 16 days of nicotine injections at 1300, 6 test days in which the rats were not disturbed, and (b) 8 days of nicotine injections at 0900 followed by 6 additional test days. The pre-drug period (PRE) is 22-24 hours following each nicotine injection. The post-drug (POST) period is 0-3 hours following each nicotine injection. The rest-of-day (ROD) period is 3-22 hours following each nicotine injection. Mean water licks that are significantly higher than acclimation water licks: \* $p < .05$ , \*\* $p < .01$ , \*\*\* $p < .001$ . Mean water licks that are significantly lower than the last 4 days of Nicotine Series 1: + $p < .05$ , ++ $p < .01$ . Mean water licks that are significantly different from the last 4 days of Nicotine Series 1: ^ $p < .05$ , ^^ $p < .01$ .

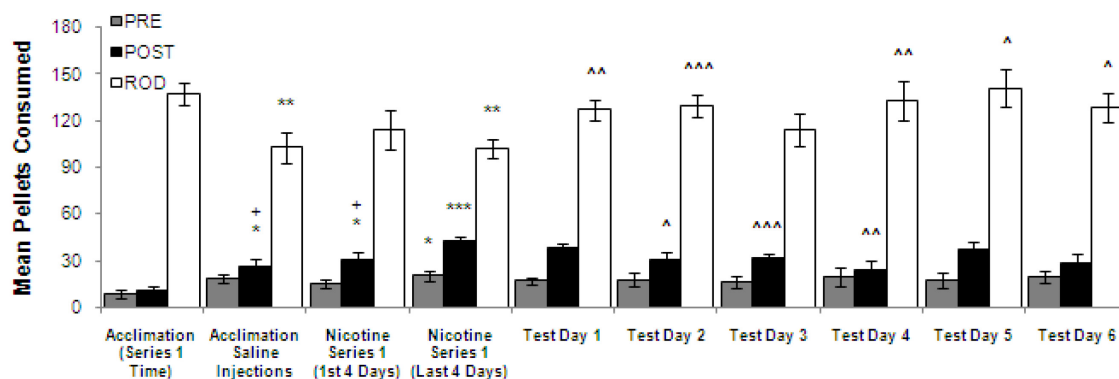


(a)

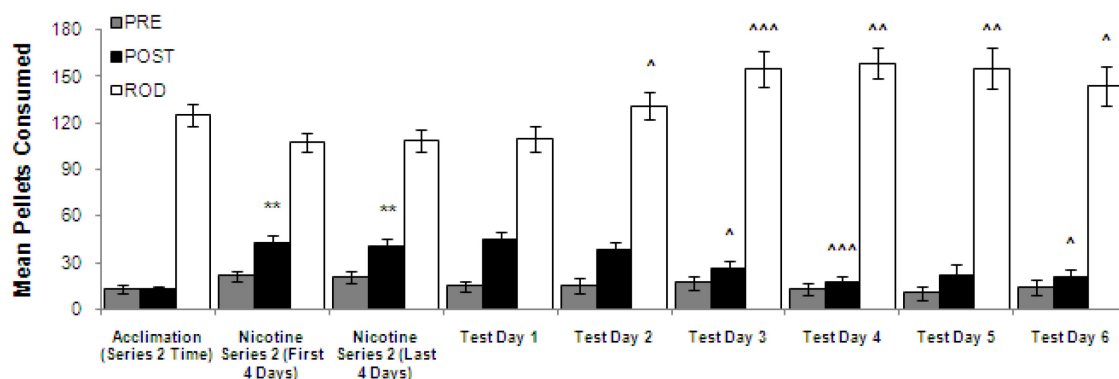


(b)

**Figure 4.** Mean head entries in the feeder for Nicotine-No Treatment control group rats throughout the 50-day study. Rats received 5 days of acclimation followed by (a) 8 days of acclimation saline injections at 1300, 16 days of nicotine injections at 1300, 6 test days in which the rats were not disturbed, and (b) 8 days of nicotine injections at 0900 followed by 6 additional test days. The pre-drug period (PRE) is 22-24 hours following each nicotine injection. The post-drug (POST) period is 0-3 hours following each nicotine injection. The rest-of-day (ROD) period is 3-22 hours following each nicotine injection. Mean head pokes that are significantly higher than acclimation head pokes: \* $p < .05$ , \*\*\* $p < .001$ . Mean head pokes that are significantly lower than the last 4 days of Nicotine Series 1: ++ $p < .01$ . Mean head pokes that are significantly different from the last 4 days of Nicotine Series 1: ^ $p < .05$ .



(a)



(b)

*Figure 5.* Mean food pellets consumed for Nicotine-No Treatment control group rats throughout the 50-day study. Rats received 5 days of acclimation followed by (a) 8 days of acclimation saline injections at 1300, 16 days of nicotine injections at 1300, 6 test days in which the rats were not disturbed, and (b) 8 days of nicotine injections at 0900 followed by 6 additional test days. The pre-drug period (PRE) is 22-24 hours following each nicotine injection. The post-drug (POST) period is 0-3 hours following each nicotine injection. The rest-of-day (ROD) period is 3-22 hours following each nicotine injection. Mean pellet consumption that is significantly higher than acclimation pellet consumption: \*  $p < .05$ , \*\*  $p < .01$ , \*\*\*  $p < .001$ . Mean pellet consumption that is significantly lower than the last 4 days of Nicotine Series 1: +  $p < .05$ . Mean pellet consumption that is significantly different from the last 4 days of Nicotine Series 1: ^  $p < .05$ , ^^  $p < .01$ , ^^^  $p < .001$ .



.134 - .786. PRE period wheel counts were also significantly higher than acclimation levels during Injection Series 2,  $F(1, 7) = 8.148, p < .05$ . Although PRE period wheel counts were significantly lower than injection series levels on Test Day 1,  $F(1, 7) = 9.300, p < .05$ , wheel counts were not significantly different from the injection series levels on Test Day 2,  $F(1, 7) = .691, p = .433$ , and on Test Day 3,  $F(1, 7) = .458, p = .520$ .

Nicotine administration did not reliably produce significant persisting pre-drug episodes of water drinking, head entries, or food pellet consumption. PRE period water licks (Figure 3) were not significantly different from acclimation levels in Injection Series 1,  $F(1, 7) = .262, p = .624$ , but were significantly higher in Injection Series 2,  $F(1, 7) = 10.426, p < .05$ . PRE period water licks persisted for two days in Test 2,  $F(1, 7) = 2.353 - 4.097, p = .083 - .169$ . In contrast, both head entries (Figure 4) and pellet consumption (Figure 5) were significantly higher than acclimation levels in the PRE period in Series 1,  $F(1, 7) = 6.181 - 6.426, p < .05$ , but not in Series 2,  $F(1, 7) = 1.052 - 2.449, p = .162 - .339$ . Both the head entries and the pellet consumption episodes persisted for all six days of Test 1,  $F(1, 7) = .005 - 2.153, p = .186 - .946$ .

Given the entrainment criteria listed above, wheel counts appeared to be the most reliable measure of episodic entrainment of activity. Although all activity measures produced significant and persisting pre- and post-nicotine episodes in either Series 1 or Series 2, only pre- and post-nicotine wheel counts were both significant and persisting in both Series 1 and Series 2. For this reason, the remaining results sections will focus on the wheel counts data, while the water- and food-directed activities will not be reported.

Although drug-induced activity episodes are usually restricted to the PRE and POST periods, activity counts in the remaining 19 hours of the day (Rest of Day or ROD) were summed and compared across the acclimation, injection series, and test phases for the No Treatment control groups. Repeated nicotine administration significantly increased ROD wheel counts in the first four days of both Injection Series 1 and Series 2 in comparison to acclimation levels,  $F(1, 7) = 6.719 - 7.097, p < .05$ , but ROD wheel counts were not significantly different from acclimation wheel levels at the end of the Injection Series,  $F(1, 7) = 3.817 - 5.116, p = .058 - .092$ . These results appear to indicate that repeated nicotine administration may initially stimulate wheel-running throughout the day (not just in the PRE and POST periods) at the beginning of the injection series, but became focused in the PRE and POST periods by the end of the injection series, possibly after the establishment of episodic entrainment.

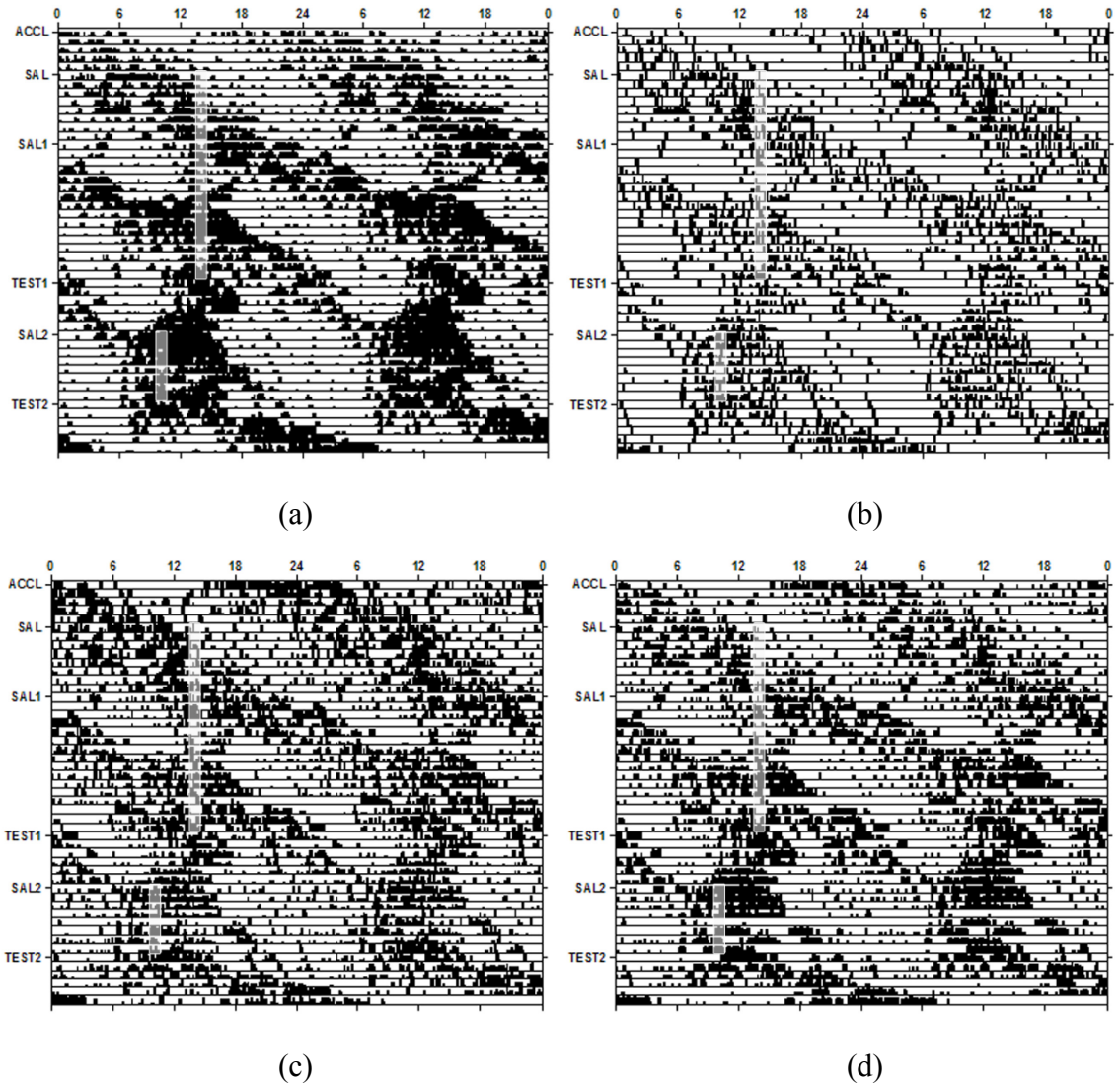
In contrast to the PRE and POST periods, ROD period wheel counts tended to increase across the test days as the PRE and POST period wheel counts decreased. During Test 1, ROD period wheel counts were significantly higher than wheel counts at the end of Injection Series 1 on Test Days 2 through 6,  $F(1, 7) = 6.693 - 32.621, p < .05$ . Similarly, during Test 2, ROD wheel counts were significantly higher than Injection Series 2 wheel counts on Test Days 3 through 5,  $F(1, 7) = 5.897 - 8.821, p < .05$ . These results appear to indicate that at the end of the Injection Series and the beginning of the Test Phases, nicotine's stimulatory effects on wheel running are concentrated in the PRE and POST periods. In contrast, ROD period wheel-running increases significantly once nicotine has been withheld for several days. This may occur because nicotine-entrained

wheel-running episodes start to free-run in the absence of the zeitgeber, although the actograms (Figure 5) do not appear to show this free-running during the Test Phases.

Finally, although persistence of nicotine-induced pre- and post-drug wheel-running episodes cannot be determined for the rats in the other eight treatment groups due to the administration of treatment injections, wheel counts for all 72 rats receiving nicotine were significantly higher in the PRE period when compared to acclimation levels during both Injection Series 1,  $F(1, 63) = 84.866, p < .001$ , and Injection Series 2,  $F(1, 63) = 69.359, p < .001$ . In addition, there was no significant (between-subjects) difference in PRE period wheel-running among the treatment groups,  $F(8, 63) = .601 - 1.604, p = .142 - .773$ .

POST period wheel counts were also significantly higher than acclimation levels in both Injection Series 1,  $F(1, 63) = 163.102, p < .001$ , and Series 2,  $F(1, 63) = 181.872, p < .001$ . POST period wheel-running was not significantly different among the treatment groups in Series 1,  $F(8, 63) = 1.587, p = .147$ , but there was a significant between-subjects effect of treatment in Series 2,  $F(8, 63) = 2.224, p < .05$ . The Naltrexone, SB-334867, and Acamprosate treatment groups (in that order) had the lowest POST period wheel counts in Series 2, while the No Treatment, Topiramate, Bupropion, and Mecamylamine groups (in that order) had the highest Series 2 POST period wheel counts.

**Entrainment to saline zeitgeber.** Previous studies have demonstrated that repetitive saline administration induces weak (but significant) pre- and post-drug activity episodes that persist for multiple days when saline is withheld (Timberlake, et al., 2009). Actograms of wheel-running, water drinking, head entries in the feeders, and food pellet

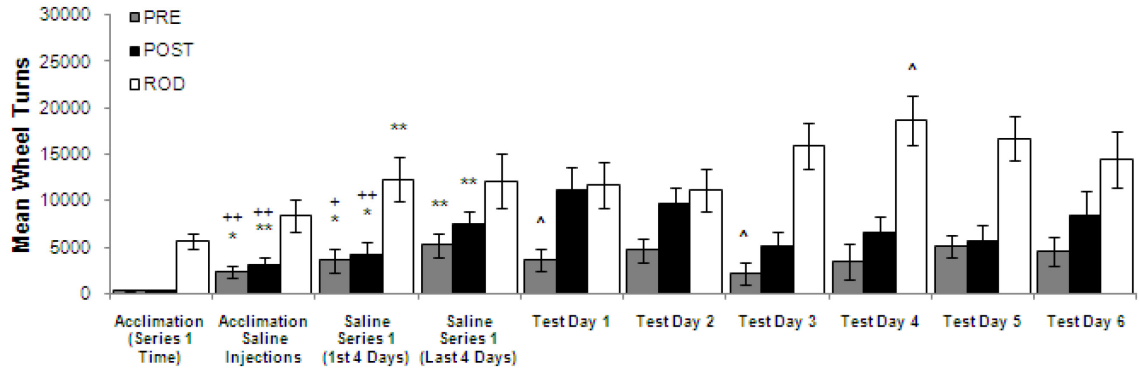


*Figure 6.* Double-plotted actograms of wheel turns (a), water bottle licks (b), head entries in the feeder (c), and food pellet consumption (d) depicted as black tick-marks for a representative rat (#16) from the Saline-No Treatment control group kept under constant dim light and rate-limited feeding. The y-axes are the days of the study with the first study day at the top of the graph, and the x-axes are the hours of the day starting at midnight (hour 0). Each horizontal line depicts two consecutive days, and the second day is repeated at the beginning of the next line. Rats in the Saline-No Treatment group received 5 days of acclimation (ACCL), 2 separate saline injection series (SAL/SAL1 and SAL2), each followed by a 6-day test phase (TEST1 & TEST2) in which the rats were not disturbed. Subcutaneous saline injections are marked with white boxes. The free-running (light-entrainable) activity rhythms are visible as diagonal bouts approximately 26 hours in length. The pre- and post-saline episodes are visible as vertical bouts before and after the administration times and persisting into the test phases.

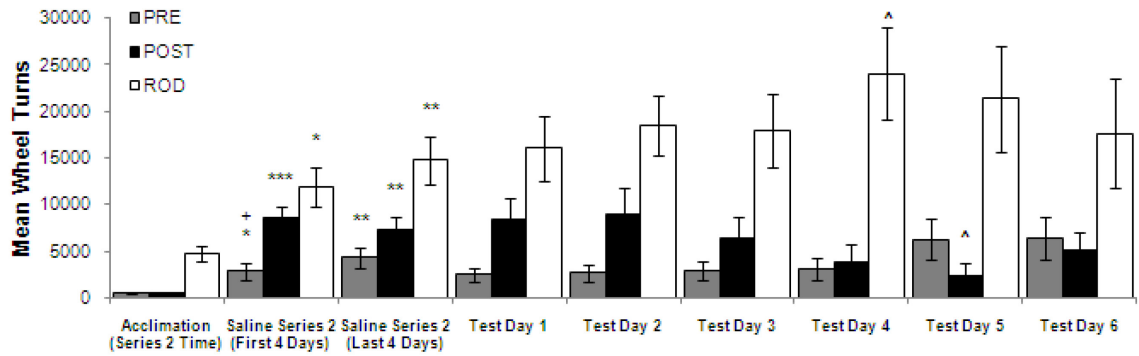
consumption for the Saline-No Treatment control group are depicted in Figure 6. As with the nicotine-induced circadian activity episodes, saline-induced episodes had period lengths ( $\tau$ ) of approximately 24 hours and were independent of the free-running activity rhythms that were approximately 25-26 hours in length.

Post-saline wheel-running episodes were significantly higher than acclimation wheel counts during the acclimation saline injections,  $F(1, 7) = 14.312, p < .01$ , and throughout both Saline Series 1 and Series 2,  $F(1, 7) = 9.405 - 39.759, p < .05$  (Figure 11). Repeated saline administration also appeared to induce locomotor sensitization during Saline Series 1, as POST period wheel counts during the last 4 days of Series 1 were significantly higher than both the first 4 days of Series 1,  $F(1, 7) = 21.223, p < .01$ , and the acclimation saline injections,  $F(1, 7) = 21.039, p < .01$ . Post-saline episodes persisted for all six days of Test Phase 1,  $F(1, 7) = .163 - 4.218, p = .079 - .698$ , and for all of Test Phase 2,  $F(1, 7) = .343 - 1.987, p = .202 - .576$ , with the exception of Test Day 5, which had significantly lower wheel counts than the end of Saline Series 2,  $F(1, 7) = 5.750, p < .05$ .

Pre-saline wheel-running activity was also significantly higher than acclimation wheel counts throughout the acclimation saline injections and both saline injection series,  $F(1, 7) = 6.665 - 15.766, p < .05$  (Figure 7). Pre-saline activity also increased as injections were repeatedly administered, as PRE period wheel counts for the acclimation saline injections were significantly lower than PRE period wheel counts during the last four days of Saline Series 1,  $F(1, 7) = 14.158, p < .01$ , and the first four days of both saline injection series were significantly lower than the last four days of the respective series,  $F(1, 7) = 12.162 - 13.440, p < .05$ . However, pre-saline wheel-running episodes



(a)



(b)

**Figure 7.** Mean wheel turns for Saline-No Treatment control group rats throughout the 50-day study. Rats received 5 days of acclimation followed by (a) 8 days of acclimation saline injections at 1300, 16 days of saline injections at 1300, 6 test days in which the rats were not disturbed, and (b) 8 days of saline injections at 0900 followed by 6 additional test days. The pre-drug period (PRE) is 22-24 hours following each saline injection. The post-drug (POST) period is 0-3 hours following each saline injection. The rest-of-day (ROD) period is 3-22 hours following each saline injection. Mean wheel turns that are significantly higher than acclimation wheel turns:  $^*p<.05$ ,  $^{**}p<.01$ ,  $^{***}p<.001$ . Mean wheel turns that are significantly lower than the last 4 days of Saline Series 1:  $^+p<.05$ ,  $^{++}p<.01$ . Mean wheel turns that are significantly different from the last 4 days of Saline Series 1:  $^{\wedge}p<.05$ .

did not persist as consistently as post-saline episodes. PRE period wheel counts were significantly lower on Test Days 1 and 3 when compared to the last 4 days of Saline Series 1,  $F(1, 7) = 6.096 - 6.750, p < .05$ , although the remaining test days were not significantly different from the last 4 days of the series,  $F(1, 7) = .025 - .865, p = .383 - .878$ . Pre-saline episodes persisted throughout Test Phase 2, as PRE period wheel counts on all six test days were not significantly different from the last 4 days of Saline Series 2,  $F(1, 7) = .565 - 4.297, p = .077 - .477$ .

Repeated saline injections had a similar effect on wheel-running activity in the remaining 19 hours of the day (ROD period) as repeated nicotine injections (Figure 11). ROD period wheel-running activity increased relative to the acclimation wheel counts, but was only significantly higher than acclimation levels during the first four days of Saline Series 1,  $F(1, 7) = 13.003, p < .01$ , and not during the acclimation saline injections,  $F(1, 7) = 4.657, p = .068$ , or the last four days of Saline Series 1,  $F(1, 7) = 5.030, p = .060$ . Over time, repeated saline injections did appear to increase total daily activity, as ROD period wheel-running activity throughout Saline Series 2 was significantly higher than acclimation wheel counts,  $F(1, 7) = 10.738 - 13.193, p < .05$ . ROD period activity for the Saline-No Treatment rats did not change significantly during most of the Test Days,  $F(1, 7) = .041 - 5.573, p = .050 - .845$ , with the exception of Test Day 4 in both Test Phase 1 and Test Phase 2, both of which had significantly higher wheel counts than the last four days of the respective Saline Injection Series,  $F(1, 7) = 5.810 - 6.954, p < .05$ .

Like the Saline-No Treatment control group, the remaining 64 rats that received the other eight treatment conditions also had significantly higher pre-saline wheel-

running episodes in Saline Series 1 than at the corresponding times during the acclimation phase,  $F(1, 63) = 47.713, p < .001$ . For the five treatment groups that received a second Saline Injection Series (No Treatment, Saline Treatment, Varenicline, Mecamylamine, and Bupropion), pre-saline wheel-running was also significantly higher than acclimation levels at the corresponding time of day,  $F(1, 35) = 31.978, p < .001$ . There was a significant between-subjects effect of the treatment groups on wheel-running in Saline Series 1,  $F(8, 63) = 4.015, p < .01$ , with the Acamprosate, Mecamylamine, and No Treatment groups having the highest levels of pre-saline wheel running.

Post-saline episodes were also significantly higher than acclimation for all 72 rats that received the saline zeitgeber in Saline Series 1,  $F(1, 63) = 101.804, p < .001$ , and Saline Series 2 for the 5 treatment groups that received a second series,  $F(1, 63) = 54.674, p < .001$ . As with the pre-saline episodes, there was a significant between-subjects effect of treatment group,  $F(8, 63) = 3.261, p < .01$ , with the Mecamylamine, Acamprosate, Bupropion, and No Treatment groups having higher overall wheel counts than the other five treatment groups.

**Summary of entrainment results.** In this paradigm, repeated nicotine administration entrained robust pre- and post-drug activity episodes to the daily administration time that persisted for approximately 3 days after nicotine was withheld. Saline administration entrained robust post-drug episodes, but weaker pre-drug episodes that did not persist after the first injection series. While water drinking, head entries in the feeder, and food consumption showed similar patterns of entrained episodes, wheel counts were consistently the most reliable measure of circadian entrainment to the drug injection times. Therefore, analysis of wheel-running data should provide the most useful



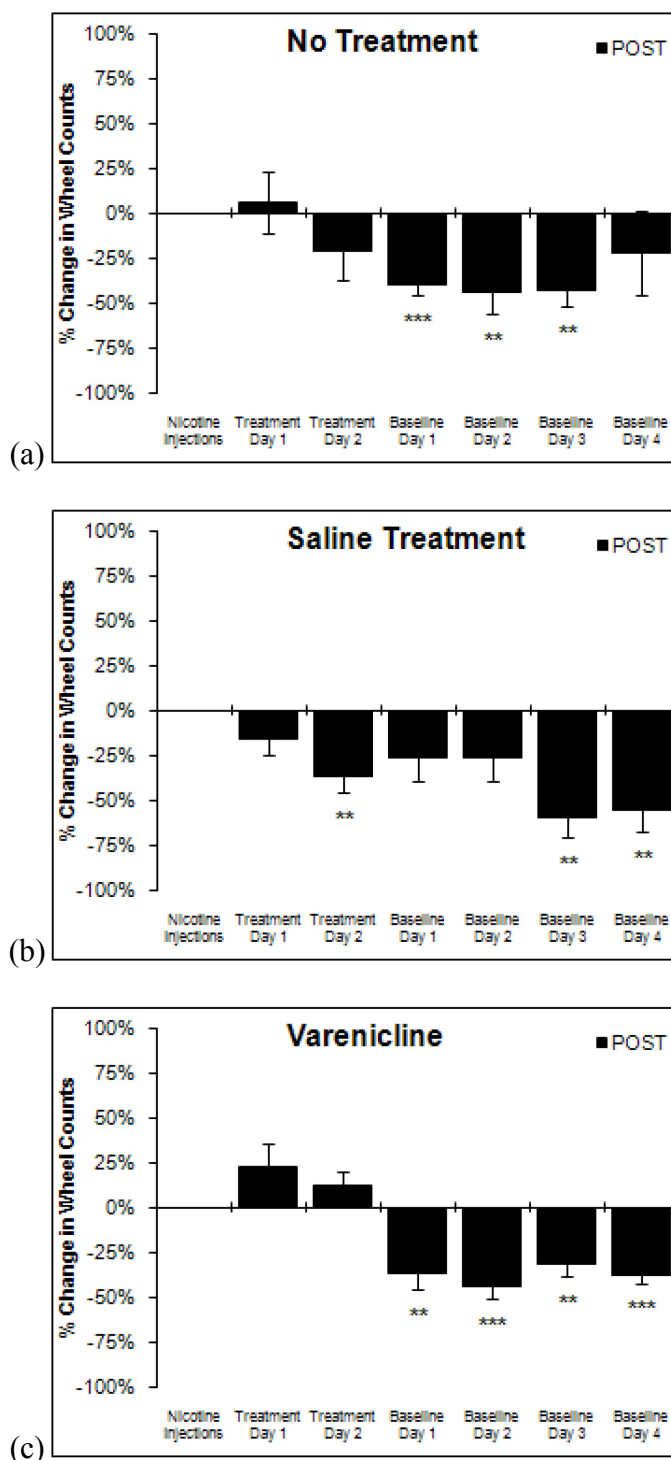
indicator of the effects of the pharmacological treatments on nicotine-induced pre- and post-drug circadian episodes.

### **Effects of Treatments on Entrained Episodes**

A total of nine treatment conditions were administered to rats that received nicotine or saline zeitgeber injections during Series 1 and Series 2. The treatments were administered on two consecutive days that immediately followed the injection series, and treatment injections were given at the same time that the zeitgeber injections had been administered in the preceding injection series. The control treatment conditions were no treatment (*i.e.*, the rats were not disturbed) and saline injections. The remaining seven treatments were injections of varenicline, mecamylamine, acamprosate, topiramate, naltrexone, SB-334867, or bupropion.

Wheel-running data were analyzed in both the PRE and the POST periods during the treatment and baseline days to assess the change in circadian activity induced by the treatments and whether the persistence of pre- and post-drug activity episodes was interrupted by the treatments. Additionally, Injection Series 1 and 2 were compared to examine whether the initial treatments had an effect on the reacquisition of pre- and post-drug activity episodes in Series 2.

**Post-nicotine activity episodes.** The effects of the nine treatments on wheel-running activity are depicted in Figure 8. As stated above, when nicotine injections are withheld, post-nicotine activity episodes tend to maintain similar magnitudes for multiple days. When no treatment was given during the two treatment days, POST period wheel-running activity was not significantly different from the wheel turns during the nicotine injection series (Figure 8a),  $F(1, 7) = .126 - 1.936, p = .207 - .733$ . On the four baseline



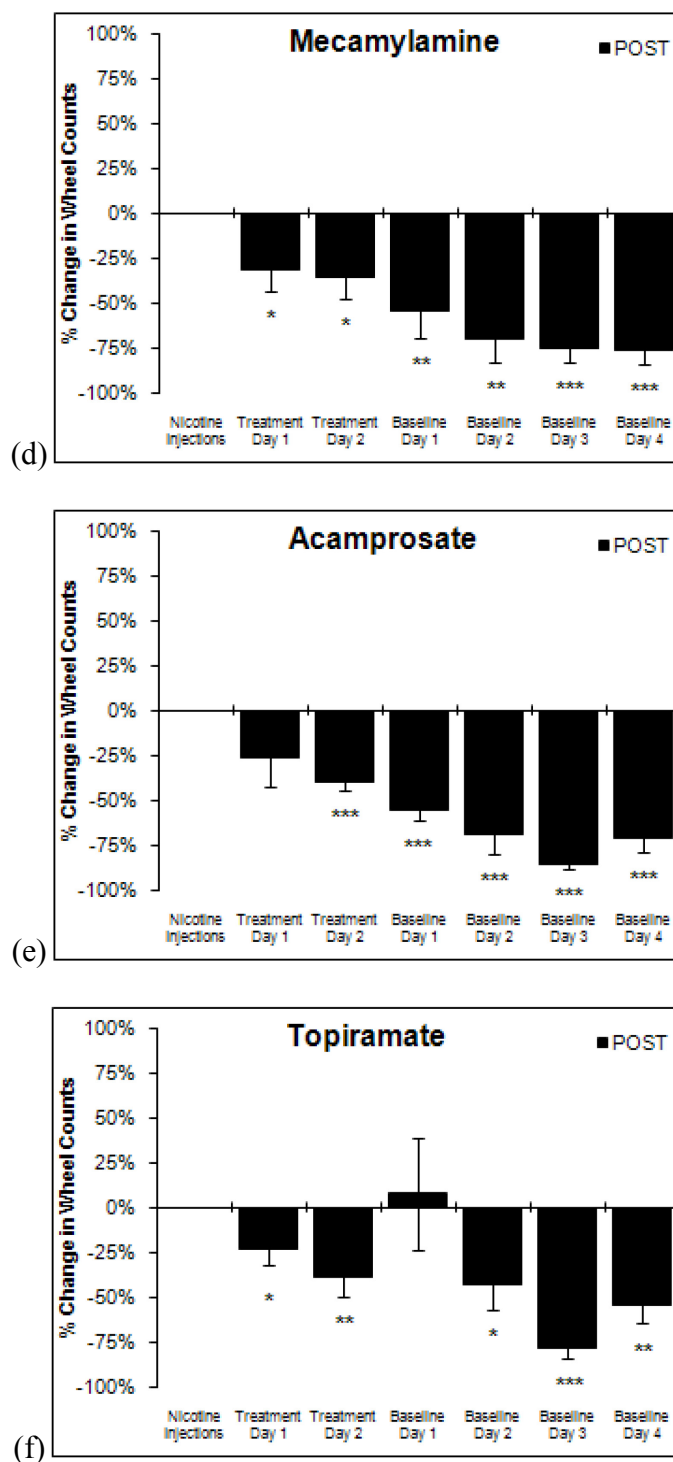
*Figure 8.* Percent change in wheel counts (± standard error) for the POST period (0-3 hours following each injection) during the Treatment and Baseline Days compared to the Nicotine Injection Series. Data are the average of Series 1 and 2. Treatments administered are labeled at the top of the individual graphs. Percent changes in wheel-running that are significantly different from zero: \* $p < .05$ , \*\* $p < .01$ , \*\*\* $p < .001$ .

days that followed the treatment days, POST period wheel running was reduced, but this reduction was only significant on Baseline Days 1-3,  $F(1, 7) = 14.380 - 50.991, p < .01$ , and not on Baseline Day 4,  $F(1, 7) = .898, p = .375$ .

Saline treatment appeared to maintain post-nicotine wheel running for slightly longer than no treatment (Figure 8b). On the treatment days, POST period activity was lower than the nicotine injection baseline, although only Treatment Day 2 was significantly lower,  $F(1, 7) = 17.684, p < .01$ , while Treatment Day 1 was not significantly different from the nicotine baseline,  $F(1, 7) = 3.197, p = .117$ . On the baseline days following the saline treatment, POST period wheel turns were not significantly lower than the nicotine injection levels on Baseline Days 1 and 2,  $F(1, 7) = 4.024 - 4.153, p = .081 - .085$ , and were significantly lower than the nicotine levels on Baseline Days 3 and 4,  $F(1, 7) = 21.723 - 32.695, p < .01$ .

The acetylcholine receptor-targeted treatments had different effects on post-nicotine wheel-running on the treatment days, but similar effects on the baseline days. Varenicline, a weak acetylcholine receptor agonist, increased wheel running during the POST period on the treatment days, but this was not a significant increase,  $F(1, 7) = 2.171 - 2.909, p = .132 - .184$  (Figure 8c). In contrast, mecamylamine, an acetylcholine receptor antagonist, significantly reduced POST period wheel running on the two treatment days,  $F(1, 7) = 6.609 - 9.592, p < .05$  (Figure 8d). Both varenicline and mecamylamine significantly reduced wheel running during the four baseline days,  $F(1, 7) = 14.831 - 117.382, p < .01$ .

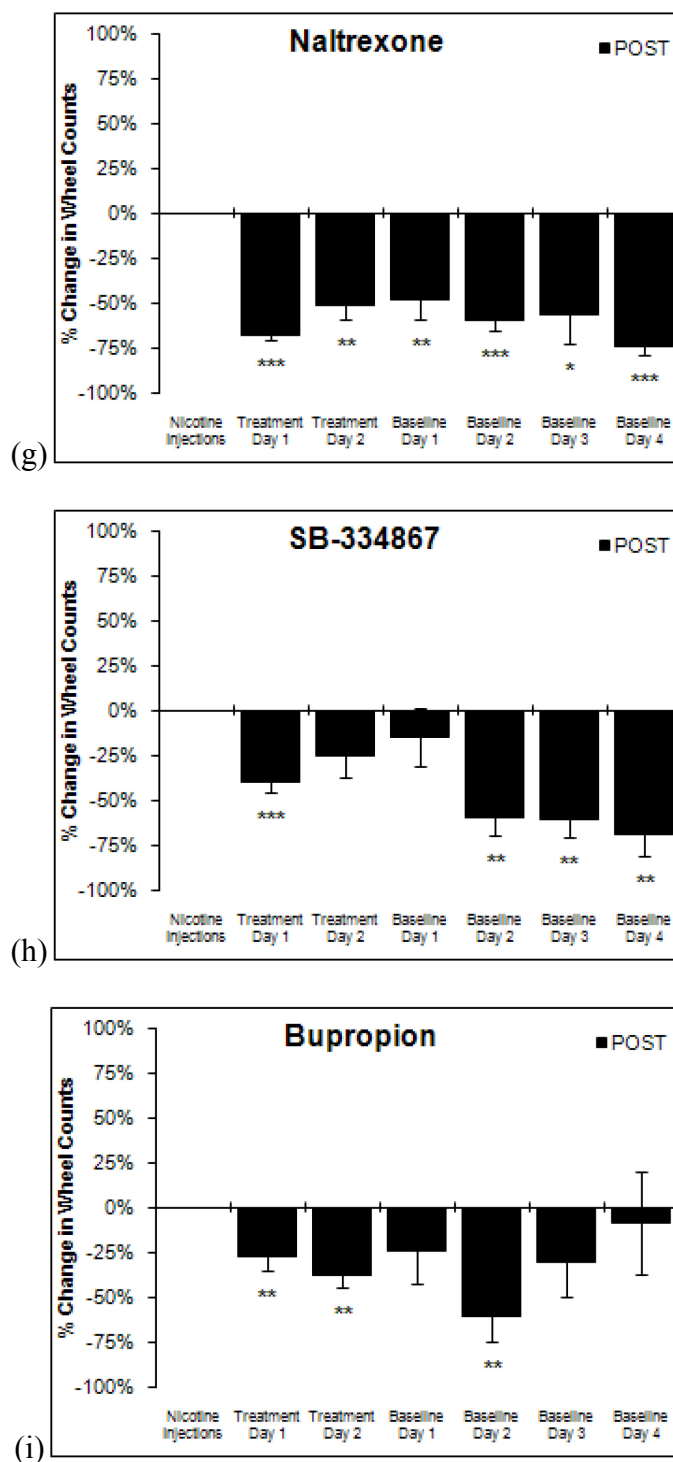
The glutamate receptor-targeted treatments both generally reduced post-nicotine activity. Acamprosate, an NDMA receptor antagonist, reduced POST period wheel



*Figure 8 (cont.).* Percent change in wheel counts ( $\pm$  standard error) for the PRE period (22-24 hours following each injection) during the Treatment and Baseline Days compared to the Nicotine Injection Series. Data are the average of Series 1 and 2. Treatments administered are labeled at the top of the individual graphs. Percent changes in wheel-running that are significantly different from zero: \* $p < .05$ , \*\* $p < .01$ , \*\*\* $p < .001$ .

running on both treatment days, although only Treatment Day 2 had a significant reduction in wheel-running,  $F(1, 7) = 60.253, p < .001$ ; Treatment Day 1 was not significantly different from the nicotine injection series levels,  $F(1, 7) = 2.559, p = .154$  (Figure 8e). Topiramate, an AMPA receptor antagonist, significantly reduced post-nicotine wheel-running on both treatment days,  $F(1, 7) = 7.136 - 14.682, p < .05$  (Figure 8f). Both acamprosate and topiramate significantly reduced wheel running throughout the baseline phase,  $F(1, 7) = 9.640 - 736.536 = p < .05$ , with the exception of topiramate on Baseline Day 1, in which wheel turns were not significantly different from the nicotine series levels,  $F(1, 7) = .061, p = .813$ .

The remaining treatments targeted opioid receptors (naltrexone), orexin receptors (SB-334867), and dopamine reuptake transporters (bupropion). Naltrexone treatment induced a strong reduction in POST period activity on both treatment days,  $F(1, 6) = 43.280 - 543.629, p < .01$ , and on all four baseline days,  $F(1, 6) = 12.281 - 276.714, p < .05$  (Figure 8g). SB-334867 treatment significantly reduced wheel counts in the POST period on Treatment Day 1,  $F(1, 7) = 45.073, p < .001$ , but not on Treatment Day 2,  $F(1, 7) = 4.466, p = .072$  (Figure 8h). On the baseline days following SB-334867 treatment, wheel turns were not significantly different from the nicotine series levels on Baseline Day 1,  $F(1, 7) = .834, p = .391$ , but were significantly lower on the remaining baseline days,  $F(1, 7) = 33.057 - 36.308, p < .01$ . Bupropion treatment significantly reduced POST period wheel-running on both treatment days,  $F(1, 7) = 12.580 - 26.746, p < .01$ , but wheel turns on the following baseline days were not significantly lower than the nicotine series levels,  $F(1, 7) = .092 - 2.792, p = .139 - .770$ , with the exception of

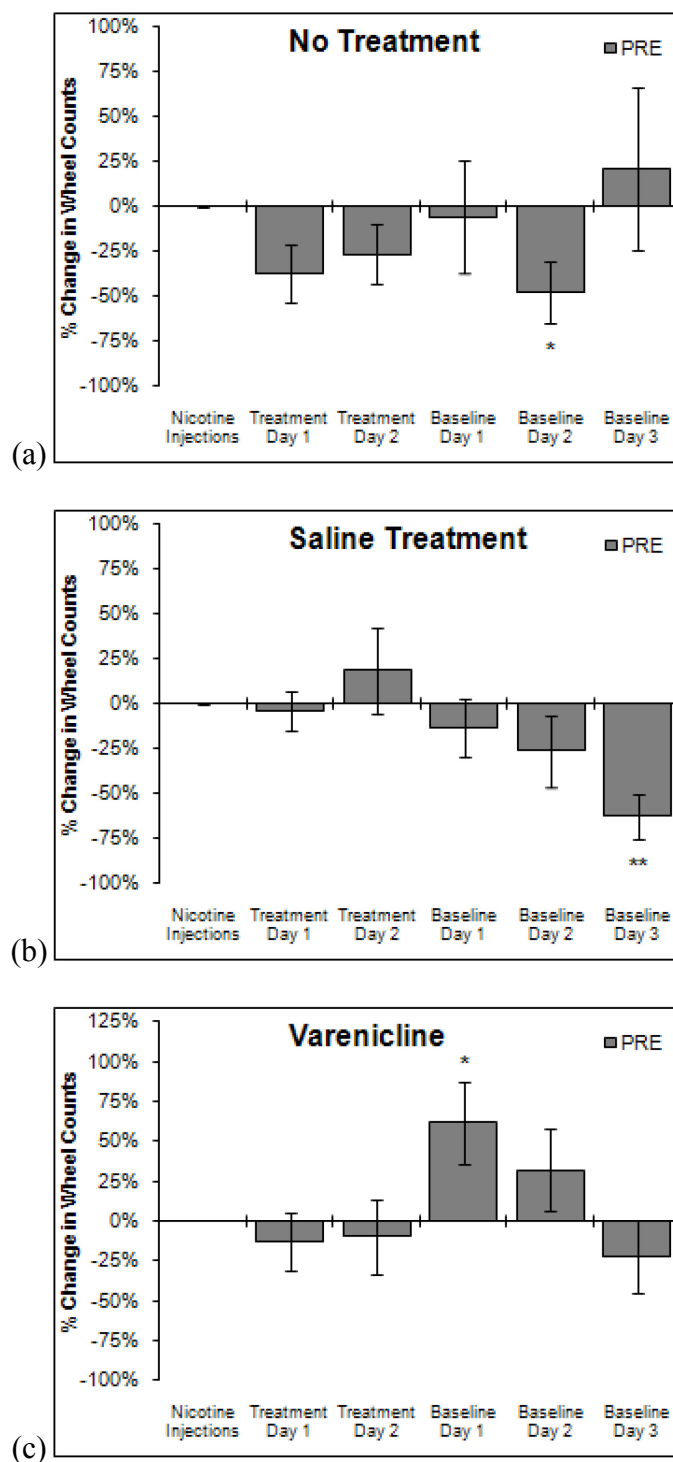


*Figure 8 (cont.).* Percent change in wheel counts ( $\pm$  standard error) for the PRE period (22-24 hours following each injection) during the Treatment and Baseline Days compared to the Nicotine Injection Series. Data are the average of Series 1 and 2. Treatments administered are labeled at the top of the individual graphs. Percent changes in wheel-running that are significantly different from zero: \* $p < .05$ , \*\* $p < .01$ , \*\*\* $p < .001$ .

Baseline Day 2, which had significantly lower wheel turns,  $F(1, 7) = 19.449, p < .01$  (Figure 12i).

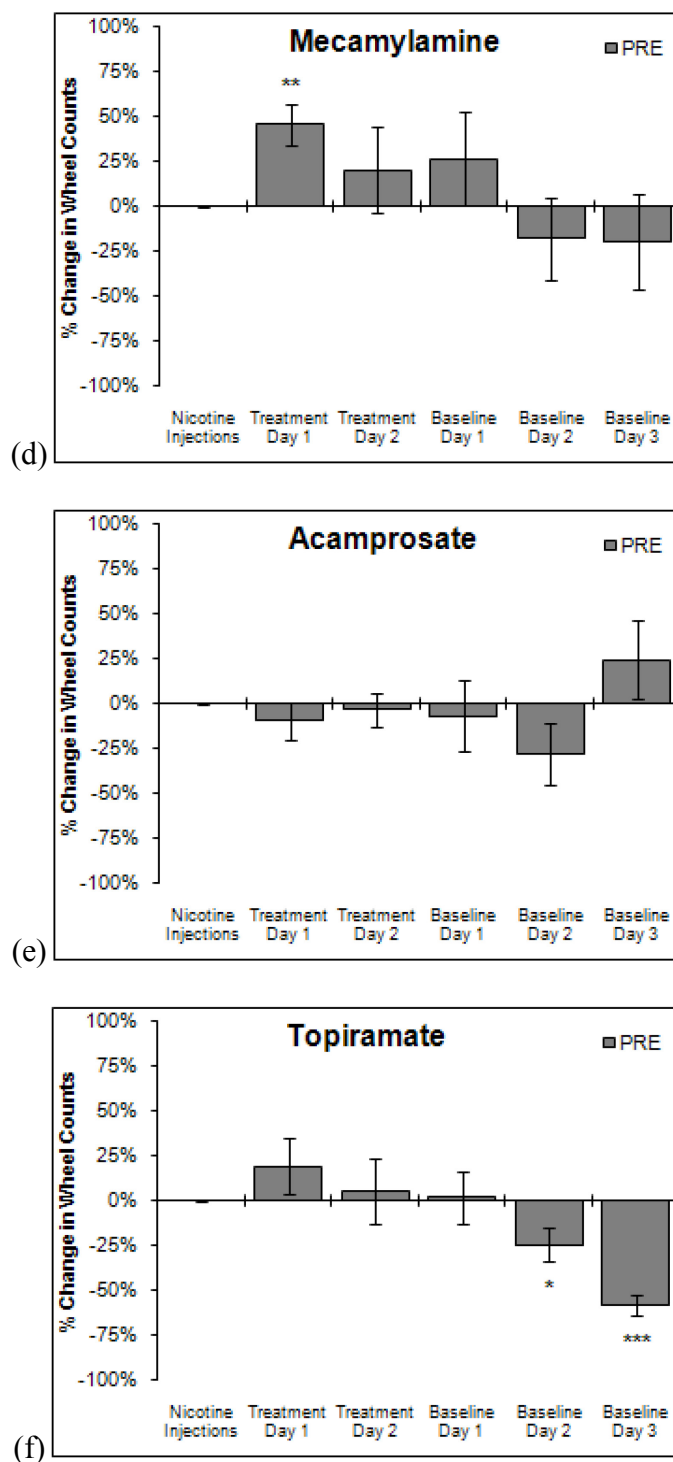
**Pre-nicotine activity episodes.** Although all of treatments and controls produced a general reduction in post-nicotine wheel-running, most treatments had very different effects on pre-nicotine activity episodes (Figure 9). When no treatment was given, PRE period wheel turns showed a slight decline across the treatment and baseline days. Except for the PRE period following Baseline Day 2,  $F(1, 7) = 7.681, p < .05$ , this decline was not significant,  $F(1, 7) = .033 - 5.386, p = .053 - .861$  (Figure 9a). Saline treatment maintained pre-nicotine wheel-running following the two treatment days, as PRE period wheel turns were not significantly different from the nicotine injection series levels,  $F(1, 7) = .133 - .624, p = .455 - .726$  (Figure 9b). Saline treatment also maintained pre-nicotine wheel turns for the first two baseline days,  $F(1, 7) = .635 - 1.750, p = .227 - .452$ , but PRE period wheel turns were significantly lower than the nicotine series levels following Baseline Day 3,  $F(1, 7) = 24.998, p < .01$ .

Varenicline and mecamylamine treatments had relatively opposite effects on pre-nicotine activity episodes. Varenicline maintained pre-nicotine wheel turns following the two treatment days,  $F(1, 7) = .191 - .529, p = .491 - .675$  (Figure 9c), while mecamylamine significantly increased PRE period wheel turns following the first treatment day,  $F(1, 7) = 15.534, p < .01$ , and induced a smaller (not significant) increase in wheel turns following the second day,  $F(1, 7) = .712, p = .427$  (Figure 9d). Following the first baseline day, rats that received varenicline treatment showed a significant increase in PRE period wheel turns,  $F(1, 7) = 5.755, p < .05$ , and PRE period wheel turns were not significantly different from the nicotine series levels for the remainder of the



*Figure 9.* Percent change in wheel counts ( $\pm$  standard error) for the PRE period (22-24 hours following each injection) during the Treatment and Baseline Days compared to the Nicotine Injection Series. Data are the average of Series 1 and 2. Treatments administered are labeled at the top of the individual graphs. Percent changes in wheel-running that are significantly different from zero: \* $p < .05$ , \*\* $p < .01$ , \*\*\* $p < .001$ .



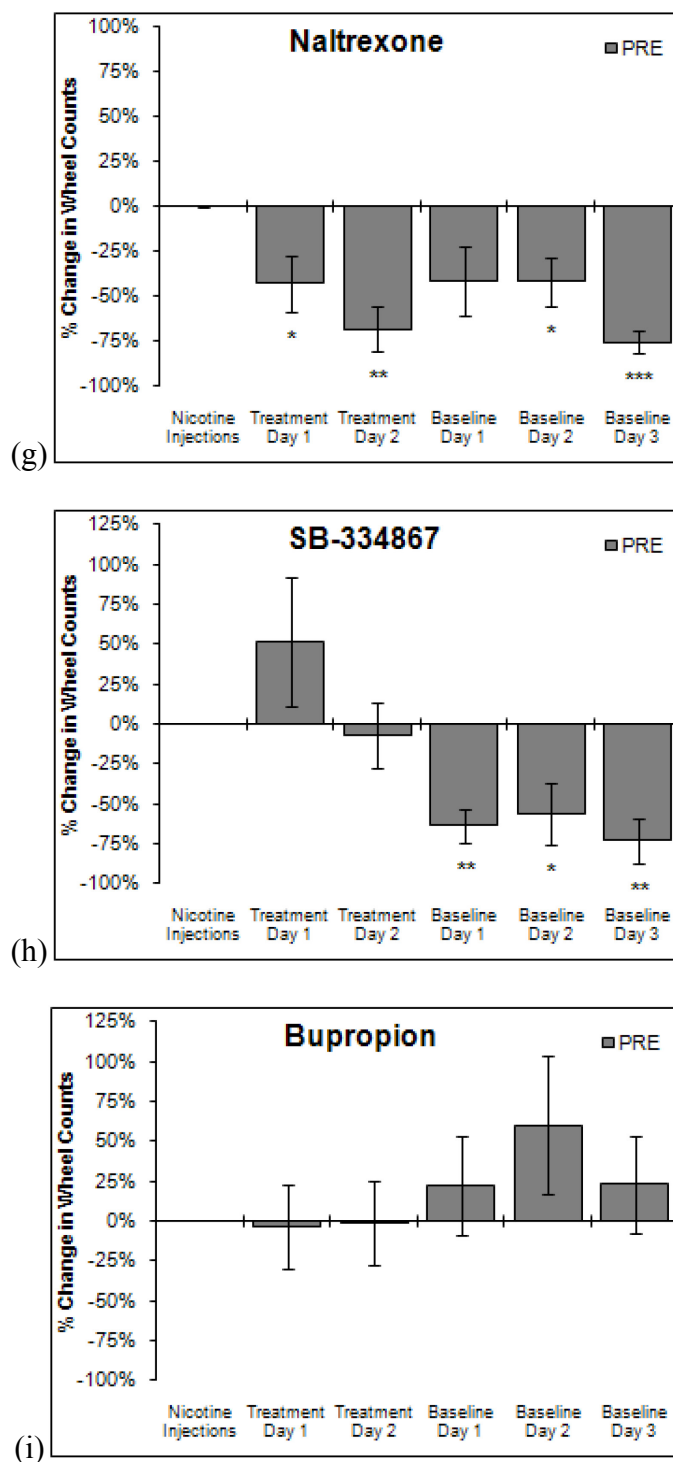


*Figure 9 (cont.).* Percent change in wheel counts ( $\pm$  standard error) for the PRE period (22-24 hours following each injection) during the Treatment and Baseline Days compared to the Nicotine Injection Series. Data are the average of Series 1 and 2. Treatments administered are labeled at the top of the individual graphs. Percent changes in wheel-running that are significantly different from zero: \* $p < .05$ , \*\* $p < .01$ , \*\*\* $p < .001$ .

baseline days,  $F(1, 7) = .935 - 1.529, p = .256 - .366$ . In contrast, rats that received mecamlamine treatment showed a gradual decline in PRE period wheel-running throughout the baseline phase, although wheel turns on these days were not significantly different from the nicotine series levels,  $F(1, 7) = .527 - 1.023, p = .345 - .491$ .

Acamprosate treatment maintained PRE period wheel turns throughout the treatment and baseline days, as wheel turns on these days were never significantly different from the nicotine injection series levels,  $F(1, 7) = .112 - 2.542, p = .155 - .748$  (Figure 9e). Like acamprosate, topiramate treatment maintained PRE period wheel-running following the two treatment days,  $F(1, 7) = .083 - 1.539, p = .255 - .781$  (Figure 9f). However, PRE period wheel turns showed a strong decline across the baseline days, being not significantly different from nicotine series levels following the first baseline day,  $F(1, 7) = .018, p = .896$ , and significantly lower than nicotine series levels on the second and third baseline days,  $F(1, 7) = 7.140 - 95.751, p < .05$ .

Of all of the treatments, naltrexone induced the strongest overall reduction in pre-nicotine wheel-running (Figure 9g). PRE period wheel turns were significantly lower than the nicotine series levels following both naltrexone treatment days,  $F(1, 6) = 7.475 - 28.337, p < .05$ , and were also significantly lower throughout the baseline days,  $F(1, 6) = 9.990 - 145.232, p < .05$ , with the exception of the PRE period following the first baseline day,  $F(1, 6) = 4.585, p = .076$ . Treatment with SB-334867 induced an increase in PRE period activity following the first treatment day and a slight decrease following the second treatment day, although these changes were not significantly different from the nicotine series wheel-running levels,  $F(1, 7) = .121 - 1.644, p = .241 - .738$  (Figure



*Figure 9 (cont.).* Percent change in wheel counts ( $\pm$  standard error) for the PRE period (22-24 hours following each injection) during the Treatment and Baseline Days compared to the Nicotine Injection Series. Data are the average of Series 1 and 2. Treatments administered are labeled at the top of the individual graphs. Percent changes in wheel-running that are significantly different from zero: \* $p < .05$ , \*\* $p < .01$ , \*\*\* $p < .001$ .

9h). However, SB-334867 treatment induced the most robust decline in PRE period activity during the baseline phase, as rats that received SB-334867 treatment were the only group to show significantly lower PRE period wheel turns following all baseline days,  $F(1, 7) = 8.692 - 35.321, p < .05$ . Finally, bupropion treatment maintained PRE period wheel turns following both treatment days,  $F(1, 7) = .002 - .020, p = .892 - .969$ , and induced a slight increase in PRE period activity across the baseline days, although this increase was not significant,  $F(1, 7) = .516 - 1.939, p = .206 - .496$  (Figure 9i).

**Reacquisition of pre- and post-nicotine episodes.** In most cases, pre- and post-nicotine episodes showed a general trend of increasing wheel-running activity counts between Nicotine Injection Series 1 and Series 2 (Figure 10). Wheel counts in the PRE and POST periods of these two injection series were compared to examine whether the administration of the treatments interfered with the reacquisition of nicotine-induced activity episodes in Series 2 after the first Treatment Phase that followed Series 1. When no treatment was administered, PRE period wheel counts were significantly higher in the first 4 days of Series 2 than in the first 4 days of Series 1,  $F(1, 7) = 7.958, p < .05$  (Figure 10a), and POST period wheel counts were also significantly higher at the beginning of Series 2 than at the beginning of Series 1,  $F(1, 7) = 18.067, p < .01$ . There were no significant differences in PRE or POST period wheel counts between the last 4 days of the two Nicotine Injection Series,  $F(1, 7) = 4.155 - 5.534, p = .051 - .081$ . Administration of most of the pharmacological treatments resulted in very similar trends in POST period wheel running between Series 1 and Series 2 as the administration of no treatment. POST period wheel counts at the beginning of Series 2 were significantly

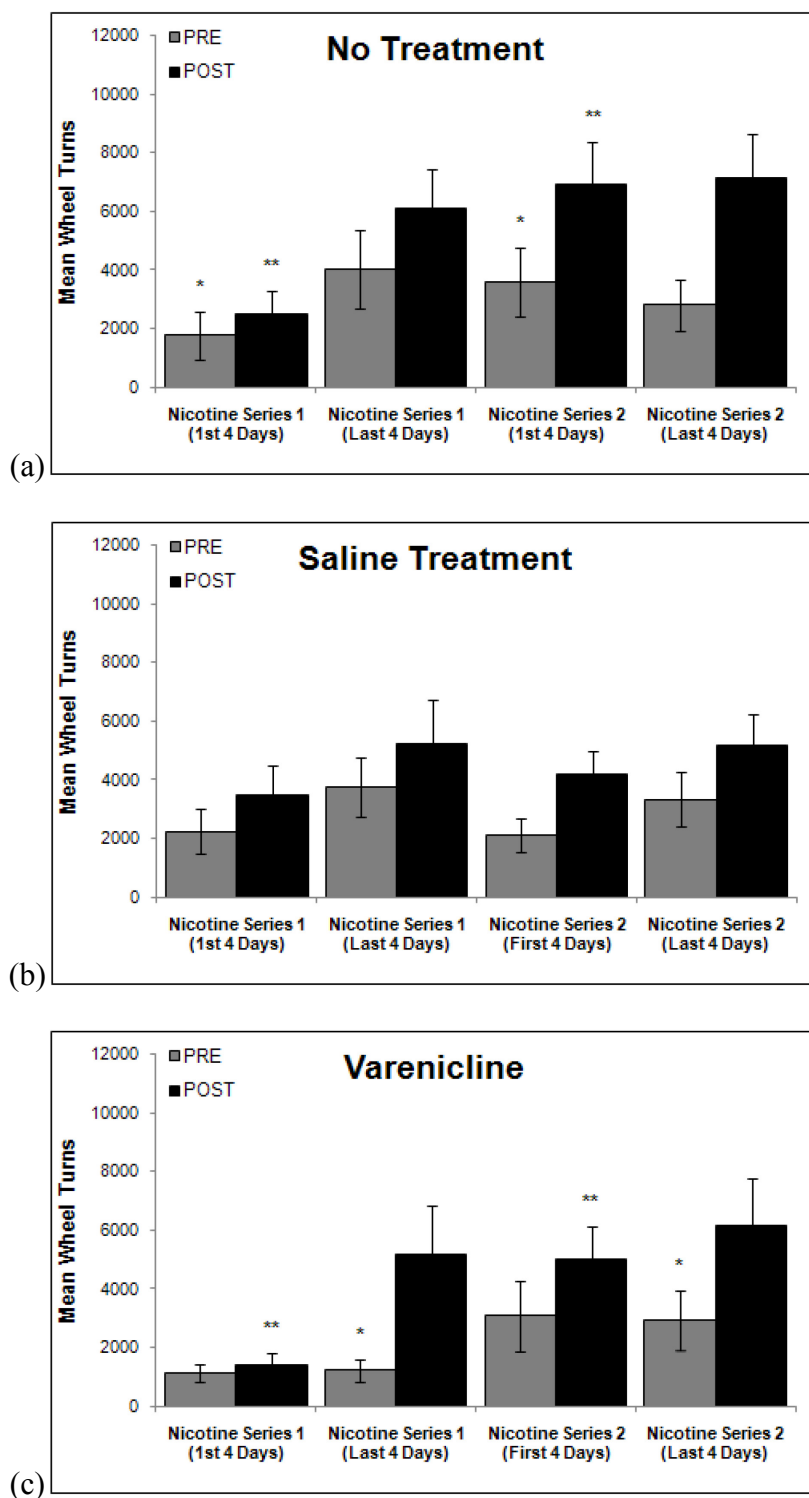


Figure 10. Mean wheel turns ( $\pm$  standard error) recorded in the PRE and POST periods during the first and last four days of Nicotine Injection Series 1 compared to Series 2, which followed the first treatment phase. Treatments administered are labeled at the top of the individual graphs. Mean wheel turns that are significantly different between Series 1 and Series 2: \* $p < .05$ , \*\* $p < .01$ .

higher than wheel counts at the beginning of Series 1 following treatment with varenicline,  $F(1, 7) = 21.789, p < .01$  (Figure 10c), mecamylamine,  $F(1, 7) = 22.107, p < .01$  (Figure 10d), acamprosate,  $F(1, 7) = 24.665, p < .01$  (Figure 10e), topiramate,  $F(1, 7) = 16.964, p < .01$  (Figure 10f), SB-334867,  $F(1, 7) = 6.997, p < .05$  (Figure 10h), and bupropion,  $F(1, 7) = 11.304, p < .05$  (Figure 10i). However, treatment with both saline,  $F(1, 7) = .702, p = .430$  (Figure 10b), and naltrexone,  $F(1, 7) = .245, p = .636$  (Figure 10g), resulted in no significant difference in POST period wheel counts between Series 1 and Series 2. There were no significant differences in POST period wheel counts between the last 4 days of Series 1 and Series 2 for any of the treatment conditions,  $F(1, 7) = .003 - 5.192, p = .057 - .960$ .

Unlike the no treatment condition, in which PRE period wheel counts were significantly higher in the first 4 days of Series 2 than Series 1, none of the pharmacological treatment conditions showed any significant differences in PRE period wheel counts between the beginning of Series 1 and Series 2,  $F(1, 7) = .018 - 4.248, p = .078 - .896$  (Figure 10). Likewise, there were no significant differences in PRE period wheel-running between the last 4 days of Series 1 and Series 2,  $F(1, 7) = .087 - 1.777, p = .224 - .776$ , with the exception of the varenicline treatment condition, in which PRE period wheel counts at the end of Series 2 were significantly higher than at the end of Series 1,  $F(1, 7) = 6.432, p < .05$  (Figure 10c).

Overall, the initial treatment conditions appeared to dampen the reacquisition of pre-nicotine activity episodes. Treatment with both saline and naltrexone also appeared to dampen the reacquisition of post-nicotine episodes. However, all of these effects appeared to be limited to the start of the second injection series, as by the end of Series 2,

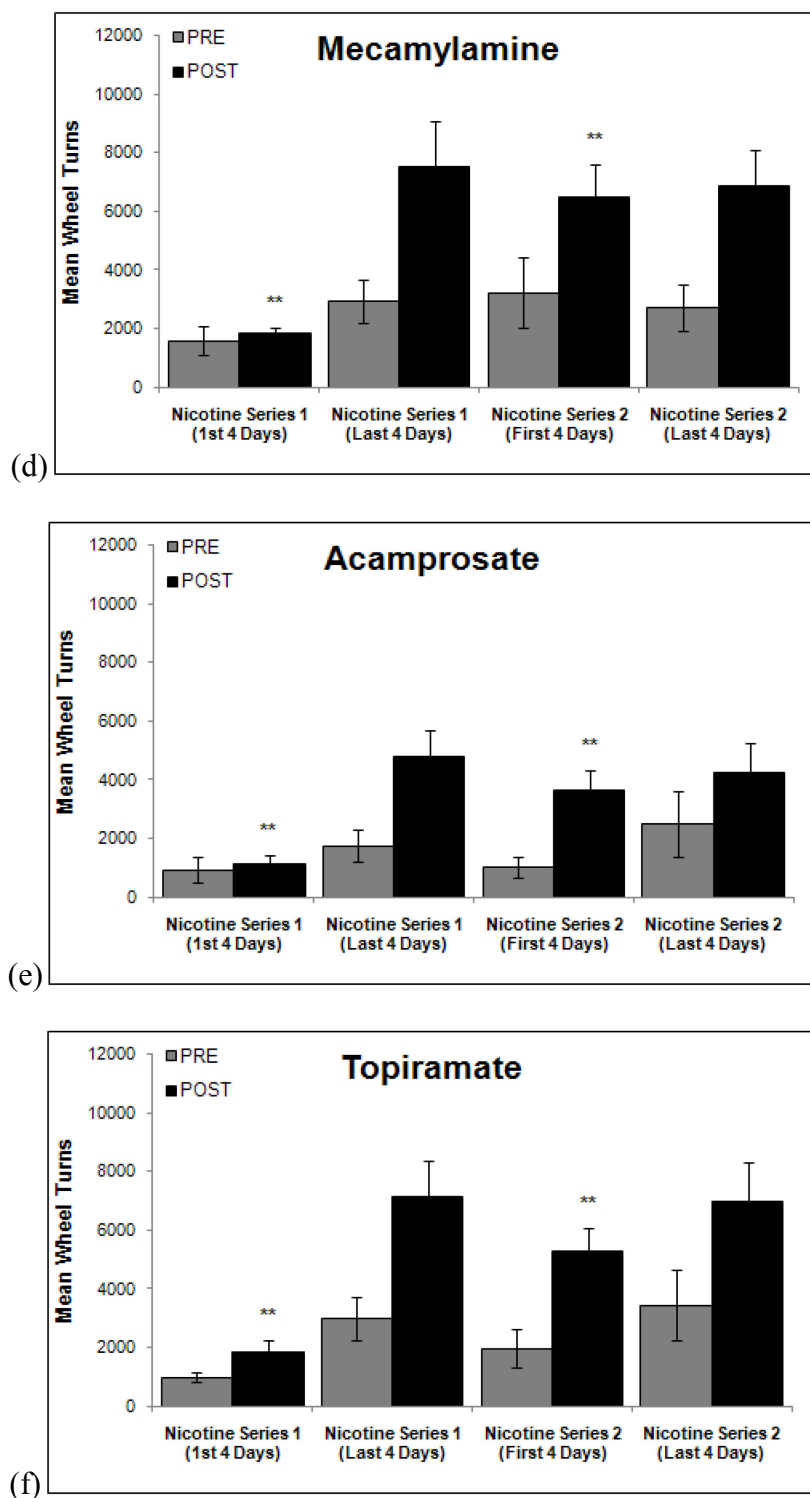


Figure 10 (cont.). Mean wheel turns ( $\pm$  standard error) recorded in the PRE and POST periods during the first and last four days of Nicotine Injection Series 1 compared to Series 2, which followed the first treatment phase. Treatments administered are labeled at the top of the individual graphs. Mean wheel turns that are significantly different between Series 1 and Series 2: \* $p < .05$ , \*\* $p < .01$ .

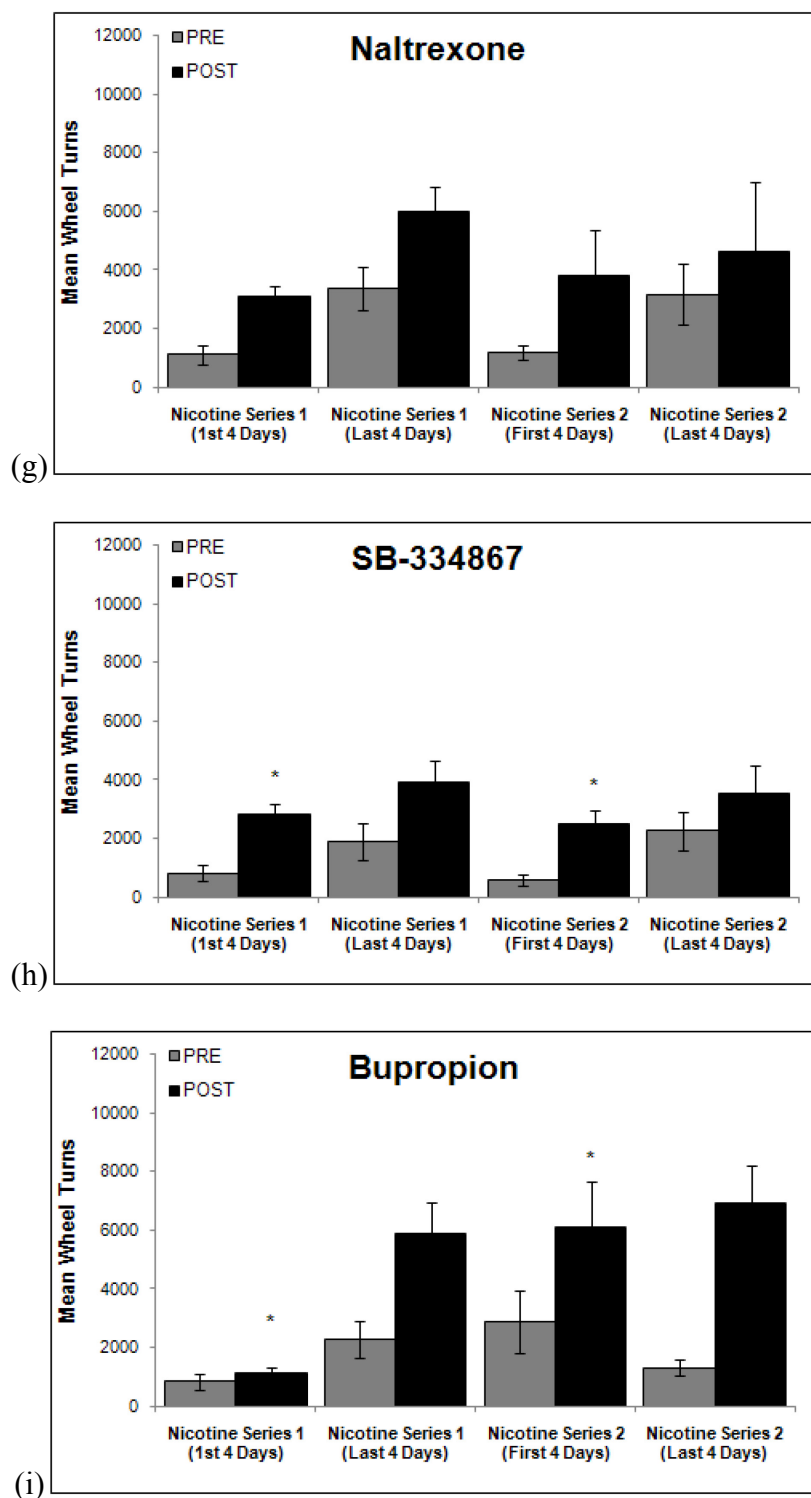


Figure 10 (cont.). Mean wheel turns ( $\pm$  standard error) recorded in the PRE and POST periods during the first and last four days of Nicotine Injection Series 1 compared to Series 2, which followed the first treatment phase. Treatments administered are labeled at the top of the individual graphs. Mean wheel turns that are significantly different between Series 1 and Series 2: \* $p < .05$ , \*\* $p < .01$ .



PRE and POST period wheel counts were equal to or (in the case of varenicline treatment) higher than wheel counts at the end of Series 1.

**Effects on post-saline activity episodes.** In general, the treatment conditions had very different effects on saline-induced pre- and post-drug activity episodes, and the rats that received the saline zeitgeber injections showed a greater amount of variability in their responses to the treatments than the rats that received the nicotine zeitgeber injections. Whereas all of the treatment conditions produced a general (although not always significant) reduction in post-drug activity levels on the treatment and baseline days, many of the treatments induced a general increase in activity counts on these days for the saline zeitgeber rats (Figure 11). Mean wheel counts for the Saline-No Treatment group were increased relative to the Saline Injection Series on both Treatment Days, but this increase was not significant,  $F(1, 7) = 1.674 - 3.345, p = .110 - .237$  (Figure 11a). Wheel counts for the Saline-No Treatment group were also not significantly different from the Saline Injection Series on all four Baseline Days,  $F(1, 7) = .001 - 1.688, p = .235 - .976$ . Rats in the Saline-Saline Treatment group showed a trend of increased wheel counts relative to the Saline Injection Series, but did not show a significant change in activity on either the Treatment or the Baseline Days,  $F(1, 7) = .010 - .854, p = .386 - .922$  (Figure 11b).

Overall, varenicline treatment induced an increase in post-saline wheel-running, and this change was significant on Treatment Day 2 and Baseline Days 1 and 3,  $F(1, 7) = 5.635 - 8.212, p < .05$ , but was not significantly different from zero on the remaining days,  $F(1, 7) = .754 - 3.986, p = .086 - .414$  (Figure 11c). Mecamylamine treatment showed an effect opposite to varenicline treatment; it induced a decline in post-saline

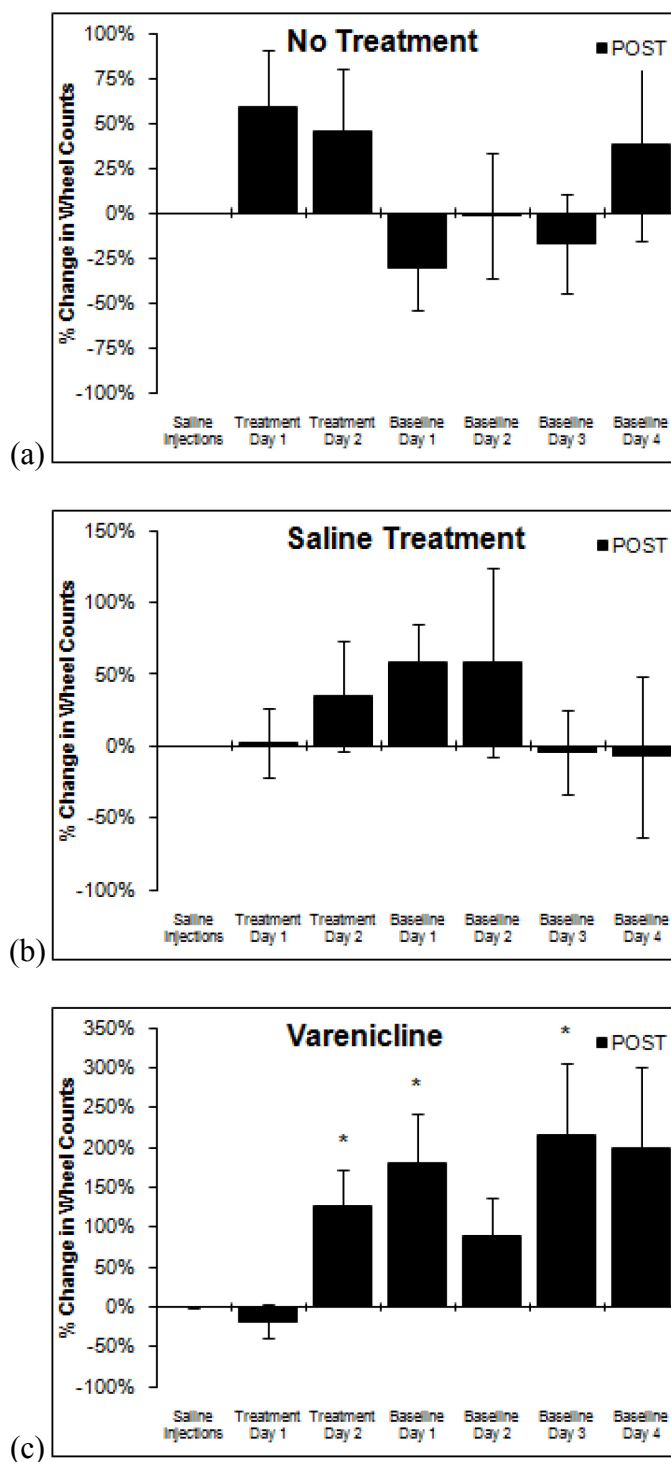


Figure 11. Percent change in wheel counts (± standard error) for the POST period (0-3 hours following each injection) during the Treatment and Baseline Days compared to Saline Injection Series 1. Treatments administered are labeled at the top of the individual graphs. Percent changes in wheel-running that are significantly different from zero: \* $p < .05$ , \*\* $p < .01$ , \*\*\* $p < .001$ .

wheel-running. Rats in the Saline-Mecamylamine treatment group did not show a significant change in wheel-running on both Treatment Days and the first Baseline Day,  $F(1, 7) = .094 - .872, p = .381 - .768$ , but they showed a significant reduction in wheel-running on Baseline Days 2 through 4,  $F(1, 7) = 6.810 - 452.101, p < .05$  (Figure 11d).

Treatment with both acamprosate and topiramate induced a general increase in POST period activity for the rats that received the saline zeitgeber. However, the percent change in wheel-running was not significant throughout the Treatment and Baseline Days for either the Saline-Acamprosate group,  $F(1, 7) = .005 - 4.762, p = .065 - .948$  (Figure 11e), or the Saline-Topiramate group,  $F(1, 7) = .076 - 3.694, p = .096 - .791$  (Figure 11f).

Naltrexone and SB-334867 both induced a general decline in POST period wheel-running activity. Rats in the Saline-Naltrexone group showed a significant reduction in wheel-running on both Treatment Days,  $F(1, 7) = 10.671 - 265.457, p < .05$  (Figure 11g). However, these rats had inconsistent wheel-running patterns on the Baseline Days, as they showed a significant increase in wheel-running on Baseline Day 1,  $F(1, 7) = 29.647, p < .01$ , a significant decrease on Baseline Day 2,  $F(1, 7) = 13.634, p < .01$ , and were not significantly different from the Saline Injection Series on Baseline Days 3 and 4,  $F(1, 7) = .021 - .501, p = .502 - .890$ . Rats in the Saline-SB-334867 group showed an overall decline in activity across the Treatment and Baseline Days, and were significantly lower than Saline Injection Series levels on Treatment Day 2 and Baseline Days 2 and 4,  $F(1, 7) = 6.652 - 11.499, p < .05$  (Figure 11h), but did not show a significant reduction in activity on the remaining days,  $F(1, 7) = .371 - 5.604, p = .050 - .562$ . Rats in the Saline-Bupropion group showed a trend of increasing activity relative to the Saline Injection

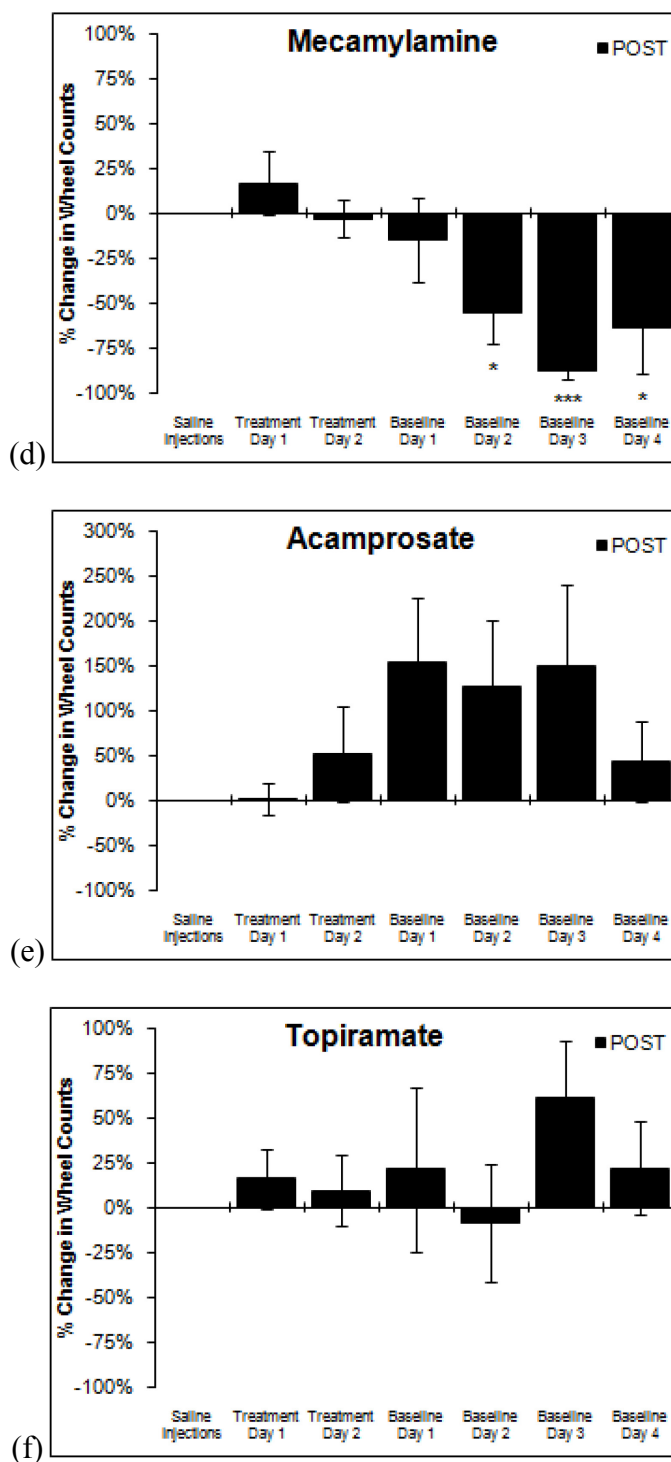
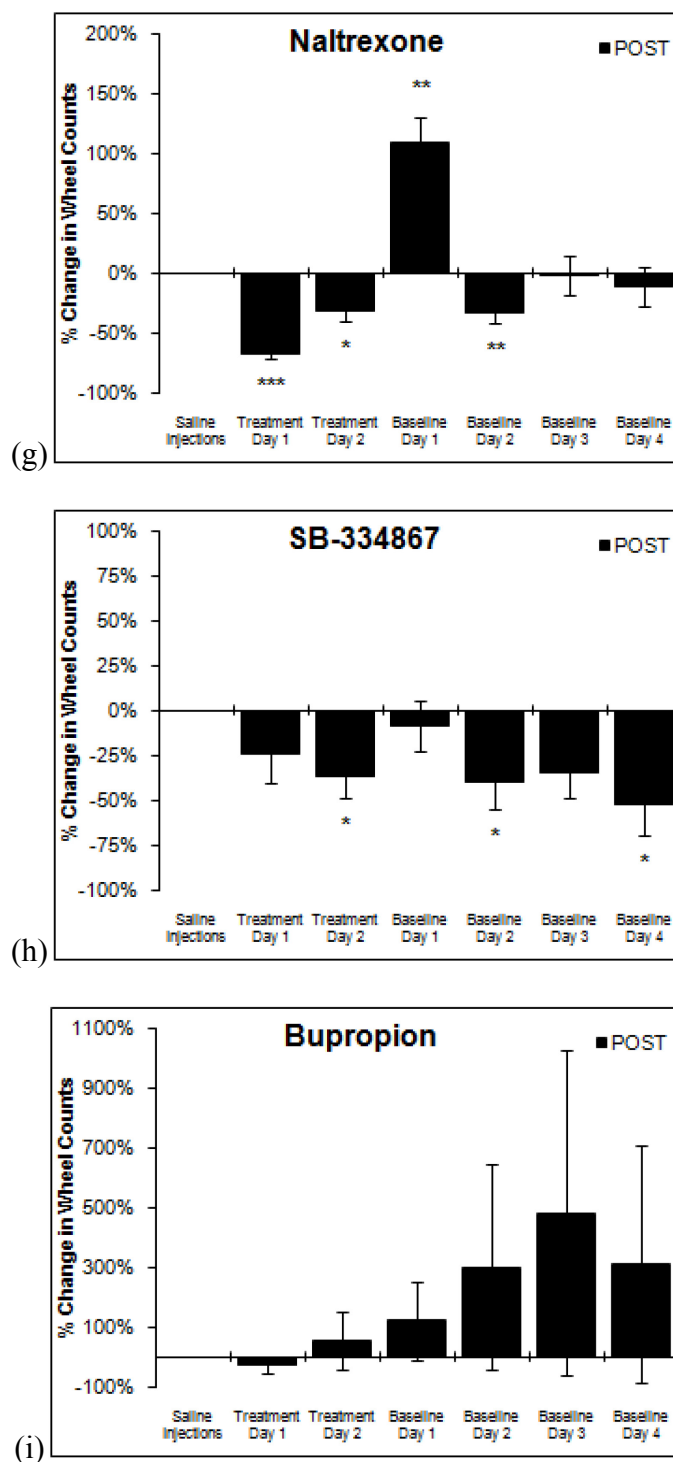


Figure 11 (cont.). Percent change in wheel counts ( $\pm$  standard error) for the POST period (0-3 hours following each injection) during the Treatment and Baseline Days compared to Saline Injection Series 1. Treatments administered are labeled at the top of the individual graphs. Percent changes in wheel-running that are significantly different from zero: \* $p < .05$ , \*\* $p < .01$ , \*\*\* $p < .001$ .

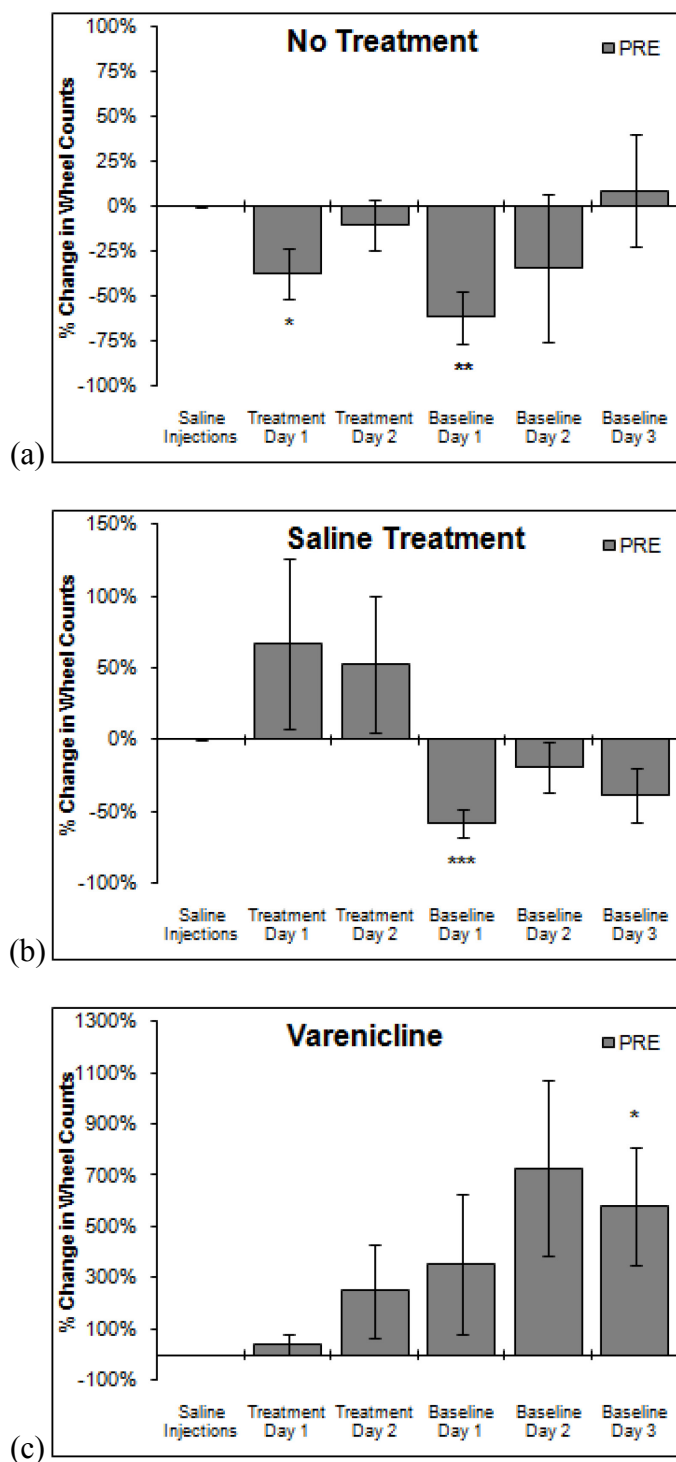


*Figure 11 (cont.).* Percent change in wheel counts ( $\pm$  standard error) for the POST period (0-3 hours following each injection) during the Treatment and Baseline Days compared to Saline Injection Series 1. Treatments administered are labeled at the top of the individual graphs. Percent changes in wheel-running that are significantly different from zero: \* $p < .05$ , \*\* $p < .01$ , \*\*\* $p < .001$ .

series across the Treatment and Baseline Days, but these changes were never significant,  $F(1, 6) = .003 - 4.285, p = .084 - .962$  (Figure 11i).

Not all of the treatment conditions had significantly different effects on post-nicotine and post-saline episodes. The groups that received No Treatment,  $F(1, 14) = .308, p = .587$ , Saline Treatment,  $F(1, 14) = 1.025, p = .329$ , Mecamylamine,  $F(1, 14) = 4.400, p = .055$ , SB-334867,  $F(1, 14) = .411, p = .532$ , and Bupropion,  $F(1, 14) = .843, p = .374$ , did not differ significantly between the rats that received the nicotine zeitgeber injections and the rats that received the saline zeitgeber injections. Significant differences between the zeitgeber groups were found for the Varenicline,  $F(1, 14) = 8.477, p < .05$ , Acamprosate,  $F(1, 14) = 7.074, p < .05$ , Topiramate,  $F(1, 14) = 9.554, p < .01$ , and Naltrexone treatment groups,  $F(1, 14) = 23.518, p < .001$ .

**Effects on pre-saline activity episodes.** As with post-saline activity episodes, most of the effects of the treatment conditions on pre-saline episodes were opposite to their effects on pre-nicotine episodes, although these differences were not always significant (Figure 12). However, the control treatment conditions had similar effects on pre-saline episodes and on pre-nicotine episodes, as wheel-running in the Saline-No Treatment group was significantly lower than Saline Injection Series levels following Treatment Day 1 and Baseline Day 1,  $F(1, 7) = 7.319 - 17.993, p < .05$ , but was not significantly different following the remaining Treatment and Baseline Days,  $F(1, 7) = .083 - .696, p = .432 - .781$  (Figure 12a). PRE period wheel running in the Saline-Saline Treatment group was not significantly different from the Saline Injection Series levels on all Treatment and Baseline Days,  $F(1, 7) = 1.146 - 4.043, p = .084 - .320$ , with the



*Figure 12.* Percent change in wheel counts (± standard error) for the PRE period (22-24 hours following each injection) during the Treatment and Baseline Days compared to Saline Injection Series 1. Treatments administered are labeled at the top of the individual graphs. Percent changes in wheel-running that are significantly different from zero: \* $p < .05$ , \*\* $p < .01$ , \*\*\* $p < .001$ .

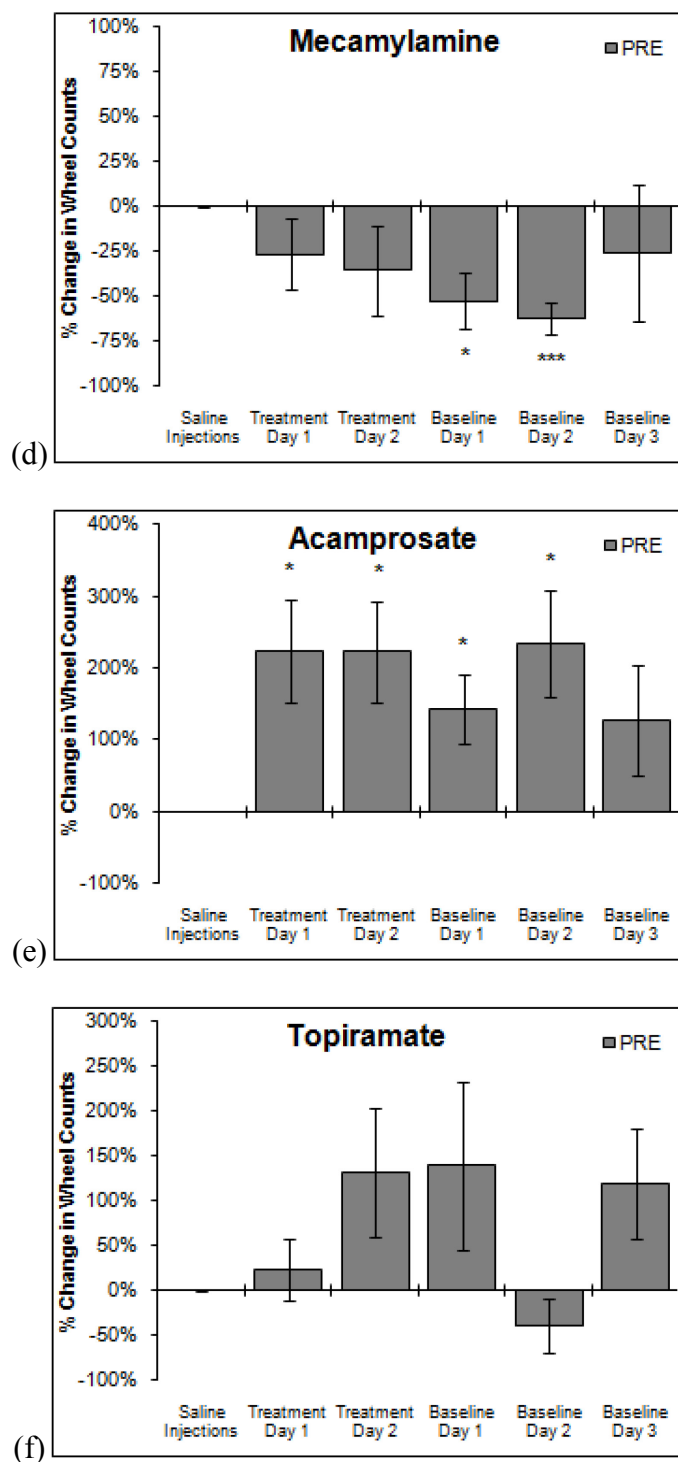
exception of the PRE period following Baseline Day 1, which was significantly lower than the Injection Series,  $F(1, 7) = 37.964, p < .001$  (Figure 12b).

As with post-saline episodes, varenicline produced an overall increase in PRE period wheel-running (Figure 12c). However, this increase was only significant following Baseline Day 3,  $F(1, 7) = 6.251, p < .05$ , and was not significantly different from the Saline Injection Series following the remaining Treatment and Baseline Days,  $F(1, 7) = .836 - 4.489, p = .072 - .391$ . Mecamylamine also had similar effects on pre-saline episodes as it did on post-saline episodes, and this effect was opposite to that of varenicline. Rats in the Saline-Mecamylamine group showed an overall reduction in PRE period wheel-running, and this change was significant on Baseline Days 1 and 2,  $F(1, 7) = 11.429 - 52.474, p < .05$ , but not on the remaining Treatment and Baseline Days,  $F(1, 7) = .466 - 1.987, p = .201 - .517$  (Figure 12d).

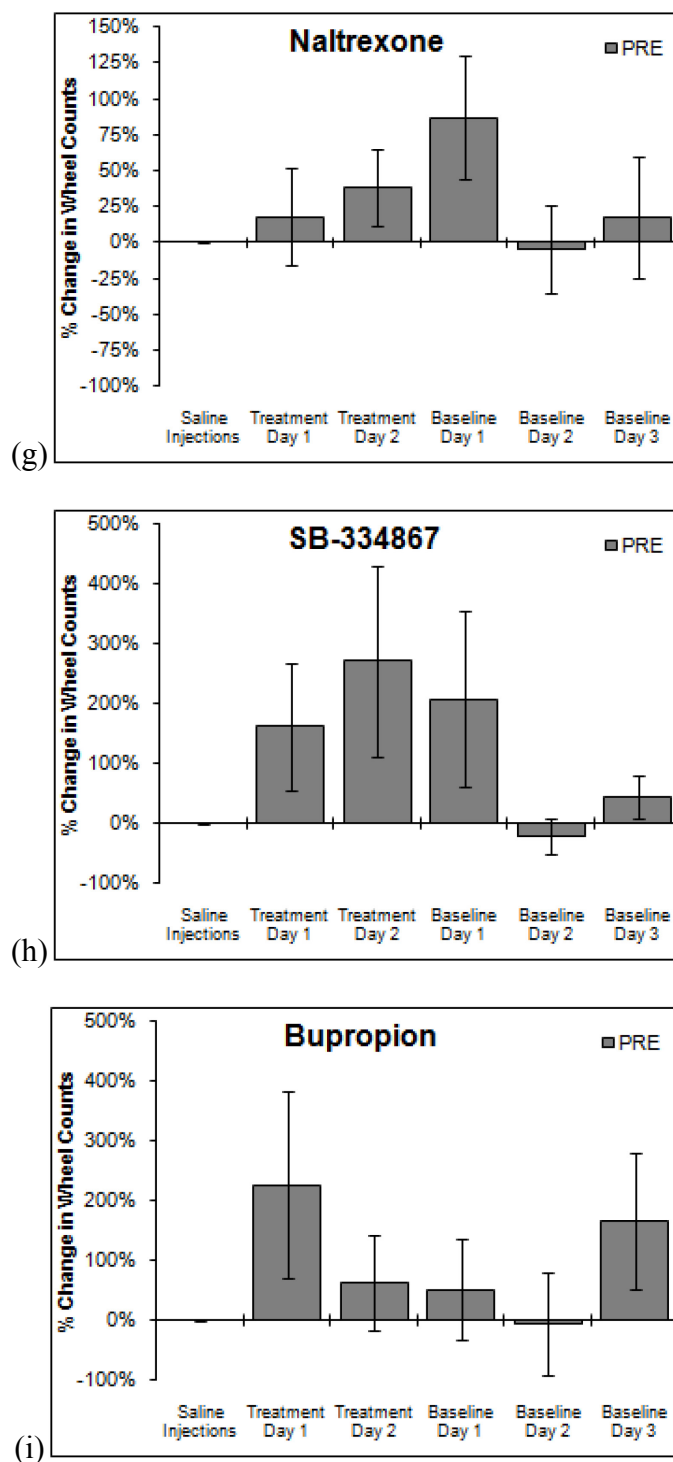
Acamprosate and topiramate both increased PRE period activity for the rats that received the saline zeitgeber injections, as they did with POST period activity. Rats in the Saline-Acamprosate group had significantly higher wheel-running in the PRE period following both Treatment Days and the first two Baseline Days,  $F(1, 7) = 9.138 - 10.061, p < .05$ , but not following Baseline Day 3,  $F(1, 7) = 2.763, p = .140$  (Figure 12e). Rats in the Saline-Topiramate group showed increases in PRE period wheel-running following most of the Treatment and Baseline Days, but these changes did not differ significantly from the Saline Injection Series,  $F(1, 7) = .434 - 3.814, p = .092 - .517$ .

Naltrexone, SB-334867, and bupropion all induced general increases in pre-saline activity episodes across the Treatment and Baseline Days (Figure 16g-i). However, these treatments did not induce significant changes in PRE period activity for any of these





*Figure 12 (cont.).* Percent change in wheel counts ( $\pm$  standard error) for the PRE period (22-24 hours following each injection) during the Treatment and Baseline Days compared to Saline Injection Series 1. Treatments administered are labeled at the top of the individual graphs. Percent changes in wheel-running that are significantly different from zero: \* $p < .05$ , \*\* $p < .01$ , \*\*\* $p < .001$ .



*Figure 12 (cont.).* Percent change in wheel counts ( $\pm$  standard error) for the PRE period (22-24 hours following each injection) during the Treatment and Baseline Days compared to Saline Injection Series 1. Treatments administered are labeled at the top of the individual graphs. Percent changes in wheel-running that are significantly different from zero: \* $p < .05$ , \*\* $p < .01$ , \*\*\* $p < .001$ .

days; naltrexone:  $F(1, 7) = .023 - 4.010, p = .085 - .883$ , SB-334867:  $F(1, 7) = .555 - 2.920, p = .131 - .480$ , bupropion:  $F(1, 7) = .632 - 1.926, p = .208 - .453$ .

In most cases, the treatments that had significantly different effects on post-nicotine, and post-saline episodes also had significantly different effects on pre-nicotine and pre-saline episodes. As with POST period activity, there were no significant difference in PRE period activity between the nicotine and saline zeitgeber groups for the No Treatment condition,  $F(1, 14) = .120, p = .734$ , Saline Treatment,  $F(1, 14) = .356, p = .560$ , Mecamylamine,  $F(1, 14) = 1.305, p = .272$ , SB-334867,  $F(1, 14) = .336, p = .572$ , and Bupropion,  $F(1, 14) = 1.010, p = .332$ . Likewise, the Acamprosate,  $F(1, 14) = 13.505, p < .01$ , Topiramate,  $F(1, 14) = 4.949, p < .05$ , and Naltrexone treatment groups,  $F(1, 13) = 13.431, p < .01$ , all showed significant differences between the nicotine and saline zeitgeber groups, as they did for POST period activity. The one exception was varenicline, which had a significant difference in POST period activity, but was not significantly different for PRE period activity,  $F(1, 14) = 4.595, p = .050$ .

**Summary of treatment results.** Most of the treatment conditions reduced post-nicotine activity that across the treatment and baseline days. In contrast, most of the treatment conditions increased post-saline activity on these days. Pre-nicotine activity levels were increased or maintained by most of the treatment conditions, with the exception of naltrexone and SB-334867. Naltrexone treatment reduced pre-nicotine wheel running on both the treatment days and most baseline days. SB-334867 treatment eliminated the persistence of pre-nicotine wheel running on the baseline days, but did not significantly lower pre-nicotine activity on the treatment days. Naltrexone and SB-334867 also appeared to diminish the reacquisition of both pre- and post-nicotine

episodes in the second Nicotine Injection Series. Overall, naltrexone and SB-334867 had the largest effects on both pre- and post-nicotine circadian activity episodes.

## Discussion

### Summary of Results

**Entrainment to nicotine and saline zeitgeber injections.** Overall, wheel counts appeared to provide the most sensitive representation of nicotine- and saline-induced circadian activity episodes. While water drinking, head entries in the feeder, and food pellet consumption showed similar daily patterns (Figure 1, Figure 6), wheel-running was the only activity measure for which significant persisting pre- and post-drug episodes were consistently recorded.

Repeated subcutaneous nicotine administration readily entrained significant post-nicotine activity episodes that persisted for at least 2 days. Significant episodes were also recorded in the PRE period (1-2 hours prior to the daily injection time) in both Nicotine Injection Series, and these episodes also persisted for multiple days, with the exception of the PRE period following the first day that nicotine was withheld during Test 2, in which PRE period wheel-running was significantly lower than wheel counts at the end of Nicotine Series 2 (Figure 2). The analysis of wheel-running in the remaining 19 hours of the day (ROD period) showed that nicotine administration initially increased the overall activity levels throughout the day, but nicotine-induced activity eventually became focused (entrained) to the PRE and POST periods, while the activity throughout the rest of the day returned to acclimation levels. These results indicate that nicotine administration in the current paradigm induced robust pre- and post-drug circadian activity episodes that are sufficient to measure the effects of the treatments.

Repeated saline administration in the same paradigm produced significant pre- and post-drug episodes, but only the post-saline episode showed reliable persistence. The

pre-saline episode did not persist following Saline Series 1, and since many of the saline zeitgeber groups in the present study did not receive a second zeitgeber injection series, it is not clear whether the effects of the treatments can be reliably interpreted for the pre-saline episodes.

**Effects of treatments on post-drug activity episodes.** The effects of the treatment on nicotine-entrained circadian activity episodes are summarized in Table 3, and the effects on saline-entrained episodes are summarized in Table 4. Some of the treatments had differential effects on the treatment days versus the baseline days, whereas others had consistent effects throughout the treatment and baseline days. For example, varenicline treatment did not produce a significant change in POST period activity on the two treatment days, but this activity was significantly lower than the nicotine injection series on all four baseline days (Figure 8c). These differential results appear to indicate that the mechanisms that produce the circadian episodes may be at least partially distinct from the mechanisms that allow these episodes to persist on a circadian schedule. In other words, the expression of the locomotor activity episodes may be governed by a set of mechanisms that is separate from the mechanisms that govern the timing of the episodes. Based on the evidence for these distinct mechanisms, the remainder of this discussion will interpret the effects of the treatments on the two treatment days as effects on the circadian locomotor activity episodes themselves, while the effects of the treatments on the baseline days will be interpreted as effects on persistence of the activity episodes.

Most of the administered treatments produced a reduction in post-nicotine activity levels during the three hours following the daily administration time. When no treatment

Table 3

*Summary of Significant Treatment Effects on Pre- and Post-Nicotine Circadian Activity**Episodes*

Treatment	PRE		POST	
	Episodes <sup>a</sup>	Persistence <sup>b</sup>	Episodes <sup>a</sup>	Persistence <sup>b</sup>
No Treatment	-	Decrease – 2 <sup>nd</sup> day	-	Decrease – 1 <sup>st</sup> – 3 <sup>rd</sup> days
Saline Treatment	-	Decrease – 3 <sup>rd</sup> day	Decrease – 2 <sup>nd</sup> day	Decrease – 3 <sup>rd</sup> & 4 <sup>th</sup> days
Varenicline	-	Increase – 1 <sup>st</sup> day	-	Decrease – all days
Mecamylamine	Increase – 1st day	-	Decrease – both days	Decrease – all days
Acamprosate	-	-	Decrease – 2 <sup>nd</sup> day	Decrease – all days
Topiramate	-	Decrease – 2 <sup>nd</sup> & 3 <sup>rd</sup> day	Decrease – both days	Decrease – 2 <sup>nd</sup> – 4 <sup>th</sup> days
Naltrexone	Decrease - both days	Decrease – 2 <sup>nd</sup> & 3 <sup>rd</sup> day	Decrease – both days	Decrease – all days
SB-334867	-	Decrease – all days	Decrease – 1 <sup>st</sup> day	Decrease – 2 <sup>nd</sup> – 4 <sup>th</sup> days
Bupropion	-	-	Decrease – both days	Decrease – 2 <sup>nd</sup> day

<sup>a</sup>Percent difference from Nicotine Injection Series during the two Treatment Days.

<sup>b</sup>Percent difference from Nicotine Injection Series during the Baseline Days. Three baseline days were measured for PRE period activity, and four baseline days were measured for POST period activity.

Table 4

*Summary of Significant Treatment Effects on Pre- and Post-Saline Circadian Activity Episodes*

Treatment	PRE		POST	
	Episodes <sup>a</sup>	Persistence <sup>b</sup>	Episodes <sup>a</sup>	Persistence <sup>b</sup>
No Treatment	Decrease – 1 <sup>st</sup> day	Decrease – 1 <sup>st</sup> day	-	-
Saline Treatment	-	Decrease – 1 <sup>st</sup> day	-	-
Varenicline	-	Increase – 3 <sup>rd</sup> day	Increase – 2 <sup>nd</sup> day	Decrease – 1 <sup>st</sup> & 3 <sup>rd</sup> days
Mecamylamine	-	Decrease – 1 <sup>st</sup> - 2 <sup>nd</sup> days	-	Decrease – 2 <sup>nd</sup> - 4 <sup>th</sup> days
Acamprosate	Increase – both days	Increase – 1 <sup>st</sup> - 2 <sup>nd</sup> days	-	-
Topiramate	-	-	-	-
Naltrexone	-	-	Decrease – both days	Increase 1 <sup>st</sup> day, Decrease 2 <sup>nd</sup> day
SB-334867	-	-	Decrease – 2 <sup>nd</sup> day	Decrease – 2 <sup>nd</sup> & 4 <sup>th</sup> days
Bupropion	-	-	-	-

<sup>a</sup>Percent difference from Saline Injection Series during the two Treatment Days.

<sup>b</sup>Percent difference from Saline Injection Series during the Baseline Days. Three baseline days were measured for PRE period activity, and four baseline days were measured for POST period activity.



was given, post-nicotine wheel-running was not significantly different from the nicotine injection series levels on the treatment days, but was significantly lower than the nicotine injection series for most of the baseline days (Figure 8a). Saline treatment appeared to maintain post-nicotine activity levels for slightly longer than no treatment, as wheel running was not significantly different from the nicotine injection series on the first treatment day as well as the first two baseline days (Figure 8b).

On the treatment days, the post-nicotine activity episodes were consistently and significantly reduced by administration of mecamylamine, topiramate, naltrexone, and bupropion (Figure 8). Acamprosate and SB-334867 significantly reduced post-nicotine wheel-running on only one of the two treatment days, and varenicline administration did not significantly alter these activity levels. Given these data, the post-nicotine episodes appear to be driven by the activation of nicotinic acetylcholine, AMPA/kainate, and  $\mu/\kappa$ -opioid receptors as well as a reduction in dopamine transmission. The less consistent results with acamprosate and SB-334867 may also indicate roles for NMDA and orexin-1 receptors in mediating post-nicotine activity episodes.

Both varenicline and mecamylamine significantly reduced post-nicotine wheel-running on all four baseline days. Since these two treatments have opposite pharmacological actions, it can be assumed that the persistence of post-nicotine episodes does not involve acetylcholine transmission. Persistence of these episodes also does not appear to involve dopamine transmission, as bupropion treatment only significantly reduced post-nicotine wheel-running on one of the baseline days. Both acamprosate and naltrexone treatment significantly reduced post-nicotine activity on all four baseline days, and both topiramate and SB-334867 significantly reduced this activity on the last three

baseline days. Therefore, persistence of post-nicotine episodes appears to be mediated by the activation of NMDA, AMPA/kainate,  $\mu/\kappa$ -opioid, and orexin-1 receptors.

Previous studies of nicotine-induced activity episodes have shown that rats will readily reacquire the expression of these episodes after nicotine has been withheld for several days (Gillman, et al., 2007; Gillman, et al., 2008). In the present study, most of the rats that received the nicotine zeitgeber showed a significant increase in post-nicotine wheel-running on the first four days of Nicotine Injection Series 2 when compared to the first four days of Series 1 (Figure 10) as the post-nicotine episodes were reacquired and quickly became entrained to the new administration time in Series 2. However, both the naltrexone and saline treatment groups did not show a significant difference in post-nicotine activity between the beginnings of the two series, which suggests that these particular treatments interfered with reacquisition of post-nicotine episodes.

In contrast to the reductions in post-nicotine episodes produced by all treatments, most of the treatments did not induce significant changes in post-saline activity episodes, although there was a great deal of variability in the effects of the treatments among individual rats (Figure 11). Only naltrexone treatment significantly reduced post-saline activity levels on both treatment days, although varenicline significantly increased post-saline activity on Treatment Day 2, and SB-334867 significantly reduced this activity on Treatment Day 2. Therefore, post-saline episodes appear to be mediated by the activation of  $\mu/\kappa$ -opioid receptors and possibly by the activation of nicotinic acetylcholine and orexin-1 receptors.

Most of the treatments did not significantly change post-saline episodes on the baseline days (Figure 11). Varenicline treatment significantly increased post-saline

wheel-running on the first and third baseline days, and mecamylamine treatment significantly reduced post-saline activity on the last three baseline days. Therefore, persistence of post-saline episodes may depend on the activation of nicotinic acetylcholine receptors. Treatment with SB-334867 significantly reduced post-saline wheel-running on the second and fourth baseline days, indicating that persistence of these episodes may also be mediated by the activation of orexin-1 receptors.

**Effects of treatments on pre-drug activity episodes.** While most of the treatments led to a decline in post-nicotine activity, the nine treatment conditions had very different effects on pre-nicotine circadian activity episodes. Most of the treatments did not induce a significant change in PRE period wheel-running after administration on the two treatment days (Figure 9). The exceptions were mecamylamine, which led to a significant increase in pre-nicotine wheel counts after the first (but not the second) treatment day, and naltrexone, which induced a significant decrease in PRE period wheel counts following both treatment days. Therefore, the expression of pre-nicotine episodes appears to be mediated by the activation of  $\mu$ - and/or  $\kappa$ -opioid receptors, and possibly by the inactivation of nicotinic acetylcholine receptors.

Treatment with acamprosate, mecamylamine, and bupropion did not significantly change pre-nicotine wheel-running following the baseline days (Figure 9). Varenicline treatment significantly increased PRE period wheel counts following the first baseline day. Both naltrexone and topiramate treatment led to significant reductions in pre-nicotine wheel-running following the last two baseline days, and treatment with SB-334867 led to significant reductions in this activity following all three baseline days. These results indicate that the persistence of pre-nicotine episodes is mediated by the

activation of  $\mu/\kappa$ -opioid, AMPA/kainite, and orexin-1 receptors. Given the effects of varenicline following the first baseline day, the persistence of these episodes may also be mediated by the activation of nicotinic acetylcholine receptors, presumably by acetylcholine instead of nicotine.

In all but one of the treatment groups, there was no significant difference in PRE period wheel counts between the last four days of Series 1 and 2 (Figure 10). The exception was the varenicline treatment group, in which pre-nicotine wheel counts at the end of Series 2 were significantly higher than at the end of Series 1. These results may indicate that varenicline treatment facilitates or enhances entrainment following a period of abstinence, but this cannot be definitively tested in these data.

As with post-saline episodes, there was a great deal of individual variability in the effects of the treatments on pre-saline episodes, but most of the treatments did not significantly change PRE period wheel counts (Figure 12). PRE period wheel counts were significantly increased following both treatment days and the first two baseline days in the Saline-Acamprosate group. The Saline-Mecamylamine group showed significant reductions in PRE period wheel-running following the first two baseline days. Following the present interpretation criteria, these results indicate that the expression of pre-saline episodes is mediated by the inactivation of NMDA receptors, and the persistence of these episodes is mediated by the activation of nicotinic acetylcholine receptors and the inactivation of NMDA receptors. However, as noted above, daily saline administration did not reliably entrain persisting pre-saline episodes after a single injection series, so it is not clear whether these results can be reliably interpreted as the effects these treatments would have on fully-entrained pre-saline circadian activity episodes.

**Summary of treatment effects.** The results of these treatment manipulations clearly show that pre- and post-nicotine circadian activity episodes are mediated by distinct neuropharmacological mechanisms, although they also appear to share some common mechanisms. Based on the pattern of treatment effects, the activation of  $\mu$ - and/or  $\kappa$ -opioid receptors appears to mediate both the expression and the persistence of pre- and post-nicotine circadian episodes, as administration of naltrexone significantly reduced both PRE and POST period wheel-running on most of the treatment and baseline days. The persistence of both pre- and post-nicotine episodes also appears to be mediated by the activation of orexin-1 receptors, as treatment with SB-334867 significantly reduced PRE and POST period wheel counts on most baseline days, but not on treatment days. Post-saline episodes were also reduced by naltrexone administration, and the persistence of post-saline episodes was reduced by both naltrexone and SB-334867, although these treatments did not significantly change pre-saline wheel running.

In addition to these common mechanisms, post-nicotine episodes appear to be driven by the activation of NMDA, AMPA/kainate, nicotinic acetylcholine, and orexin-1 receptors, and these receptors also appear to play a role in the persistence of these episodes. These results are not surprising given that nicotine administration is known to engage cholinergic and glutamatergic mechanisms (Benowitz, 2008; Reid, et al., 2000). In contrast, pre-nicotine episodes appear to be driven by inactivated nicotinic receptors in addition to  $\mu/\kappa$ -opioid receptors, and the persistence of pre-nicotine episodes appears to be driven by the activation of AMPA/kainate receptors in addition to opioid and orexin-1 receptors. Surprisingly, neither the expression nor the persistence of both pre- and post-nicotine episodes appears to involve dopamine transmission, as bupropion administration

did not significantly alter pre- or post-nicotine wheel-running throughout all treatment and baseline days.

Finally, as noted in the introduction, it should be noted that the present study was limited to single, high doses of nicotine and the treatment drugs. Therefore, many of the treatments that did not significantly affect nicotine-induced circadian activity episodes may have significant effects if administered at higher or lower doses. Future studies will need to address these limitations and examine a range of doses for all of the substances used in the present research.

### **Proposed Mechanisms and Significance**

**Anatomical mechanisms.** The present study was not designed to specifically examine the involvement of discrete brain regions in the expression of pre- and post-drug circadian activity episodes. Rather, it was designed to provide a basic overview of the neurotransmitter systems involved in the generation and timing of these circadian episodes. The results of the present research allow the formation of several hypotheses that can be more directly tested in future studies. As studies of drug-induced circadian activity episodes have to date been restricted to intact, behaving animals, the hypotheses proposed in this section are partially based on the findings of studies of food-anticipatory activity and the methamphetamine-sensitive circadian oscillator combined with studies of drug abuse pharmacology that did not utilize a circadian biological perspective.

Before discussing the anatomical mechanisms that mediate pre- and post-drug circadian activity episodes, it seems important to clarify the significance and differences between these episodes. Post-drug circadian episodes undoubtedly reflect the acute, immediate effects of the drugs. A great deal is known about the brain regions that are

affected *after* a drug is administered such as the VTA, the nucleus accumbens, the amygdala, and the pre-frontal cortex (Kalivas, et al., 2005; Kalivas & Volkow, 2005). However, it is not clear why a circadian timing system would be engaged in the post-drug period, particularly if an independent mechanism controls the timing of the pre-drug episodes.

There appear to be two basic conceptual approaches to analyzing the causation and function of the post-drug episode. First, consider that administration of a drug of abuse is an enormously noxious event for many systems of the body. As a drug is repeatedly administered over time, a number of compensatory changes occur in metabolism, receptor sensitivity, and other physiological processes that lead to drug tolerance (Ramos, Siegel, & Bueno, 2002; Stewart & Badiani, 1993). Entrainment of the post-drug circadian episode therefore may ensure that these compensatory changes occur at the time(s) of day when administration is most likely to occur. The importance of these compensatory changes appears to be a likely explanation of why emergency room admissions for drug overdoses tend to show an exogenous circadian rhythm that peaks in the early evening (Erickson, et al., 1998; Morris, 1987; Raymond, et al., 1992). If drug users are accustomed to administering their drugs on a regular daily schedule, then taking drugs at a different time of day when these compensatory mechanisms are not engaged may lead to the symptoms of an overdose.

If the pre- and post-drug episodes are timed by common mechanisms, a second function of the post drug circadian episode may be to initiate the timing of the pre-drug episode. The pre-drug episode may represent a circadian-based drug craving, a drug-anticipatory activity rhythm that is analogous to food-anticipatory activity, or a circadian

drug-seeking motivation. While craving, anticipation, and motivational seeking sound like very similar behaviors, each has been linked to different neural circuits.

Drug craving has been linked to the activity of the insula, which integrates interceptive information from the periphery with emotional information from limbic and cortical circuits (Naqvi & Bechara, 2009; Paulus, Tapert, & Schulteis, 2009). Drug anticipation, if analogous to food anticipation, is a circadian rhythm of heightened activity entrained to and emerging a few hours before a daily drug administration time (Mistlberger, 1994, 2009). The mechanisms that mediate food anticipation have not been definitively isolated, but peripheral clock mechanisms and the dorsomedial hypothalamic nucleus appear to play roles in the expression of this behavior (Davidson, 2009; Escobar, Cailotto, Angeles-Castellanos, Delgado, & Buijs, 2009). Drug-seeking motivation is typically measured as active lever pressing in drug self-administration paradigm and reflects the “work” that drug users perform to successfully obtain and administer their drugs (Everitt, Dickinson, & Robbins, 2001). Drug-seeking motivation has been linked to the transmission of dopamine in the nucleus accumbens from neurons originating in the VTA, and the motor output of this motivation appears to be due to the disinhibition of motor neurons in the ventral pallidum that occurs when dopamine is transmitted in the accumbens (Berridge, 2009). Pre-drug circadian activity episodes may reflect a combination of these behaviors, and therefore the expression of pre-drug activity episodes may be mediated in any or all of these neural regions.

The combined results of the treatment manipulations in the present study suggest that the expression of drug-entrainable circadian activity episodes is controlled by timing mechanisms within endogenous opioid-transmitting cells. Further, it appears that the



persisting expression of these rhythms is driven by the activity of both orexin-transmitting and opioid-transmitting neurons. Specifically, this orexin transmission appears to involve the orexin-1 receptor, and this endogenous opioid transmission appears to involve the  $\mu$ - and/or  $\kappa$ -opioid receptors.

The origin of these orexin neurons appears easy to locate, as orexin-transmitting neurons originate mainly in the lateral hypothalamus and the adjacent perifornical area and dorsomedial hypothalamic nucleus (Date, et al., 1999; Peyron, et al., 1998). The lateral hypothalamus receives GABA projections directly from the nucleus accumbens, and the firing of these inhibitory projections appears to be inhibited by the transmission of dopamine that results from the administration of a drug of abuse (Kelley, 2004; Stratford & Kelley, 1999). Therefore, administration of these drugs also activates lateral hypothalamic neurons, and if this administration occurs on a circadian schedule, it would presumably entrain molecular clock mechanisms within the cells in this region. Further, there is an orexin projection from the lateral hypothalamus to dopaminergic neurons of the ventral tegmental area that express the orexin-1 receptor (Borgland, et al., 2006). When drug use ceases, entrained clock mechanisms in the lateral hypothalamus may continue to oscillate and continue to activate this tegmental projection pathway, thereby facilitating a weakened but persisting circadian activity episode that presumably reflects a persisting circadian drug-seeking motivation.

An entrained clock mechanism in the lateral hypothalamus may also contribute to drug craving, as there are orexin projections from this region to neurons in the insular cortex that also express the orexin-1 receptor (Hollander, et al., 2008; Peyron, et al., 1998), and these neurons may continue to be activated on a circadian schedule after drug

use ceases. The lateral hypothalamus also receives inputs from the dorsomedial hypothalamus, which appears to play a role in circadian food anticipation and may therefore have a role in circadian drug anticipation (Yoshida, McCormack, España, Crocker, & Scammell, 2006).

The locations of the endogenous opioid timing mechanisms that are entrained by drugs of abuse are more difficult to isolate, as there are several different types of endogenous opioids that activate the  $\mu$  and  $\kappa$  receptors, and these receptors are expressed in numerous brain regions (Mansour, et al., 1994). However, there are two opioid pathways that have been identified as important for the reinforcing effects of non-opioid addictive drugs. First, there are dynorphin-transmitting neurons in the nucleus accumbens that project to dopaminergic neurons of the ventral tegmental area that express  $\kappa$ -opioid receptors (Shippenberg & Rea, 1997). These dynorphin neurons are activated when dopamine is transmitted to the nucleus accumbens from the VTA and are part of a negative feedback loop in which the firing of these neurons inhibits the firing of the VTA dopamine neurons. This negative feedback loop is believed to have a neuroprotective function that dampens the dopamine transmission that occurs to repeated rewards, and in the case of drug addiction, this feedback loop is believed to dampen the rewarding effects of non-drug rewards (Nestler, 2004).

Despite their functions, these dynorphin neurons are probably not part of the mechanisms that time the expression of pre- and post-drug circadian episodes. As the activation of these neurons inhibits the firing of VTA dopamine neurons, circadian activation of these neurons would presumably lead to a period of reduced activity, rather than the robust circadian activity episodes that precede and follow drug administration.

Further, naltrexone is a  $\kappa$ -opioid antagonist, so its administration would prevent these neurons from transmitting dynorphin to the VTA dopamine neurons, and therefore would presumably produce a rise in activity. However, naltrexone administration reliably and significantly *reduced* circadian activity episodes, so it can be assumed that these episodes are not mediated by these dynorphin neurons that originate in the nucleus accumbens.

The second endogenous opioid pathway that appears to play a role in drug addiction is a  $\beta$ -endorphin pathway that originates in the arcuate nucleus of the hypothalamus and projects to the nucleus accumbens, which expresses both  $\mu$  and  $\kappa$  opioid receptors (Gianoulakis, 2009). Drugs of abuse stimulate these arcuate neurons to transmit  $\beta$ -endorphin to the nucleus accumbens shell, and this process has been shown to induce locomotion (Sanchis-Segura, Correa, & Aragon, 2000; Sanchis-Segura, Correa, Miquel, & Aragon, 2005). Further, the arcuate nucleus transmits multiple neuropeptides to the lateral hypothalamus, including neuropeptide Y, agouti-related peptide, and  $\alpha$ -melanin stimulating hormone (Elias, et al., 1998), and in return, the lateral hypothalamus transmits orexin to the arcuate nucleus (Date, et al., 1999). This orexin projection has been found to stimulate GABAergic cells in the arcuate nucleus that have been linked to food intake (Burdakov, Liss, & Ashcroft, 2003). Interestingly, orexin transmission in this pathway is mediated by the orexin-2 receptor and therefore would not be blocked by the administration of SB-334867. The main function of the arcuate nucleus appears to be the maintenance of energy homeostasis, and this region receives and transmits a number of food- and energy-related signals (Cone, et al., 2001). Endogenous circadian rhythms are often considered to be a form of temporal physiological homeostasis (Moore-Ede, 1986), so it is not too difficult to imagine that the arcuate nucleus would possess timing

mechanisms that help to mediate circadian reward-seeking behavior and compensatory changes to daily drug administration.

In summary, present evidence from this and other studies indicates that the expression and timing of pre- and post-drug circadian activity episodes are mediated by a network of neurons that includes the dopamine neurons in the VTA/nucleus accumbens, the orexin neurons in the lateral hypothalamus, and the  $\beta$ -endorphin neurons in the arcuate nucleus of the hypothalamus. The proposed model of the functioning of this network works in the following way: Administration of a drug of abuse stimulates the transmission of dopamine from neurons that originate in the ventral tegmental area and project to the nucleus accumbens. This dopamine transmission disinhibits and therefore activates the orexin neurons of the lateral hypothalamus. These neurons transmit orexin to numerous neural structures, including the VTA and the arcuate nucleus of the hypothalamus.  $\beta$ -endorphin neurons in the arcuate nucleus are also activated by drug administration, and these neurons transmit this endogenous opioid to the nucleus accumbens shell. Both the transmission of orexin to the VTA dopamine neurons and the transmission of  $\beta$ -endorphin to the nucleus accumbens lead to heightened levels of locomotor activity. Thus, when a drug of abuse is repeatedly administered on a daily schedule, molecular timing mechanisms are entrained within these opioid and orexin neurons that facilitate circadian rhythms of motivational and compensatory responses that continue to oscillate for several days after drug use is stopped.

**Molecular timing mechanisms.** The actograms of the rats in the present study (Figure 1, Figure 6) clearly show that two circadian timing mechanisms are operating independently with distinct periods. As the rats were kept under constant light, the light-

entrainable activity rhythm free-ran on a schedule of approximately 25-26 hours while repeated nicotine administration entrained pre- and post-drug episodes on a 24-hour schedule. Based on the results of the present study and previous studies of the effects of drugs of abuse on circadian timing systems, there are two hypotheses that can be formed as to which molecular timing mechanisms govern these differentiable circadian rhythms.

The first hypothesis is that the known circadian clock genes, *BMAL1*, *CLOCK/NPAS2*, *Period*, and *Cryptochrome*, mediate the expression of both light-entrainable and drug-entrainable circadian rhythms. If this hypothesis is true, these two rhythms are likely governed by the expression of clock genes in separate brain regions or at least in separate cells. The light-entrainable rhythms are undoubtedly mediated by the activity of SCN neurons, and given the results of the present study, the drug-entrainable rhythms are likely mediated by  $\beta$ -endorphin neurons in the arcuate nucleus and orexin neurons in the lateral hypothalamus.

As stated in the introduction, both *Per2* mutants and *Cry1/Cry2* double knockout mice have been shown to have greatly reduced food-anticipatory rhythms (Feillet, et al., 2006; Iijima, et al., 2005). If the molecular mechanisms that govern drug-induced circadian activity episodes are the same mechanisms that govern food-anticipatory circadian rhythms, then pre-drug episodes are likely mediated by the expression of *Period:Cryptochrome* heterodimers in the orexin and endorphin neurons listed above. In contrast, *CLOCK* mutant mice show regular food-anticipatory rhythms (Pitts, et al., 2003), but these animals do show robust sensitization to cocaine and increased activity in VTA dopamine neurons (McClung, et al., 2005). As sensitization is measured as a post-

drug effect (*i.e.*, after the drug is administered), post-drug episodes may be governed by the expression of CLOCK:BMAL1 heterodimers in the ventral tegmental area.

An alternate hypothesis is that the known circadian clock genes mediate light-entrainable circadian rhythms while an unknown set of timing genes mediate drug-entrainable circadian rhythms. At present, evidence from several different studies suggests that this second hypothesis is more likely to be true. Chronic administration of several drugs of abuse has been shown to eliminate the periodicity of several of the known clock genes in hypothalamic regions, including the arcuate nucleus (Chen, et al., 2004; Li, et al., 2009); therefore, it is unlikely that these genes could mediate the expression of drug-entrained circadian activity episodes. Further, both the methamphetamine-sensitive circadian oscillator (Mohawk, et al., 2009) and the food-entrainable oscillator (Storch & Weitz, 2009) have been shown to be able to operate without the functioning of all of the known circadian clock genes. If drug-entrainable circadian activity episodes are indeed mediated by an undiscovered set of molecular timing mechanisms, the best place to look for these mechanisms would appear to be the endogenous opioid and orexin neurons discussed above.

### **Treatment Implications**

The expression and persistence of post-nicotine circadian activity episodes were significantly reduced in almost all of the treatment conditions in the present study. The persistence of these episodes was significantly reduced both by mecamylamine, a drug that can increase nicotine craving in smokers (Nemeth-Coslett, et al., 1986), and by varenicline, a drug that can alleviate nicotine craving in smokers (Rollema, et al., 2007).

Due to these conflicting results, post-nicotine circadian episodes do not appear to be an accurate indicator of the efficacy of smoking cessation treatments.

As stated earlier, pre-drug circadian episodes appear to represent a circadian-based craving, anticipation, and/or seeking response that would presumably be an important target for treatments in the early stages of drug abstinence, particularly for smokers who show strong circadian rhythms of smoking behavior. Based on this assumption, several of the pharmacological treatments currently available for nicotine addiction, including varenicline, bupropion, and topiramate, appear to exacerbate this circadian activity, and therefore may be more efficacious if administered after a smoker has been abstinent for a week or more. All of these smoking cessation treatments have been reported to alleviate craving in human smokers, but none have shown 100% efficacy. The results of the present study also imply that the “cold turkey” method of smoking cessation may be better than some forms of pharmacologically-assisted cessation in the early stages of abstinence.

An alternative interpretation of these results is that pre-nicotine episodes may not represent a form of craving, as varenicline, bupropion, and topiramate have been shown to alleviate nicotine cravings, and each of these treatments increased pre-nicotine activity. However, treatment with mecamylamine, which does exacerbate nicotine cravings, significantly increased the expression of pre-nicotine wheel-running in the present study. Therefore, it seems likely that the therapeutic efficacy of varenicline, bupropion, and topiramate may be limited to stress-, cue-, and drug-induced craving and not to circadian-based craving. Finally, the results of the present study suggest that naltrexone and/or SB-334867 should be considered for the treatment of circadian-based nicotine craving,

particularly in the first week of abstinence and particularly for smokers who show strong circadian smoking patterns.



## Conclusion

In this and previous studies, nicotine and other drugs of abuse have been shown to entrain robust pre- and post-drug circadian activity episodes when repeatedly administered at a consistent time of day. These episodes persist for several days after drug use ceases, and their periodicity is independent of the free-running light-entrainable locomotor activity rhythm. Pre-drug episodes may be a useful behavioral target for the treatment of drug addiction, as they appear to represent a circadian-based drug craving, anticipation, and/or seeking motivation. Several pharmacological treatments that are currently prescribed to alleviate the nicotine craving associated with smoking cessation appear to exacerbate pre-nicotine episodes. Two treatment drugs that significantly reduced the expression and/or persistence of pre-nicotine activity episodes were the  $\mu$ - $\kappa$ -opioid receptor antagonist naltrexone and the orexin-1 antagonist SB-334867. These drugs also had similar effects on post-nicotine episodes.

Overall, there are two major conclusions that can be drawn from this work. First, naltrexone and SB-334867 may be efficacious in the treatment of nicotine and other drug addictions, particularly for drug users who show strong circadian rhythmicity in their drug consumption. Second, the timing of pre- and post-nicotine circadian activity episodes appears to be mediated by currently undiscovered molecular clock mechanisms that are at least partially separate from the known circadian clock genes and are likely located in  $\beta$ -endorphin-transmitting neurons in the arcuate nucleus and orexin-transmitting neurons in the lateral hypothalamus.

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**Doctor of Philosophy Program in Neuroscience & Psychology** 2005-present

*University of Guelph, Guelph, ON, Canada*

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**AWARDS**

Graduate Research Fellowship, Indiana University Bloomington 2008-2010  
Integrative Predoctoral Training in Drug Abuse Research Project (1 of 2 awarded)

Best Student Poster Award, American Psychological Association 2008  
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Graduate Research Fellowship, Indiana University Bloomington 2005-2008

International Entrance Scholarship, University of Guelph 1999-2000

**TEACHING EXPERIENCE**

*Department of Psychological & Brain Sciences, Indiana University, Bloomington, IN*

**Graduate Laboratory Instructor** - "Methods in Psychology" 2008

Developed laboratory syllabus and grading rubrics, collaborated on assignment curriculum, instructed two weekly laboratory sections, met with students upon request, graded all written work, and administered final course grades.

*Center for the Integrative Study of Animal Behavior, Indiana University, Bloomington, IN*

**Graduate Coordinator** – Research Experience for Undergraduates Program 2008

Mentored undergraduate students performing research projects, assisted in organization of undergraduate research conference.

**RELATED EXPERIENCE**

*Charles River Laboratories Preclinical Services – Pennsylvania Division, Horsham, PA*

**Laboratory Technician I-III** - Reproductive Toxicology, Neurotoxicology 2003-2005

Performed technical procedures and daily animal care and maintenance for reproductive toxicology and neurotoxicology studies.

## PUBLICATIONS

### **Research Papers:**

Gillman, A.G., Leffel, J.K., Kosobud, A.E.K., & Timberlake, W. (2009). Fentanyl, but not haloperidol, entrains persisting circadian activity episodes when administered at 24- and 31-hour intervals. *Behavioural Brain Research*, 205(1), 102-114.

Gillman, A.G., Kosobud, A.E.K., & Timberlake, W. (2008). Pre- and post-nicotine circadian rhythms can be differentiated by a paired environmental cue. *Physiology and Behavior*, 93(1-2), 337-350.

Gillman, A.G., Kosobud, A. E. K., & Timberlake, W. (2010). Effects of multiple daily nicotine administrations on pre- and post-nicotine circadian activity episodes in rats. *Behavioral Neuroscience (submitted)*.

### **Reviews:**

Kosobud, A.E.K, Gillman, A.G, Leffel, J.K., Pecoraro, N.C., Rebec, G.V., & Timberlake, W.D. (2007). Drugs of abuse can entrain circadian rhythms. *TheScientificWorldJOURNAL*, 7(S2), 203-212.

### **Abstracts:**

Gillman, A.G., Kosobud, A.E.K., & Timberlake, W. (2009). Pre- and post-nicotine circadian activity episodes are differentially affected by pharmacological treatments for drug abuse. Abstract for poster presentation, American Psychological Association Annual Meeting, Toronto, ON and Society for Neuroscience Annual Meeting, Chicago, IL.

Kosobud, A.E., Gillman, A.G., Leffel, J.K., Rebec, G.V., Timberlake, W. (2009). Alcohol entrains circadian rhythms. Abstract for poster presentation, Society for Neuroscience Annual Meeting, Chicago, IL.

Timberlake, W.D., Gillman, A.G., Leffel, J.K., Kosobud, A.E.K. (2009). A 31-hr administration schedule for drugs of abuse shows one-trial entrainment of circadian activity episodes instead of anticipatory conditioning of activity. Abstract for poster presentation, Society for Neuroscience Annual Meeting, Chicago, IL.

Gillman, A.G., Kosobud, A.E.K., & Timberlake, W. (2008). Amplitudes of nicotine-evoked activity episodes decline across the day independent of dosage. Abstract for poster presentation, American Psychological Association Annual Meeting, Boston, MA, and Society for Neuroscience Annual Meeting, Washington, D.C.

Gillman, A.G., Leffel, J.K., Kosobud, A.E.K., & Timberlake, W. (2008). Rats anticipate the effects of addictive drugs in a circadian pattern. Abstract for poster presentation, Society for Research on Biological Rhythms Biennial Meeting,

Sandestin, FL, and Center for the Integrative Study of Animal Behavior  
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Gillman, A.G. & Timberlake, W. (2008). Rats anticipate the effects of addictive drugs in a circadian pattern. Abstract for platform presentation, Winter Conference on Animal Learning and Behavior, Winter Park, CO, and Tri-State Animal Learning and Behavior Conference, Lexington, KY.

Timberlake, W., Gillman, A.G., Leffel, J.K., & Kosobud, A.E.K. (2007). Nicotine and fentanyl entrain circadian activity patterns in rats under both 24- and 31-hour schedules. Abstract for poster presentation, Society for Neuroscience Annual Meeting, San Diego, CA.

Gillman, A.G. & Timberlake, W. (2007). Nicotine-induced circadian activity patterns under fixed and variable zeitgebers. Abstract for poster presentation at American Psychological Association Annual Meeting, San Francisco, CA, and Society for Neuroscience Annual Meeting, San Diego, CA.

Gillman, A.G. & Timberlake, W. (2007). Nicotine-induced circadian activity patterns under fixed and variable zeitgebers. Abstract for platform presentation at Tri-State Animal Learning Conference, West Lafayette, IN, and Center for the Integrative Study of Animal Behavior Conference, Bloomington, IN.

Gillman, A.G., Kosobud, A.E.K., & Timberlake, W. (2007). Pre- and post-nicotine circadian activity rhythms are differentiated by a paired auditory stimulus. Abstract for poster presentation, Nebraska Symposium on Motivation, Lincoln, NE.

Gillman, A.G. & Timberlake, W. (2006). Auditory stimuli paired with the effects of nicotine injections interfere with anticipatory activity. Abstract for poster presentation at Animal Behavior Society Annual Meeting, Snowbird, UT, and Society for Neuroscience Annual Meeting, Atlanta, GA.

Gillman, A.G. & Timberlake, W. (2006). The role of circadian rhythms and Pavlovian conditioning in nicotine addiction. Abstract for platform presentation at Tri-State Animal Learning Conference, Indianapolis, IN, and Center for the Integrative Study of Animal Behavior Annual Conference, Bloomington, IN.

#### PROFESSIONAL CERTIFICATIONS

American Association for Laboratory Animal Science  
Assistant Laboratory Animal Technician (ALAT)

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#### PROFESSIONAL ASSOCIATIONS

American Association for the Advancement of Science

American Psychological Association (Graduate Student Affiliate)

Division 6: Behavioral Neuroscience and Comparative Psychology  
Executive Committee Student Representative

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Representative to the General Assembly – Psychology Graduate Program 2007-  
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