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# The Limited Effects of REM Sleep Deprivation on the Acquisition, Extinction, and reinstatement of a methamphetamine-induced Conditioned Place

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# THE LIMITED EFFECTS OF REM SLEEP DEPRIVATION ON THE ACQUISITION, EXTINCTION, AND REINSTATEMENT OF A METHAMPHETAMINE-INDUCED CONDITIONED PLACE PREFERENCE

by

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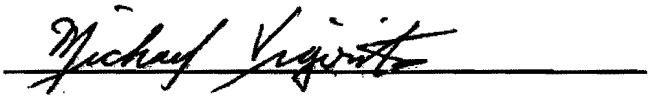

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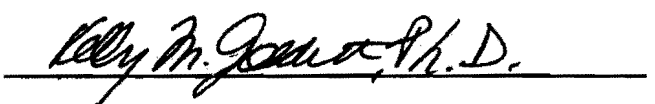

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Dedication

*To my family, friends, and girlfriend for their unwavering love, support, and encouragement*

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## Abstract

The abuse of psychostimulant drugs is a national health concern. Methamphetamine (METH) is a psychostimulant that is frequently abused due to its strong potency. The conditioned place preference (CPP) paradigm serves as an animal model of subjective drug effects that utilizes Pavlovian contextual conditioning to assess the rewarding properties of a drug by pairing it with a specific environment. Rapid-eye movement (REM) sleep is a stage of sleep with unique characteristics that has been linked to learning and memory in studies that have demonstrated REM sleep deprivation (RSD)-induced impairments of these faculties (e.g. Alvarenga et al., 2008; Ishikawa et al., 2006; Smith & Rose, 1995; Smith et al., 1998). The goal of the present study was to investigate the effects of RSD on drug preference and locomotor activity by examining the effects of RSD during the acquisition phase on the acquisition, extinction, and reinstatement of a METH-induced CPP. Acquisition consisted of sixteen days of alternating injections of either METH or saline that were administered every other day and then subsequent isolation to a particular chamber in the CPP apparatus. After METH administration, rats were either deprived of REM sleep for six hours or were allowed to sleep undisturbed in a control condition for six hours. The day after conditioning was concluded, preference for the METH-paired chamber and its possible alteration by RSD were assessed. During the subsequent phase of extinction, all rats were given free access to the entire apparatus until no chamber preference was apparent. Finally, in order to measure the effects of a stressor on the reinstatement of a CPP, rats received either ten unsignaled low-voltage footshocks or no shock treatment. All rats were then tested for preference one final time to evaluate whether the stressor had facilitated a reinstatement of preference for the METH chamber. Results showed that a METH-induced CPP was established in both groups, but RSD ultimately had no effect on either drug preference or locomotor activity during the acquisition, extinction, or reinstatement phase. These results suggest that six hours of RSD during acquisition may not be sufficient for affecting contextual conditioning and that six hours of RSD that occur every four days may not have an effect on the formation of, abstinence from, or relapse to METH addiction.

# The Limited Effects of REM Sleep Deprivation on the Acquisition, Extinction, and Reinstatement of a Methamphetamine-Induced Conditioned Place Preference

The abuse of psychostimulant drugs is a public health concern in the United States. One such drug of abuse – and the particular drug that will be investigated in this study – is methamphetamine (METH), a more synthetic version of its derivative, amphetamine (AMPH). METH is known to be a "street drug" and is commonly synthesized in underground, illegal laboratories. Its rates of addiction have increased throughout the years as well, with adolescents being especially vulnerable to its addictive properties (Zakharova et al., 2009). According to the 2009 National Survey of Drug Abuse and Health, the number of METH users in the U.S. population rose from 2.5 in 2008 to 2.8 percent in 2009 – more specifically, from 314,000 reported users to 502,000 reported users within one year. METH addiction is not just a problem in the United States, however; it is a global problem as well. According to Cruickshank and Dyer (2009), METH is the second most popular illicit drug abused worldwide.

The model that will be used to investigate drug addiction in this particular study is the conditioned place preference (CPP) paradigm, a Pavlovian animal model of subjective drug effects that uses the concept of contextual conditioning to investigate the associations between an unconditioned stimulus (US) – the drug – and a conditioned stimulus (CS) – the environment in which the drug is consumed. CPP studies examine the subjective drug effects that an organism experiences in a particular environment after consuming a drug and assess how the association that the organism creates between the drug properties and the environment contribute to addiction formation. Previous research has demonstrated a link between sleep and the CPP (Sharp, 2012; Shi et al., 2011), which will be further explored in the present study

## Drug Addiction and Methamphetamine

*Drug Addiction and Its Underlying Neural Mechanisms*

Repeated use of a drug can lead to drug addiction – a state in which the body is so dependent on the drug that extended withdrawal from it will cause unpleasant symptoms (Meyer & Quenzer, 2005, p. 190). Addiction is often characterized by an intense, compulsive desire, or craving, for the abused drug that will motivate an individual to seek out the drug, despite acknowledgement of the harmful consequences that may arise as a result of prolonged use (p. 190). In one model of drug addiction, the positive reinforcement model, it is said that an addict will seek out a drug in order to reinstate – and maintain as long as possible – the pleasurable rewarding feelings that are associated with use of the drug (p. 198).

The striatum and the core and shell of the nucleus accumbens have been implicated in reinforcing the pleasurable effects of a drug (e.g. Everitt & Robbins, 2005). Increased firing of dopamine (DA) neurons in the midbrain and in the nucleus accumbens core has been shown to occur in response to Pavlovian conditioned stimuli (e.g. an environment), indicating that this brain area and particular neurotransmitter play a critical role in drug addiction. Everitt and Robbins (2005) also report that lesions of the nucleus accumbens core or infusions of DA receptor antagonists during drug conditioning impairs the acquisition of a conditioned response, whereas, infusions of these antagonists into the nucleus accumbens core after conditioning impairs reconsolidation of the reward memory associated with the acquisition of the conditioned response. The nucleus accumbens core has also been implicated in the motivated behavior that is exhibited during drug-seeking (Robbins et al., 2008). The mesocorticolimbic DA pathway (which projects into the nucleus accumbens shell) has been implicated in mediating the increasing rate of a response that is made to the drug (Everitt & Robbins, 2005; Koob, 2005). So

it appears that the nucleus accumbens core seems to be responsible for the desire and motivation for drug-seeking, while the nucleus accumbens shell is responsible for increasing the rate of drug-seeking behavior itself. Additionally, the amygdala (Everitt & Robbins, 2005; Koob, 2005; Koob, 2009; Robbins et al., 2008) and the prefrontal cortex (Everitt & Robbins, 2005; Koob, 2005; Robbins et al., 2008) have also been said to play a critical role in drug addiction. Koob (2005) emphasizes the role of the amygdala during reinstatement – relapse to drug-taking behavior that was previously extinguished – of an addiction, in particular. Koob makes an interesting distinction by claiming that drug addiction is initially mediated by positive reinforcement (e.g. the addictive behavior is sustained by the pleasurable feelings associated with the drug), but after prolonged drug use (to the point where physical dependence is reached), addiction is mediated by negative reinforcement (e.g. the desire to remove unpleasant physical symptoms that are experienced during withdrawal). Koob (2009) claims that the amygdala plays a critical role in the latter case, especially during times when stress is experienced during withdrawal. In response to stress hormones (e.g. corticotropin-releasing factor; CRF) being released during withdrawal, the amygdala is activated and is thus potentially responsible for causing the drive to alleviate the symptoms by seeking out the drug. Aversive withdrawal symptoms have even been shown to be alleviated by stress hormone antagonists (Koob, 2005). Therefore, it is possible that a combination of CRF secretion and activation of the amygdala may be responsible for relapse to addiction after a period of abstinence. The prefrontal cortex (PFC) is typically responsible for the goal-directed behavior that is involved in the addiction – namely, it is involved in actually *choosing* to take the drug again. The PFC's role is said to be responsible for the development of "habits," which, as defined by Everitt and Robbins (2005), is the persisting strength of a craving for a drug even after the drug itself has been devalued. So, even

when an addict's craving has been momentarily satiated by consumption of the drug, the persistent desire for more is mediated by the PFC.

It is important to note that most research (Everitt & Robbins, 2005; Koob, 2005; Koob, 2009; Robbins et al., 2008), acknowledges that different brain structures contribute to different aspects of drug addiction; there does not seem to be a particular brain structure that is the "center of addiction." Rather, it is an interconnected circuit of structures, typically mediated by DA, that work together to produce and reinforce drug addiction.

*Reward Memory and Drug Abuse*

In addition to examining drug abuse and addiction within a learning context (e.g. the positive reinforcement and negative reinforcement models discussed in Koob, 2005), they can also be examined from the perspective of memory formation. Reward memory often occurs as a result of associative learning. After repeated experiences with a drug, a person learns to associate the pleasurable (or rewarding) effects of the drug with the presentation of the drug. By learning what to expect after consuming the drug, the individual establishes reward memory – namely, recalling that "taking this drug makes me feel good." The reward memory elicited by presentation of the drug is then reconsolidated; this occurs after each time that the memory is retrieved and reactivated (Lee et al., 2006). As mentioned previously, the nucleus accumbens core and DA release are critical neuroanatomical and neurochemical substrates for the acquisition of a conditioned drug response (CR; Flagel et al., 2011). Lee and colleagues (2006) investigated whether blocking the reconsolidation of reward memories that were elicited by the presence of cocaine before the reactivation of those memories would impair subsequent reinstatement. After being conditioned to self-administer cocaine in the presence of a CS (a light), rats were injected with Zif268 ASO/ missense oligodeoxynucleotides (MSO) – an enzyme

that targets and breaks down the protein, Zif268, which has been linked to diminished fear conditioning (Lee et al., 2005) – into the basolateral amygdala to inhibit reconsolidation of reward memory before being presented with the light again. A single infusion of Zif268 ASO three days after the last self-administration of cocaine during conditioning eliminated any further cue-induced drug seeking (Lee et al., 2006). In other words, the association between the light and reward memory was abolished by inhibition of protein synthesis in the basolateral amygdala, which severely impaired the maintenance of the reward memory. These data support the claim that addiction may be directly linked to the successful retrieval and reconsolidation of a drug reward memory. However, further research needs to be conducted in order to determine whether impairing the reconsolidation of a reward memory is sufficient to override the unpleasant withdrawal symptoms that facilitate relapse – namely, the part of drug addiction that is facilitated by negative reinforcement.

*Methamphetamine*

METH is a synthetic psychostimulant drug that is derived from amphetamine (AMPH); however it has a more potent effect on the central nervous system than AMPH does (Meyer & Quenzer, 2005, p. 294). Due to its potency, it tends to be favored by substance abusers and is often ingested orally, snorted, injected intravenously, or smoked in order to achieve a "high." (p. 294). As with most drugs of abuse, repeated abuse of METH eventually leads to addiction to the drug.

Due to the similarity in chemical structure that METH shares with DA (Cruickshank & Dyer, 2009; Meyer & Quenzer, 2005, p. 292), the psychostimulant serves as an indirect agonist at DA receptors, in addition to serving as one at serotonin (5-HT) and norepinephrine (NE) receptors (Cruickshank & Dyer, 2009). METH molecules substitute for neurotransmitter

transporters – namely, the DA transporter (DAT), norepinephrine transporter (NET), serotonin transporter (SERT), and the vesicular monoamine transporter-2 (VMAT-2; Cruickshank & Dyer, 2009; Sulzer et al., 2005) – in the presynaptic membrane. METH molecules also disrupt the pH level that is responsible for maintaining storage of the neurotransmitters in their respective synaptic vesicles. By doing this, METH reverses the function of the neurotransmitter transporters and causes the release of DA, 5-HT, and NE into the synapse, which then bind to and activate postsynaptic receptors (Cruickshank & Dyer, 2009; Sulzer et al., 2005). METH maintains the release of these neurotransmitters by serving its secondary role as a monoamine oxidase inhibitor (Sulzer et al., 2005).

Each circuit within the brain contains a particular neurotransmitter that facilitates communication within that circuit. Drugs target circuits that contain specific neurotransmitters. Since different brain circuits can possess a common neurotransmitter, a drug that targets a particular neurotransmitter can simultaneously affect multiple circuits; thus, a variety of brain areas are susceptible to the effects of a drug. As mentioned previously, METH primarily targets DA, 5-HT, and NE circuits. DA circuits include the mesolimbic, mesocortical and nigrostriatal pathways (Cruickshank & Dyer, 2009), with the mesolimbic circuit also playing a role in reward memory. Serotonergic pathways are widely distributed throughout the brain and target various areas. NE pathways are prominent in the medial basal forebrain, hippocampus, and PFC (Cruickshank & Dyer, 2009). These structures provide a neurobiological substrate for the effects of METH on arousal, memory, and other cognitive functions. The dosage of METH determines the behavioral effects that manifest themselves as a result of METH's influence on these particular brain areas. Acute to moderate doses of METH produce responses such as reduced fatigue, heightened arousal, increased confidence, reduction in sleep time, and short-term

improvement in certain cognitive domains (e.g. sustained attention; Cruickshank & Dyer, 2009; Meyer & Quenzer, 2005, p. 295). Conversely, chronic doses of METH produce aversive physiological responses such as tachycardia, hypertension, and increased rates of breathing, as well as psychotic responses such as visual and auditory hallucinations, paranoia associated with delusions of persecution, and disorderly behavior (Cruickshank & Dyer, 2009; Meyer & Quenzer, 2005, p.295).

Research has also yielded data on other properties of METH, such as its ability to alter the perceived rewarding effect of the drug by enhancing the perceived rewarding effects of subsequent METH administration after a neurotoxic dose (a dose that causes damage at the neuronal level, often manifesting itself in the form of severe depletions of neurotransmitter levels) of it has been administered. This was demonstrated in a study conducted by Gehrke and colleagues (2003), in which the researchers divided a sample of Sprague-Dawley rats into four groups. All groups were pre-treated with four injections of 10.0 mg/kg of METH spaced two hours apart, ensuring that all rats had received a neurotoxic dose of METH before beginning the acquisition phase of the CPP. During the acquisition phase of the METH-induced CPP, three groups were conditioned with METH (either 0.1, 0.3, or 1.0 mg/kg) and saline in an alternating eight-day cycle, while the fourth group received saline on all eight days. Results showed that the rats that received 0.3 mg/kg of METH during the acquisition phase spent significantly more time in the METH-paired chamber than they did in the saline-paired chamber (Gehrke et al., 2003), compared to the other three groups. All rats were sacrificed after CPP data collection, and their brains were removed to measure levels of DA and 5-HT. Results showed that rats that were pre-treated with neurotoxic levels of METH had significantly lower levels of DA than did controls in the striatum, nucleus accumbens, and PFC. There were significantly lower levels of 5-HT in

these areas as well (Gehrke et al., 2003). These data suggest that neurotoxic doses of METH enhanced the METH-induced CPP, which may explain the "snowballing" effect of METH use – that is, severely depleted levels of DA and 5-HT may increase the rewarding effect, which may fuel escalated METH use (Gehrke et al., 2003). As a result, it is possible that neurotoxic doses of METH enhance reward learning for the drug by enabling the organism to more quickly learn the rewarding properties of it and to more effectively recognize and anticipate those rewarding effects during future exposures. METH-induced CPP has also been demonstrated to be affected by other psychostimulants. Lan and colleagues (2009) demonstrated that a combined administration of morphine and METH resulted in a greater METH-induced CPP and a longer-lasting CPP than when either morphine or METH was administered alone, thus providing data about the synergistic mechanisms that underlie polydrug abuse as well as providing data about METH's ability to be enhanced by other psychostimulants.

## REM Sleep, Memory, and Learning

### *Characteristics of REM Sleep*

Rapid Eye Movement (REM) sleep is a period within the sleep cycle that is characterized by increased heart rate and breathing, atonia (the loss of muscle tone), rapid back-and-forth movements of the eyes, and brain wave patterns that resemble those exhibited during wakefulness (Meyer & Quenzer, 2005, p. 425). During atonia, the skeletal musculature becomes paralyzed, making it completely rigid. REM sleep typically occurs about four or five times each night, increasing in duration with each cycle, and is often the period of sleep associated with dreaming (p. 425). Since the body is paralyzed during REM sleep, yet the brain is as active as it is during wakefulness, this stage of sleep is also sometimes referred to as "paradoxical sleep"

(Alvarenga et al., 2008; Hernandez & Abel, 2011; Martins et al., 2008; Silva et al., 2004; Smith & Rose, 1994; Smith et al., 1998; Yang et al., 2008a).

*The Neurobiology of Sleep and Memory*

Two primary theories have been proposed about the neurological relationship between sleep and memory (Diekelmann & Born, 2009; Hernandez & Abel, 2011). The first is the "active systems" theory, which proposes that cellular activity that occurred during the acquisition of learned events during wakefulness repeats itself during sleep in order to recapitulate learned events (and thus, lead to effective consolidation of them; Hernandez & Abel, 2011). The other theory is "synaptic homeostasis," which suggests that during sleep, synapses that underwent weak encoding during the day are eliminated (a form of synaptic pruning), so that the information from the more efficient encoding of the stronger synapses can be processed more efficiently into long-term memory (Hernandez & Abel, 2011). It has also been suggested that the cAMP-PKA-CREB (3'-5'-cyclic adenosine monophosphate; protein kinase A; cAMP response element binding protein) pathway may be a molecular underpinning for these theories, since this pathway is activated during the reconsolidation of memories (Hernandez & Abel, 2011). In the context of REM sleep, they suggest that lower levels of NE binding throughout the brain may also be responsible for the memory impairments that occur during REM deprivation (Hernandez & Abel, 2011). Tasks that require more complex forms of learning and memory, such as complex maze learning (Henneven & Leconte, 1977), discriminative learning and probability learning (Henneven & Leconte, 1977), instrumental conditioning (Peigneux et al., 2001), and contextual fear conditioning (Vecsey et al., 2009) have been demonstrated to be particularly sensitive to REM deprivation (Hernandez & Abel, 2011). This suggests that more complex forms of associative learning are more susceptible to impairments caused by REM deprivation.

REM sleep has also been associated with increased levels of DA, particularly in the ventral tegmental area (VTA) of the brain (Dahan et al., 2007; Martins et al., 2008). Therefore, it may be possible that reduced NE levels and decreased DA levels that can occur as a result of REM deprivation may be associated with memory impairment.

REM sleep deprivation (RSD) has been shown to impair learning and memory by adversely impacting long-term potentiation (LTP). LTP is a cellular model for learning and memory consolidation that proposes that frequent stimulation of neurons facilitates development of more efficient synaptic transmission between them, which, in turn, facilitates learning (Ishikawa et al., 2006). Ishikawa and colleagues (2006) demonstrated that 24 hours of RSD after induction of LTP impaired the subsequent maintenance of the LTP in the perforant path-dentate gyrus pathway. In addition, a 4-hour period of uninterrupted sleep, which was presumed to include a REM rebound, after 48 hours of RSD could not reverse this impairment of LTP. Taken together, these findings demonstrate that REM sleep after LTP induction is critical for maintaining learning and that it is necessary for REM sleep to occur immediately after induction of LTP. This supports some findings about post-learning REM sleep being critical in retaining a learned task after acquisition of it (Alvarenga et al., 2008; Silva et al., 2004); however, it also contradicts some findings, such as Alvarenga and colleagues' claim (2008) that a 24-hour period of REM rebound was sufficient for reversing the mnemonic impairments caused by RSD. Due to these contradictions in the current literature, further research is necessary to determine whether or not REM rebound actually does reverse this impairment. If it does, further research could also investigate the duration of the REM rebound that is required to do so.

*REM Sleep, Learning, and Memory*

An extensive amount of research on both humans (e.g. Gann et al., 2001; Gillin et al., 1994; Saxvig et al., 2009) and on animals (Albert et al., 1970; Alvarenga et al., 2008; Hanlon et al., 2010; Silva et al., 2004; Silvestri, 2005) has been conducted on the associations between REM sleep and memory, learning, motivation, and drug dependence. Since the current study utilized rats, the findings of animal studies will be primarily discussed.

It has been well-established that REM sleep plays a critical role in memory function and learning. For example, Alvarenga and colleagues (2008) demonstrated that 96 hours of RSD impaired the acquisition, consolidation, and retrieval of a discriminative avoidance task in rats. However, these same impairments were reversed by a 24-hour period of recovery sleep (which presumably consisted of longer periods of REM sleep due to prior deprivation), thereby further demonstrating the role that REM sleep plays in the various stages of memory (Alvarenga et al., 2008). The connection between REM sleep and acquisition is also demonstrated by the existence of post-learning "REM windows" (Smith et al., 2004) that occur after acquisition (Alvarenga et al., 2008; Smith et al., 2004). Periods of REM sleep increase immediately after the acquisition of a new learned response, suggesting that REM sleep aids in the consolidation of this new material (Diekelmann & Born, 2010). Other research has demonstrated the temporal role that RSD plays in memory deficits. In other words, depending on *when* a subject is deprived of REM sleep, different outcomes can result. For example, it has been demonstrated that depriving a mouse of REM sleep 72 hours prior to a memory test significantly impaired memory retention (Silva et al., 2004). Post-learning RSD does not seem to impair short-term retention of a task, but it does seem to impair the consolidation of that task into long-term memory (Silva et al., 2004). Silva and colleagues (2004) further suggest that the neurological mechanisms for the post-learning-RSD

disruption of long-term memory consolidation of the task could possibly be attributed to RSD disrupting the neural and molecular mechanisms that actively maintain a memory trace before it is fully stored into long-term memory, thus providing a possible mechanism for explaining how RSD that occurs after acquisition of a task directly impacts the subsequent consolidation of that task into long-term memory.

In addition to the acquisition, consolidation, and retrieval of learned tasks, RSD has also been shown to impair the extinction of learned responses. Silvestri (2005) showed that 6 hours of RSD immediately after acquisition of fear conditioning impaired the extinction of a cued conditioning task but did not impair the extinction of a contextual conditioning task in a sample of rats. This implies that in the context of this particular study – which will be using a CPP apparatus (a form of contextual conditioning) to investigate the effects of RSD on the acquisition and extinction of a drug-induced CPP – it is possible that RSD may not impair the extinction of the CPP response.

As mentioned previously, the CPP paradigm operates primarily on the principles of contextual conditioning; namely, the animal becomes conditioned to experiencing a drug's rewarding effects within a particular chamber (context) in which the drug was repeatedly administered – to the point where it will seek to experience those effects by going to that chamber even when not previously administered with the drug. While previous literature seems to suggest that RSD may not affect the extinction of a contextual memory (e.g. Silvestri, 2005), other research has suggested that RSD may impair the acquisition, or formation, of a contextual memory. Ruskin and colleagues (2004), for example, found that 72 hours of RSD prior to contextual fear conditioning training impaired acquisition of the fear memory, as indicated by a significant deficit in freezing behavior when the deprived animals were exposed to the

conditioned context 24 hours later. It should be noted, though, that Ruskin and colleagues' (2004) sleep deprivation paradigm also included heavy reduction in non-REM sleep in addition to complete elimination of REM sleep. In addition, Graves and colleagues (2003) found that depriving a rat of sleep for five hours immediately after contextual fear conditioning training impaired the acquisition and consolidation of the fear memory; however, this was total sleep deprivation and not specifically RSD.

The relationship between RSD and contextual conditioning remains relatively unexplored; however, some results from studies that have reported RSD-induced impairments of spatial learning (e.g. Haguewoud et al., 2010; Smith & Rose, 1995; Smith et al., 1998) merit more research on how RSD impacts contextual conditioning. Contextual conditioning and spatial learning are two forms of learning that both rely on the hippocampus (for a review on the role of the hippocampus in contextual conditioning, Holland & Bouton, 1999). Previous research has demonstrated an acquisition impairment of a Morris water maze task (a form of spatial learning) when rats were subjected to four hours of RSD four hours after acquisition training (Smith & Rose, 1995). In addition, RSD has also been shown to impair acquisition of a different spatial task, the radial arm maze, when subjects were deprived of REM sleep immediately after training (Smith et al., 1998). Neurological explanations for these spatial memory impairments have implicated the hippocampus as a target on which RSD operates. Reduced activity at α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, particularly at the GluR1 receptor, in the hippocampus (Haguewoud et al., 2010) and decreased membrane excitability of pyramidal neurons in the CA1 section of the hippocampus (Yang et al., 2008a) have been linked to RSD. Since RSD-induced impairments of hippocampal function have been linked to impairments of spatial learning, it is quite possible that decrements in

hippocampal function impair contextual conditioning as well. Therefore, within the context of the present study, it is possible that RSD will impair the contextual conditioning that underlies the CPP paradigm. This rationale is further bolstered by studies that have found the hippocampus to be one of the brain structures activated during contextual conditioning to METH in mice (Rhodes et al., 2005) as well as by those that have found that orexin receptors in the dorsal hippocampus are linked to the acquisition of a morphine-induced CPP (Riahi et al., 2013) In addition, previous research that has examined the relationship between sleep deprivation (REM and non-REM) and contextual conditioning have primarily used fear conditioning protocols (e.g. Graves et al., 2003; Ruskin et al., 2004; Silvestri, 2005). The CPP paradigm is a form of contextual conditioning that assesses reward memory. Since the relationship between RSD and contextual reward conditioning remains relatively unexplored, the present study also sought to examine whether there was a relationship between these two factors.

*REM Sleep and Motivation for Reward*

As mentioned previously, the reward learning and memory that underlie drug addiction are primarily mediated by the dopaminergic mesocorticolimbic pathway that spans from the VTA to the nucleus accumbens. Research has demonstrated that increased DA firing in the form of a "bursting pattern" occurs in the VTA during REM sleep and that this rate of firing is similar to that which is exhibited in the presence of a rewarding, appetitive stimulus – such as a preferred food – during wakefulness (Dahan et al., 2007). In the context of drug addiction, it is therefore possible that this increased dopaminergic activity in the VTA could be the mechanism that consolidates drug reward memory during REM sleep. This also suggests that depriving an organism of REM sleep could interfere with the consolidation of drug reward memory by

inhibiting or preventing this "burst" of synaptic DA levels, thus impairing the rate at which an addiction is acquired.

After repeated pairings with a US, a neutral stimulus becomes a CS. In the context of reward learning, this CS also becomes an "incentive stimulus" (Flagel et al., 2011), which motivates the organism to seek out the stimulus in order to experience its rewarding effects. Therefore, depriving an organism of REM sleep produces a deficit in this motivation to seek out the incentivizing stimulus, which could possibly lead to reduced exposure to the stimulus. Hanlon and colleagues (2010) demonstrated this in a study that found RSD-induced impairments in motivation for food reward in rats. In a progressive ratio operant task, rats had to press a lever a certain number of times in order to receive a food pellet. Since the schedule of reinforcement was progressive, the number of lever presses required for reinforcement (pellet distribution) increased after each reinforcement. This measured motivation and display of effort in attaining a reward (food). The final ratio completed by the rat before the session ended was called the "break-point." RSD rats (which were deprived of REM sleep for 120 hours) demonstrated lower break-points at the end of their sessions in comparison to the control rats that followed a regular, uninterrupted sleep schedule, indicating reduced motivation and reward-seeking behavior. However, this reduction was reversed by direct injection of AMPH into the nucleus accumbens (Hanlon et al., 2010). Since food reward and drug reward (Everitt & Robbins, 2005; Robbins et al., 2008) both seem to share the nucleus accumbens as a source for motivated behavior, it is possible that RSD may be able to impair reinstatement of a drug-induced CPP, since it may affect dopaminergic transmission in the nucleus accumbens (Everitt & Robbins, 2005; Hanlon et al., 2010, Robbins et al., 2008). Thus, within the context of drug addiction, it is possible that RSD could therefore reduce an organism's motivation to seek out and experience the rewarding

properties of a drug, which would reduce the amount of exposure to the drug and potentially delay the acquisition of an addiction.

Interestingly, however, data has also shown that sleep deprivation may actually sensitize positive appraisals of rewarding stimuli, thus increasing motivation to seek them out. Gujar and colleagues (2011) found that sleep-deprived people tended to rate the pleasantness of a visual image more highly than controls. Functional magnetic resonance imaging (fMRI) revealed that there was more significant activation in the mesolimbic regions of the brain – which, as previously mentioned, mediate reward-reinforced behaviors (Cruickshank & Dyer, 2009) – of the sleep-deprived people than in the brains of the controls. Thus, the authors hypothesize that sleep deprivation may actually increase reactivity to pleasurable stimuli through the enhancing of the mesolimbic pathways, creating a positive bias that reinforces future exposures to the same stimuli (Gujar et al., 2011). It is important to note, though, that Gujar and colleagues (2011) did not specifically deprive people of REM sleep; the subjects in their study were subjected to general sleep deprivation (e.g. affecting all stages of sleep). However, Albert and colleagues (1970) demonstrated in their research that RSD increased levels of short-term activity in rats, possibly due to a sensitization to environmental stimuli. If further research can demonstrate that RSD sensitizes responses to environmental stimuli, it is possible that there may be a link between that and the biased positive appraisal of visual stimuli that occurs after sleep deprivation, as stated by Gujar and colleagues (2011). In the context of drug addiction, this would imply that RSD may sensitize the appraisal of the drug and produce *more* of an incentive to seek it out.

*REM Sleep Deprivation and Drug Addiction*

Sleep deprivation and drug addiction have been shown to be associated with each other, particularly in the case of relapse. According to research, sleep disturbance is an indicator of

drug relapse, meaning that the more that a person is REM-deprived, the more likely he or she is to relapse (Brower & Perron, 2010; Gillin et al., 1994). Decreased REM sleep causes an increase in "REM sleep pressure" – the combined index of REM latency, REM density, and the amount of time spent in REM sleep (Gann et al., 2001). Gann and colleagues (2001) also noted an association between increased REM sleep and likelihood for relapse in nondepressed alcoholic patients, which Brower and Perron (2010) hypothesized could possibly be generalized as an indicator for relapse to psychoactive substances as well. If this is the case, then it is possible that REM sleep deprivation can actually induce relapse, rather than prevent it. This potentially contradicts the literature that claims that REM sleep impairs the reconsolidation of a drug memory, and thus, reduces the likelihood of relapse.

Shi and colleagues (2011) demonstrated that sleep plays a critical role in morphine reward memory reconsolidation. After being totally sleep-deprived for six hours, rats underwent a morphine-induced CPP test. Results indicated that sleep deprivation had no effect on later retrieval of the memory; however, six hours of sleep deprivation after exposure to the morphine-paired chamber significantly impaired the reconsolidation of the reward memory, which was indicated by the inability to express a morphine-induced CPP during a subsequent preference test (Shi et al., 2011). Thus, it appears that sleep deprivation that occurs after the acquisition of a drug-induced CPP does impair its reconsolidation, and thus, subsequent expression. However, it should be noted that total sleep deprivation, rather than specifically RSD, was implemented in this study. The present study will attempt to investigate whether the effects of only RSD produce similar effects in the impairment of drug reward memory reconsolidation by using the CPP paradigm.

## The Conditioned Place Preference (CPP) Paradigm

The conditioned place preference (CPP) is a paradigm that is used to investigate the rewarding effects of drugs and in animal models – usually rats and mice – of neural mechanisms implicated in addiction. The subjective effects of drugs experienced by the user are an important contributor to the formation of an addiction, and the CPP examines these subjective effects in relation to the environment in which the effects are repeatedly experienced. This particular paradigm employs the principles of Pavlovian classical conditioning to assess the effects of environmental cues on the conditioned responses to drug presentations. In a typical drug-induced CPP experiment, a three-chambered apparatus (usually two larger chambers divided by a smaller third middle chamber), in which each chamber contains distinctive contextual stimuli (e.g. differing visual or tactile cues), is utilized. During the acquisition phase of the experiment, the animal repeatedly receives an injection of a particular drug (unconditioned stimulus; US) prior to placement in one of the chambers and repeatedly receives an injection of saline prior to placement in the other chamber. After multiple drug-environmental pairings, the distinctive stimuli in the drug-paired chamber become conditioned stimuli (CS). During the testing phase, the animal is given unrestricted access to the entire apparatus; however, this time, the drug is not presented at all. The time that the animal spends in each chamber is measured, and if the animal spends more time in the drug-paired chamber than it did in the saline-paired chamber, then it is said that the animal has developed a preference for that chamber due to its learning of the association between the rewarding properties of the drug and the chamber. The development of a CPP is indicative of the animal's associative learning between the CS (the chamber's environment) and the US (the drug; Bardo & Bevins, 2000). Research has also demonstrated that drugs are not the only US that when paired with a context, can elicit a preference. Access to

various natural appetitive stimuli (i.e. US's) can be used to establish a preference, including food Spyraki et al., 1982), social interaction (Calcagnetti & Schechter, 1992), and opportunity to copulate (Meisel et al., 1996). Polston and colleagues (2011) demonstrated that even complex contextual stimuli, such as auditory cues in the form of classical music, were sufficient for establishing CPP to METH.

*Habituation and Baseline*

The first phase of the CPP procedure is habituation and baseline. During habituation, the subjects have free access to all chambers of the CPP apparatus in order to become familiar with it. This negates the effects of novelty-seeking during subsequent training, since a new unfamiliar environment can cause the subject to explore it, which would affect the amount of time that it spends in a particular chamber. Habituation is followed by baseline testing, in which the subjects have free access to all of the chambers again, but this time, the amount of time that they each spend in each chamber is recorded. The baseline data are used for determining if there is a pre-existing preference for a particular chamber. If there is, according to the biased protocol (Aguilar, 2009), the drug is paired with the less-preferred chamber. If there is no pre-existing preference, the drug-chamber pairings are made randomly. The baseline data is used after the CPP test as a reference to determine if there is a difference in the time spent in the drug-paired chamber and, therefore, whether or not a CPP had been established after conditioning.

As explained by Aguilar and colleagues (2009), the CPP has three different types of conditioning protocols that can be implemented during the acquisition phase. The first is the "biased protocol," in which the drug is typically paired with the chamber that the rats spent less time in during the baseline phase (A more detailed explanation of the phases of a CPP will be explained later.) The second is the "unbiased protocol," in which the drug is randomly assigned

to either chamber. The third is the "balanced protocol," in which some of the rats are conditioned to the drug in one chamber, while the other rats are conditioned to the drug in the opposite chamber (Aguilar et al., 2009).

*Acquisition*

The acquisition phase of the CPP begins after the collection of baseline data. Subjects are administered a drug and are then confined to a single chamber. Drug-chamber pairings are alternated with saline-chamber pairings, in which animals receive injections of saline and are confined to the other chamber of the CPP apparatus. The number of drug and chamber pairings, as well as the duration of each pairing and the delay between each pairing, can vary based on the type and dose of drug being administered. After the conditioning trials, the subjects typically learn to associate the drug-paired chamber with the rewarding effects of the drug and are conditioned to prefer that chamber. It is then that a test for preference (a CPP test) is conducted to see if the subjects successfully acquired a preference for the drug-paired chamber. During the test phase, the subjects once again have free access to all chambers of the apparatus. As with baseline, time spent in each chamber is recorded. If the subjects spend more time in the drug-paired chamber, then they are considered to have successfully acquired a CPP.

*Extinction*

During the extinction phase, subjects are isolated to the drug-paired chamber in a drug-free state. After several days of extinction sessions, the subjects learn that the chamber is no longer associated with the effects of the drug. Tests for preference similar to those conducted after the conditioning phase are conducted throughout the extinction trials in order to monitor whether extinction of the preference is occurring. When the subjects are no longer spending significantly more time in the drug-paired chamber than they are in the saline-paired chamber,

extinction has occurred. Alternatively, extinction can also occur by allowing subjects free access to the entire apparatus without administering the drug beforehand. After initially spending more time in the previously drug-paired chamber and not experiencing the drug's rewarding effects there, the animal will eventually spend less time in that chamber. The acquired preference learned during conditioning has been extinguished, and the subjects have learned that the chamber is now no longer paired with the drug.

*Reinstatement*

After some time has passed – typically a week – a final preference test is given after subjects are exposed to either a non-contingent priming dose of the drug or an unsignaled stressor in order to reinstate preference to the previously-drug-paired chamber (Aguilar et al., 2009). This simulates drug addiction relapses that human addicts sometimes suffer when they experience stress after a period of abstinence from the drug. The craving they experience magnifies in intensity after being exposed to stress or to a small sample of the drug. In the context of a CPP, reinstatement is said to have occurred when there is a significant difference between the amount of time spent in the drug-paired chamber and the saline-paired chamber during the reinstatement test. Alternatively, reinstatement can be assessed by comparing time spent in the drug-paired chamber at the end of extinction and after reinstatement (Aguilar et al., 2009).

*Advantages and Disadvantages of Using the CPP Paradigm*

There are advantages and disadvantages of using the CPP paradigm for investigating the rewarding properties of drugs and their subsequent effects on behavior. In a review, Carr and colleagues (1989) indicated several advantages of the CPP, including the following: it is sensitive to low doses of a drug; it can be obtained using only a single drug-pairing, which Bardo

and Bevins (2000) claim is very useful, since it eliminates the effects of tolerance and sensitization that may arise as a result of repeated administrations of the drug during the self-administration procedure; it can measure both reward and aversion to a drug; it is tested when the subject is in a drug-free state, which provides more external validity to the results; it does not require surgery; and it controls for drug dosage (Carr et al., 1989). In addition, it is a flexible enough procedure that can be used with a variety of animal subjects; and it typically yields a monophasic dose-effect curve (meaning that the dose-response relationship of the drug typically changes in one direction) which simplifies statistical analysis and provides more definitive information about whether or not drug reward is increasing or decreasing as time goes on (Bardo & Bevins, 2000).

The paradigm is not without its limitations, however. One such limitation is the possibility that novelty-seeking behavior caused by the administration of the drug itself can impair familiarization to the drug-paired chamber and, thus, present a confound (Bardo & Bevins, 2000). In other words, the administration of the drug in the chamber actually prevents the rat from familiarizing itself with the "true" nature of the compartment, because it is always intoxicated during acquisition; therefore, it is difficult to determine whether true preference or simple novelty-seeking is responsible for increased time spent in the drug-paired chamber during testing day. However, it should also be noted that habituation to the apparatus is a useful way to eliminate this confound, since the animal is experiencing both chambers in a drug-free state, Another limitation is the difficulty to generate dose-effect information (Aguilar et al., 2009; Bardo & Bevins, 2000) due to the between-groups nature of the design and the inability to change doses of the drug during the acquisition phase (Bardo & Bevins, 2000). Due to this limitation, many pharmacological questions about the dose-effect curve of the drug cannot be

answered simply by a CPP (Bardo & Bevins, 2000). Bardo and Bevins (2000) also claim that CPP does not truly simulate drug-taking behavior by human addicts, since it does not involve self-administration. Although many addicts do usually acquire a drug in a particular context, the drug is typically administered by the individual; this behavior is not accounted for in the CPP paradigm. Finally, Aguilar and colleagues (2009) indicate a critical difference in drug dosage during the acquisition phase and during the reinstatement test of a CPP. A chronic dose of the drug is received during the acquisition phase, while an acute, priming dose is received during reinstatement. This limits research into the effects of a chronic dose during reinstatement (Aguilar et al., 2009). In other words, does reinstatement occur differently when a chronic, as opposed to an acute, dose of the drug is presented to an individual who has recently abstained from it?

Despite the limitations of the CPP paradigm, it has been generally agreed upon that it is a very useful method for investigating drug reward and behavior and that the paradigm's benefits outweigh its limitations (Aguilar et al., 2009; Bardo et al., 1993; Bardo & Bevins, 2000).

*METH-Induced CPP*

Many studies have demonstrated the effectiveness of METH in the production of a robust CPP (Gehrke et al., 2003; Kuo et al., 2011; Lan, et al., 2009; Polston et al., 2011; Zakharova et al., 2009). A METH-induced CPP follows the same protocol as a regular CPP; the drug that is used to produce a conditioned response to an associated chamber is METH. A meta-analysis conducted by Bardo and colleagues (1993) provided data about some of the variables that can affect the outcome of a CPP that uses an opiate or stimulant drug. For example, they found that Sprague-Dawley rats and Wistar rats were significantly more sensitive to the effects of an AMPH-induced CPP than rats of other strains (Bardo et al., 1993). In addition, rats seemed more

likely to develop a drug-induced CPP when the apparatus contained three chambers, instead of two (Bardo et al., 1993).

In a neurological context, research has demonstrated that brain areas that are normally activated by METH exposure are also activated with a contextual cue that was paired with the drug itself. Rhodes and colleagues (2005) measured levels of c-Fos protein in several brain areas of mice that had been placed in an environment in which they previously received METH. The PFC, orbitofrontal cortex, and cingulate cortex demonstrated significant levels of activation, as indicated by high levels of c-Fos protein. These areas have also been implicated in cravings, motivation for drug-seeking, and acquisition of drug reward memory (Everitts & Robbins, 2005; Robbins et al., 2008), demonstrating not only the addictive power of METH but also that contextual cues for METH can elicit similar levels of brain activation that direct exposure to METH also elicits (Rhodes et al., 2005). The mediodorsal nucleus of the thalamus (MD) has also been implicated as playing a critical role in METH-induced CPP, as indicated by impaired METH-induced CPP memory retrieval that was caused by lesions to the MD (Kuo et al., 2011). Specifically, this brain area is responsible for the memory retrieval of the learned association between the rewarding effects of METH and environment, suggesting that the MD may also play a critical role in relapse of METH addicts (Kuo et al., 2011). On the neurochemical level, in addition to DA, glutamate has also been implicated in psychostimulant addiction. Gass and colleagues (2009) found that MTEP (3-((2-methyl-l,3-thiazol-4-yl)ethynyl)pyridine, a selective type 5 metabotropic glutamate receptor (mGluR5) antagonist, significantly reduced the reinforcing effects of METH as well as the effects of cue- and drug-induced reinstatement.

The purpose of this study is to further expand on the work of Sharp (2012), which found that RSD did not affect extinction rate during a METH-induced CPP. More broadly, she

concluded that RSD does not always impair learning (Sharp, 2012). In addition, one of the limitations that Sharp (2012) mentioned in her study was that her data lacked sufficient statistical power due to a small sample size. The current study utilized a larger sample of rats in order to increase power. In addition, the current study REM-deprived the rats during the acquisition phase of the CPP, rather than during the extinction phase like in Sharp's (2012) study, in order to assess whether depriving the rats during a different phase of the CPP would produce effects on the other phases. More broadly, this study investigated how RSD affects all phases of a METH-induced CPP – acquisition, extinction, and reinstatement – rather than just on a single phase within the paradigm. Thus, the hypothesis for this study is that RSD will impair the acquisition, extinction, and reinstatement of a METH-induced CPP.

## Method

### Subjects

Twenty-four experimentally-naïve Sprague-Dawley rats were used for this study. All rats were fed on an *ad libitum* schedule. The rats were obtained at approximately 50 days old. They were housed in the Jubilee Hall vivarium on a 12/12h light/dark cycle. Approval of the Seton Hall Institutional Animal Care and Use Committee was obtained before the start of any experimental procedures. Due to the unexpected illness and subsequent euthanasia of five of the subjects throughout the course of the experiment, the number of rats varied at different time points, as noted in appropriate figure legends.

### Apparatus

*CPP Apparatus*

Two identical three-chambered CPP apparatuses (76 cm x 30.5 cm x 30.5 cm) were used (Figure 1). Each was made of plastic and contained removable panels that served as partitions for separating the apparatus into three chambers. The two chambers at each end were 30.5 cm x 30.5 cm, with the center chamber 30.5 x 15cm. In each apparatus, two chambers served as the contextual cues during conditioning. One chamber contained black parallel stripes on its walls to serve as a discriminative stimulus, while the other chamber contained black circles on its walls. In addition to the visual stimuli, olfactory discriminative stimuli were also used in each of the larger chambers (Sharp, 2012). In one chamber, a cotton ball containing three drops of pure mint extract was taped to the lid, while a cotton ball containing three drops of pure lemon extract was attached in the same fashion to the lid of the other chamber. Each box contained two removable panels (10 cm x 13 cm) that served as doorways between the chambers, which allowed the rats access to all the chambers. The middle chamber that separated the two larger chambers contained

no visual or olfactory stimuli. Two stopwatches were used to record time spent in both chambers. A camera mounted on the ceiling above the CPP apparatus was used to record the rats' locomotor behaviors throughout the experiments.

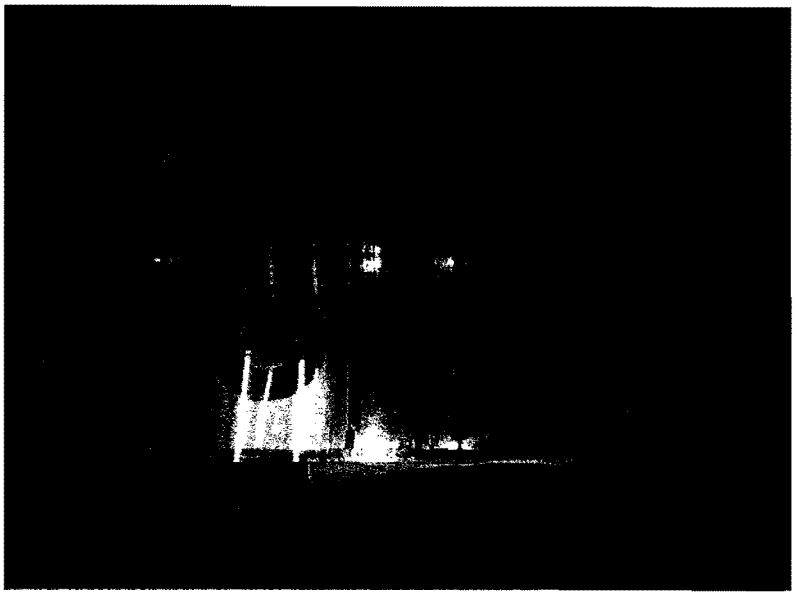

**Figure 1.** Photograph of the CPP apparatus used in this study

*REM Sleep Deprivation Apparatus*

The apparatus used to deprive the rats of REM sleep was similar to the "inverted flowerpot technique" described by Mendelson and colleagues (1974). Inverted flowerpots 10 cm in diameter and 14 cm tall were placed in cylindrical containers that were 33 cm in diameter and 47 cm tall and were filled with water up to 7 cm below the rim of the flowerpot (as shown in Figure 2 with a stuffed animal used as a model). This water level was enough to ensure that the rats' tails did not touch the water if they extended below the edge of the rim of the flowerpot, eliminating any potential thermoregulatory confounds that may have arisen as a result of the rats having their tails in water for an extended period of time (Walsh et al., 2011). When they entered the REM phase of sleep, muscle atonia caused the rats to begin to lose balance (Mendelson et al.,

1974). Either the sensation of falling off the flowerpot or the actual falling into the water awakened the rat, thereby preventing the occurrence of REM sleep. The rats in the control group were placed on inverted pie plates that were 20 cm in diameter (Silvestri, 2005), which were large enough such that they could center REM sleep without interruption. This procedure has been shown to produce selective deprivation of REM sleep but to leave non-REM sleep unaffected (Mendelson et al., 1974).

**Figure 2.** RSD apparatus with a stuffed animal rat model sitting on the inverted flowerpot

*Shock Apparatus*

Stress-induced reinstatement was conducted through the use of two operant conditioning chambers (23 cm x 18 cm x 23.5 cm) made of Plexiglas sides and containing a metal grid floor, which were used to deliver footshocks to the rats. There were levers inside the chambers, but they were not equipped to perform any actions. Shocks (1.0 mA) were produced by an ENV-414

shocker/distributor (MED Associates, Inc., Georgia, VT), and footshock presentations were controlled by a computer program using MED-PC (MED Associates, Inc. Georgia, VT).

**Drugs**

Each rat received 1.0 mg/kg of methamphetamine via intra-peritoneal (ip.) injection during each day of the acquisition phase of the CPP. A meta-analysis conducted by Bardo and colleagues (1993) found that the ip.-route of AMPH administration produced CPPs with larger effect sizes to various drugs, including heroin, cocaine, and amphetamine.

**Procedure**

This experiment investigated the effects of RSD that occurred during the acquisition of a METH-induced CPP in a sample of 19 Sprague-Dawley rats. Three primary measures were calculated in this experiment. The first was chamber preference, in which time spent in each chamber was used to calculate preference proportion scores (to be described in more detail later) – a quantifiable measure of chamber preference. The second was locomotor activity. Due to METH's nature as a stimulant, the crossover activity (i.e. movement from one chamber to the opposite chamber) of the rats was assessed to note any changes in activity level that occur in response to exposure to the drug chamber. The final measure was body weight, which served as a physiological indicator of the rats' physical health throughout the experiment. A timeline and summary of the procedures involved in each phase of this experiment can be viewed in Table 1.

During the first phase of the CPP, baseline, each rat was placed into the CPP apparatus and allowed to roam freely for 15 minutes to become familiarized with all of the chambers within the apparatus. Time spent in each chamber, as well as the number of times that the rat crossed over from one of the two main chambers to the other, was recorded. A rat was considered to be inside a chamber when its entire body (excluding its tail) was inside. This

procedure was repeated for five days in order to ensure that the rats became familiarized with the apparatus, thus minimizing the potentially confounding effects of novelty-seeking during the acquisition phase. These data were analyzed to determine if there was a pre-existing preference for a particular chamber. The biased protocol (Aguilar et al., 2009) was employed in this study. Therefore, if a rat exhibited a preference for a chamber, the non-preferred chamber was paired with the drug and the preferred chamber was paired with saline. If a rat did not exhibit a preference, the drug-paired chamber was randomly assigned. After baseline data were analyzed, it was determined that seven rats would undergo the biased protocol during the acquisition phase, while the remaining twelve would be randomly assigned to the METH-paired chamber.

After baseline, the rats were randomly assigned to one of two groups – nine rats to a REM-deprived (RSD) group and ten to a control group. As described earlier, rats in the RSD group slept on inverted flowerpots that were surrounded by water during the acquisition phase, while the rats in the control group slept on larger inverted pie plates that would not disrupt REM.

During the acquisition phase, all rats received 1 mg/kg (ip) injection of METH and spent 30 minutes in their respective drug-paired chambers (Sharp, 2012). On alternating days, the rats received saline injections in the same volume and in the same route of administration and spent 30 minutes in the opposite chamber. The rats were randomly assigned to one of two squads prior to acquisition – ten in one squad and nine in the other. The squads alternated receiving injections on alternating days; thus, each rat in each squad underwent eight treatment days (four days each of METH and saline), making the acquisition phase last a total of 16 days. Immediately after each METH treatment, the RSD rats (n = 3 in each squad) underwent six hours of RSD by being placed on the inverted flowerpots that were surrounded by water, while the controls were placed on pie plates in the same environment (as described by Silvestri, 2005). Due to a limited number

of RSD apparatuses, no rats were deprived after saline treatment. All rats were placed back into their cages and left undisturbed until the CPP test.

The CPP test occurred the day after the final day of acquisition for the second squad of rats. This phase of the CPP lasted for two days – one day of testing for each squad. All of the rats were allowed to freely access all chambers of the apparatus for 15 minutes – just as was the case during baseline – and time spent in each chamber and number of crossovers were recorded. This data was used to calculate *preference proportion scores* (PPS). Preference proportion score is a measure of level of preference for a particular chamber within the CPP apparatus. It is calculated by dividing the seconds spent in the drug-paired chamber (D) by the seconds spent in both the drug-paired and saline-paired (S) chambers [PPS = D/(D+S)]. A PPS of 0.5 indicated that there was no preference for either chamber. A PPS greater than 0.5 indicated that there was a preference for the drug-paired chamber. A PPS less than 0.5 indicated that there was a preference for the saline-paired chamber. In other words, a preference proportion score that was greater than 0.5 indicated a successful acquisition of the CPP.

After the CPP test, the rats were left undisturbed in their cages for 96 hours. Extinction then began, in which the rats were allowed to freely access all chambers of the apparatus for 15 minutes, and time spent in each chamber and the number of crossovers were recorded. This time, however, the rats did not receive any prior METH or saline injection. PPS was calculated at the end of each day of extinction to monitor the occurrence of extinction. CPP extinction was operationally defined as a rat attaining a PPS less than or equal to 0.53 for three consecutive days, indicating a period of no preference for the previously METH-paired chamber. This phase continued until each rat extinguished its response, which amounted to 33 days in total.

Due to the large variability within the extinction rates of the subjects, the final phase of the CPP, reinstatement, occurred twenty days after extinction. This was determined by separating the subjects into four different cohorts that were matched for the week that the subject successfully extinguished its CPP. Reinstatement testing occurred twenty days after the final subject in each cohort extinguished its CPP. A stress-induced reinstatement was used for this study. Therefore, of the nine rats in the RSD group, five were randomly assigned to the shock group, while the other four were assigned to the no shock group. Additionally, of the ten rats in the control group, five were assigned to the shock group, while the other five were assigned to the no shock group. Due to the malfunction of the shock apparatus on one day of testing, however, a "control/shock" rat had to be moved to the "control/no shock" group, changing the n's of the groups to 4 and 6, respectively. In addition, technical error that occurred on one of the testing days lost the data of four rats – one from each group. Thus, only the data from fifteen rats were analyzed for this particular phase of the experiment. Of the rats that underwent the biased protocol during acquisition, two were in the RSD/Shock group and three were in the Control/No Shock group. Of the rats that underwent random assignment to a METH-paired chamber during acquisition, two were in the RSD/Shock group; three were in the RSD/No Shock group; three were in the Control/Shock group; and two were in the Control/No Shock group. In total, the groups were as follows: RSD/Shock (n = 4); RSD/No Shock (n = 3); Control/Shock (n = 3); and Control/No Shock (n = 5).

Each rat was placed into the shock apparatus, but only seven of them were shocked. The other rats remained in the chambers for an equivalent period of time but did not receive any shock. The rats that were shocked received ten unsignaled footshocks (1.0 mA), that lasted 0.5 seconds each (DiFeo, 2011; Sharp, 2012) for 35 minutes. Immediately, after the shock session, a

final CPP test was conducted to determine whether or not reinstatement had occurred. If the rats spent more time in the chamber that was previously paired with METH, then a reinstatement of the CPP occurred.

**Table 1.** Experimental timeline and summary of procedure. Note: ("Base." = Baseline; "Acq." = Acquisition; "Ext."= Extinction; "Reinst."= Reinstatement)

| Day | Days 1-5 | Days 6-8 | Days 9-24 | Day 25 or 26 | Days 27-30 | Days 31-63 | Day 57, 63, 68, or 77 |
|---|---|---|---|---|---|---|---|
| **Phase** | **BASE.**<br><br>15 min<br><br>Free access to all chambers | **Delay** | **ACQ.**<br><br>Isolation to respective chamber for 30 min. each day<br><br>Alternate between METH (4 days) and saline(4 days)<br><br>RSD rats undergo 6 hours of RSD immediately after each METH treatment<br><br>Control rats undergo control sleep condition for 6 hours (pie plate)<br><br>Saline-treated rats are returned to home cages | **CPP TEST**<br><br>15 min. | **Delay** | **EXT.**<br><br>15 min. each day<br><br>Free access to all chambers of apparatus without prior drug/saline administration<br><br>Extinction is defined as three consecutive days of no displayed preference for the previously METH-paired chamber | **REINST.**<br><br>35 min. of shock or no shock<br>+<br>CPP Test (15 min.) |

**Data Analysis**

A paired-samples *t*-test was used to compare differences in PPS observed at baseline and at the end of acquisition in order to determine whether a CPP to the METH-paired chamber had been successfully established. Independent-samples *t*-tests were used to assess whether there were differences in PPS between the RSD rats and the control rats at the end of acquisition and whether differences in time required to establish successful extinction were dependent on sleep condition. In addition, bivariate correlational analyses were conducted in order to assess whether there were any associations between days required to establish successful extinction and PPS at the end of acquisition as well as PPS at the end of reinstatement. The effects of sleep condition during the acquisition phase and the shock condition during the reinstatement test on reinstatement PPS were assessed using a two-way between-groups analysis of variance (ANOVA). In addition, independent-samples *t*-tests were conducted to assess the specific effects of the shock condition on each sleep group (RSD and control).

Locomotor activity was assessed by analyzing crossover data. Due to the different numbers of days in each experimental phase, two 4 X 2 repeated-measures ANOVAs were conducted to assess whether locomotor activity changed by phase of the experiment or by sleep condition. One analysis analyzed locomotor activity on the first day of each experimental phase while the other analyzed activity on the last day of each experimental phase. In addition, bivariate correlational analyses were conducted in order to determine whether there were any associations between rate of extinction and locomotor activity during either the CPP test or the reinstatement test. In order to determine whether prior sleep condition or shock condition had any effect on crossover activity during reinstatement, a two-way between-groups ANOVA was conducted.

Finally, in order to assess the possible effects of sleep condition on body weight, a physiological indicator of health in the rat, a 5 X 2 repeated-measures ANOVA was conducted. A probability of $p < 0.05$ was considered to be statistically significant for all analyses.

## Results

### Effects of RSD on Acquisition

In order to determine whether a successful CPP to the METH-paired chamber had been established, PPS calculated at the end of the fifth day of baseline (B5) and at the end of the acquisition phase (CPPPPS) were compared. The rats displayed a higher PPS during the CPP test after acquisition [$M_{CPPPPS} = 0.62$, $SE = 0.06$; Figure 3] compared to the fifth day of baseline [$M_{B5} = 0.51$, $SE = 0.04$; Figure 3]. A paired-samples $t$-test confirmed that the rats spent significantly more time in the METH-paired chamber at the end of the acquisition phase than they did at the end of the fifth day of baseline [$t(18) = 2.46$, $p = 0.02$, $d = 0.56$; Figure 3], demonstrating a successful acquisition of a METH-induced CPP with a moderate effect size. As mentioned previously, a PPS > 0.5 indicates a preference for the METH-paired chamber. When compared by sleep condition, both groups displayed a PPS that indicated successful CPP acquisition [$M_{RSD} = 0.66$, $SE = 0.05$; $M_{Control} = 0.59$, $SE = 0.05$; Figure 4]. Although the control group appeared to display less of a preference for the METH-paired chamber, an independent-samples $t$-test confirmed that this difference in PPS between the two groups was not significant [$t(17) = -0.57$, $p = 0.58$, $d = -0.28$; Figure 4]. Thus, RSD during the acquisition phase did not affect acquisition of the CPP.

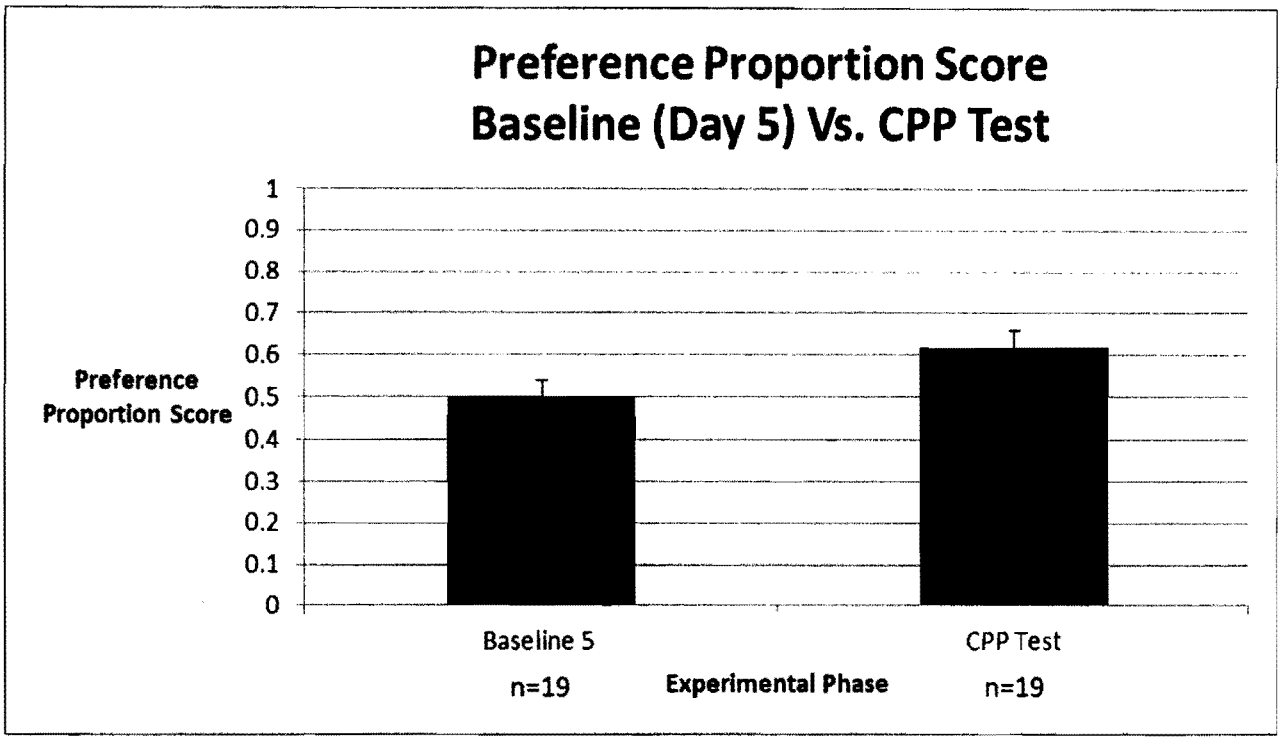


**Figure 3.** Differences in PPS between baseline and the CPP test at the end of acquisition

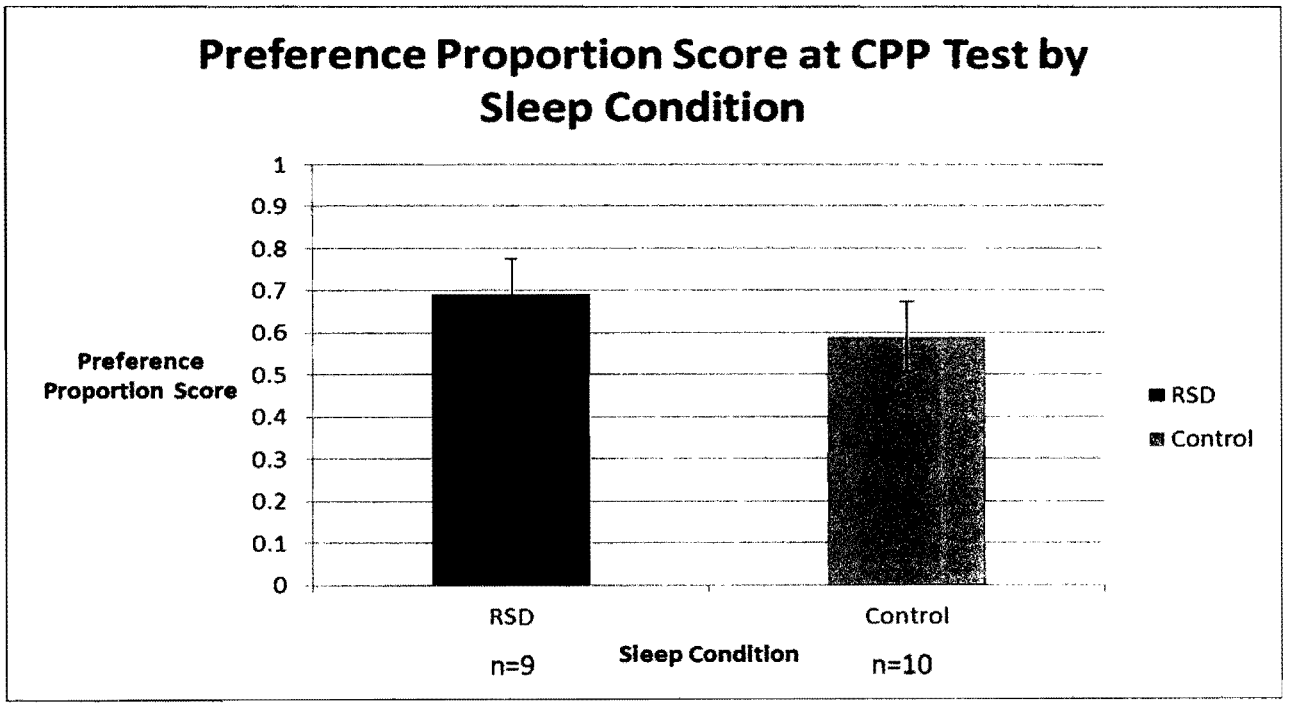


**Figure 4.** PPS at CPP Test by sleep condition

**Effects of RSD on Extinction**

The extinction phase began 96 hours after the CPP test at the end of the acquisition phase. Successful extinction, as mentioned previously, was operationally defined as three consecutive days of no preference for the chamber that was previously paired with METH. Measures of central tendency revealed that rats in both groups took an average of almost 14 days [$M_{RSD}$ = 13.67, $M_{Control}$ = 13.90; range: 3-33] to extinguish their CPPs (Figures 5 and 6) and also that *most* rats in each group took 14 days to extinguish [$Mode_{RSD}$ = 14; $Mode_{Control}$ = 14; Figures 5 and 6]. In order to determine whether prior RSD during the acquisition phase affected the rate at which extinction was established (i.e. how many days it took for the rat to extinguish the CPP), an independent-samples *t*-test was conducted, which revealed that RSD during acquisition had no effect on extinction rate [$t(17) = 0.061$, $p = 0.95$, $d = 0.02$; $M_{RSD}$ = 13.67, $SD$ = 7.28; $M_{Control}$ = 13.90, $SD$ = 9.07; Figures 5 and 6].

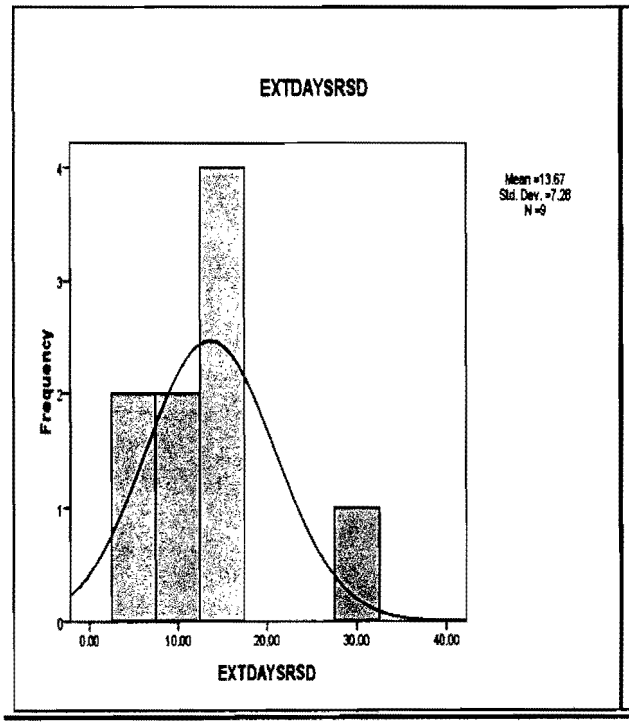


**Figure 5.** Frequency distribution of rate of extinction in RSD subjects

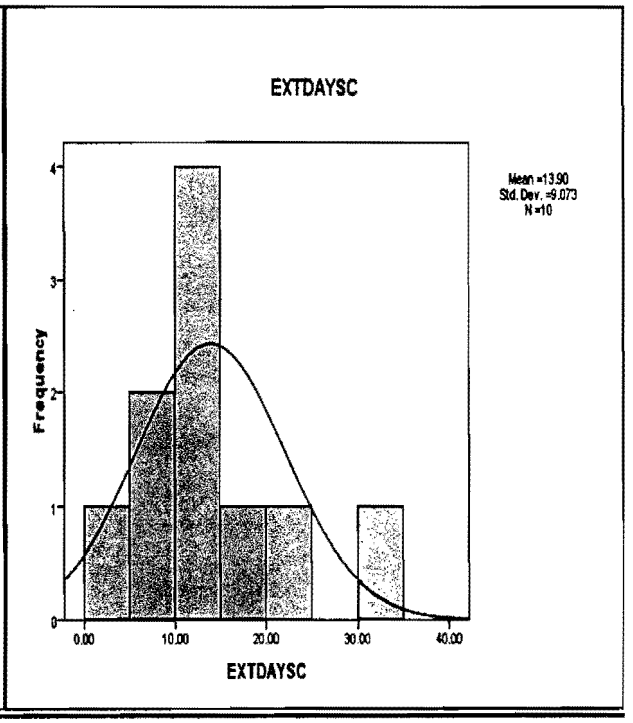


**Figure 6.** Frequency distribution of rate of extinction in control subjects

As evident in figures 5 and 6, a large amount of variability was present in the rate at which subjects extinguished their CPP. Therefore, bivariate correlational analyses using Pearson's product-moment coefficients were conducted to determine whether there was a relationship between the PPS at the end of the acquisition phase or at the reinstatement test (the data of which is reviewed in the next section) and the number of days it took for each subject to extinguish its CPP. The analyses revealed that there was no significant correlation between preference for the METH-paired chamber at the end of the acquisition phase and number of days needed to reach extinction [$r = 0.05, p = 0.85$; Figure 7] nor was there a significant correlation between preference for the METH-paired chamber at the reinstatement test and number of days needed to reach extinction [$r = 0.11, p = 0.73$; Figure 8]. Collectively, these results indicate that the rate of extinction in each rat was not associated with drug preference prior to extinction or to strength of reinstatement.

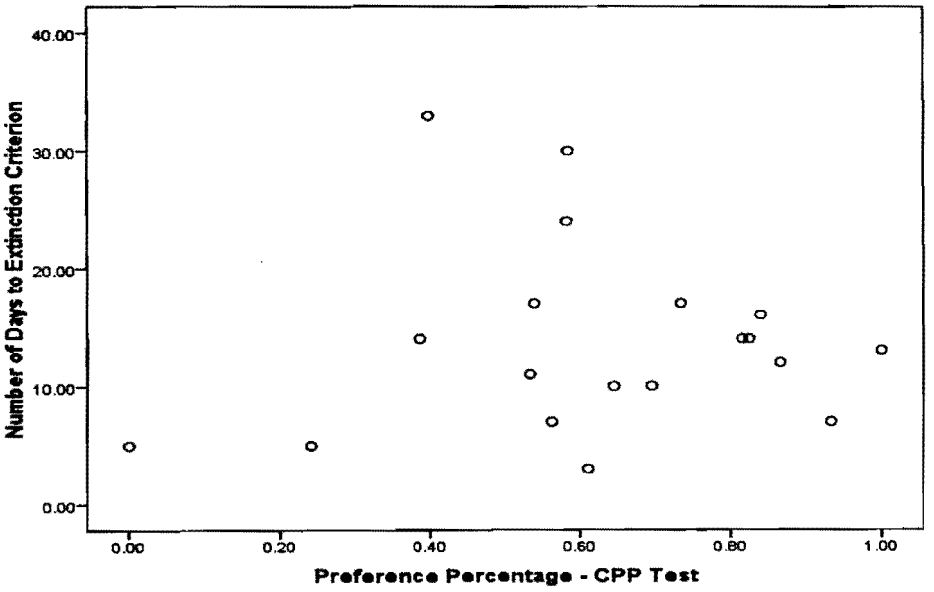


**Figure 7.** Scatterplot of the relationship between PPS during the CPP test and number of days needed to reach extinction

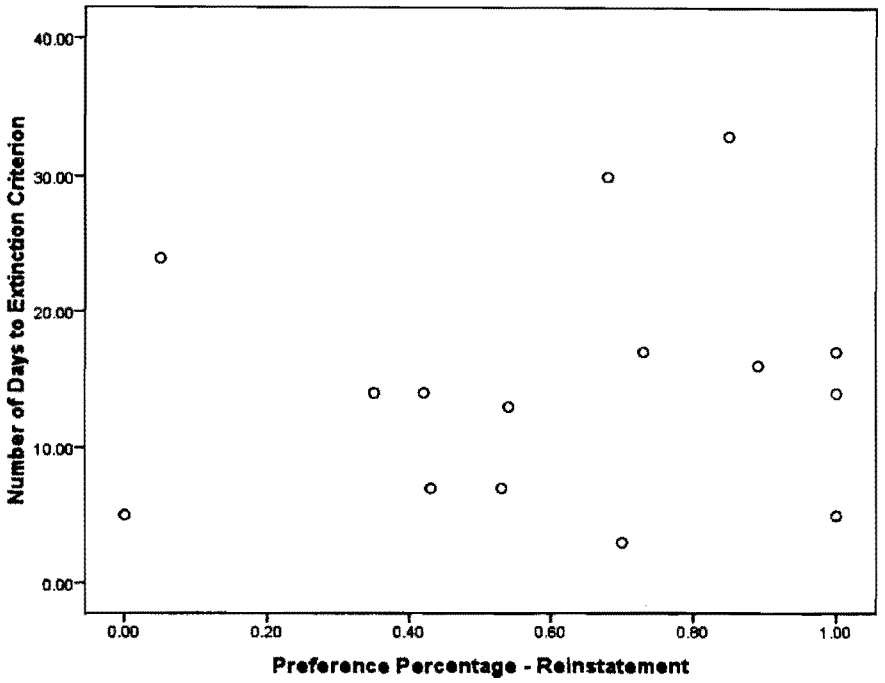


**Figure 8.** Scatterplot of the relationship between PPS during the reinstatement test and number of days needed to reach extinction

**Effects of RSD on Reinstatement**

The initial experimental design included a one-week delay between extinction and stress-induced reinstatement. However, due to the large and unanticipated variability within the extinction rates of the subjects, a delay of approximately 20 days was implemented. Due to technical error on one of the reinstatement days, the data of four subjects were lost; thus, data from only 15 subjects were analyzed for the reinstatement phase of the experiment. The combination of sleep condition and shock condition produced four groups during the reinstatement test: RSD/Shock (n = 4), RSD/No Shock (n = 3), Control/Shock (n = 3), and Control/No Shock (n = 5).

In order to determine the effects of sleep condition during acquisition and the shock condition during reinstatement on the METH-chamber preferences that were displayed during the reinstatement test, a 2 X 2 (sleep condition; shock condition) between-groups ANOVA was conducted. Results of the analysis demonstrated that there was no main effect of either prior sleep condition [$F < 1, p = 0.36, \eta^2 = 0.01$] or shock condition [$F < 1, p = 0.87, \eta^2 < 0.001$] and that the interaction between the conditions was also not significant [$F < 1, p = 0.44, \eta^2 = 0.01$; Figure 9]. The lack of a main effect of shock condition, which is apparent in the lack of a difference in mean PPS for each of the four groups [$M_{RSD/Shock} = 0.66$, $SE = 0.13$; $M_{RSD/NoShock} = 0.77$, $SE = 0.13$; $M_{Control/Shock} = 0.63$, $SE = 0.29$; $M_{Control/Shock} = 0.46$, $SE = 0.29$; Figure 9], indicates that the footshocks did not successfully induce reinstatement of the CPP; thus, stress-induced reinstatement did not seem to occur in this experiment. In addition, independent-samples *t*-tests computed separately for each sleep condition similarly demonstrated that the shock treatment did not reinstate a preference for the METH-paired chamber [RSD: $t(5) = 0.55, p = 0.61, d = 0.40$; Control: $t(6) = -0.61, p = 0.56, d = -0.41$ ; Figure 9]. Finally, paired-samples *t*-tests were computed for each reinstatement group to determine whether there was any change in preference during reinstatement relative to the groups' baseline preference, but none of those comparisons were significant ($p > 0.05$ for all analyses). Collectively, the results of these analyses further demonstrate that the shock was unsuccessful in reinstating a preference in either sleep condition.

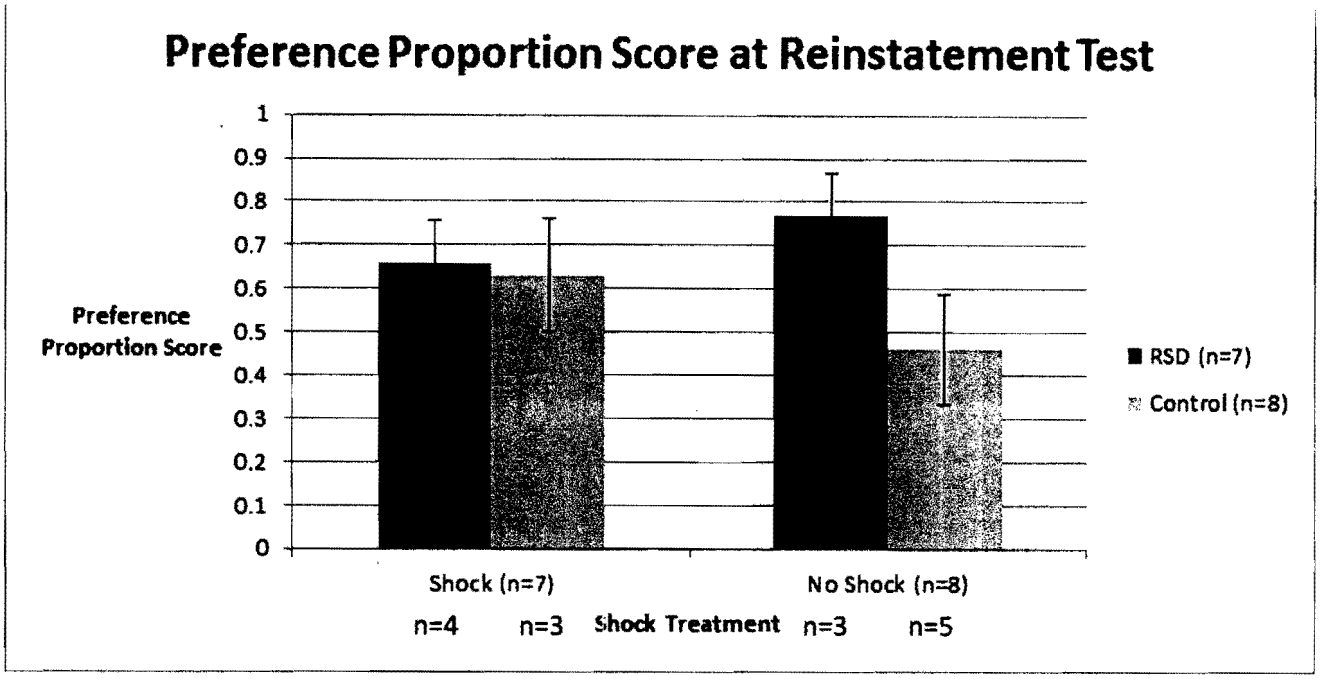


**Figure 9.** Results of PPS at reinstatement test

### Crossover Locomotor Activity

Locomotor activity was measured by observing the number of crossovers that subjects made between chambers. Two 4 (phase: baseline, CPP test, extinction, reinstatement) X 2 (sleep condition: RSD, control) repeated-measures ANOVAs were conducted in order to assess whether any significant changes in locomotor activity occurred throughout the experiment and whether sleep condition influenced these changes. Acquisition was not included in the analyses because crossover activity was not measured during that particular phase (rats were isolated to chambers during this phase). Due to each of the phases lasting a different number of days, the first analysis assessed crossover activity on the first day of baseline, the CPP test at the end of acquisition, the first day of extinction, and the reinstatement test, while the second analysis assessed crossover activity on the final day of baseline, the CPP test at the end of acquisition, the final day of extinction, and the reinstatement test.

The 4 X 2 ANOVA conducted on the first day of each phase revealed that there was a main effect of experimental phase on locomotor activity [$F(3,13) = 44.28$, $p < 0.001$, $\eta^2 = 0.76$; Figure 10]. The eta-squared value ($\eta^2 = 0.76$) also indicates that this had a large effect (Cohen, 1988). Follow-up paired-samples *t*-tests revealed that there was a significant decrease in locomotor activity from the first day of baseline to the CPP test [$t(18) = 6.77$, $p < 0.001$, $d = 2.24$; $M_{Baseline} = 17.16$, $SD = 4.29$; $M_{CPP} = 6.95$, $SD = 4.81$; Figure 10], a significant increase in locomotor activity from the CPP test to the first day of extinction [$t(18) = -2.80$, $p = 0.01$, $d = -0.47$; $M_{CPP} = 6.95$, $SD = 4.81$; $M_{Extinction} = 9.11$, $SD = 4.46$; Figure 10], and a significant decrease in locomotor activity from the first day of extinction to the reinstatement test [$t(14) = 3.46$, $p = 0.004$, $d = 1.22$; $M_{Extinction} = 8.40$, $SD = 4.22$; $M_{Reinstatement} = 3.87$, $SD = 3.09$; Figure 10]. Sleep condition did not have a main effect [$F < 1$, $p = 0.78$, $\eta^2 = 0.006$; Figure 10], and the interaction between experimental phase and sleep condition was also not significant [$F(3,13) = 1.08$, $p = 0.37$, $\eta^2 = 0.02$; Figure 10]. These results indicate that there were significant changes in locomotor activity across several phases of the experiment, but these changes were not influenced by sleep condition.

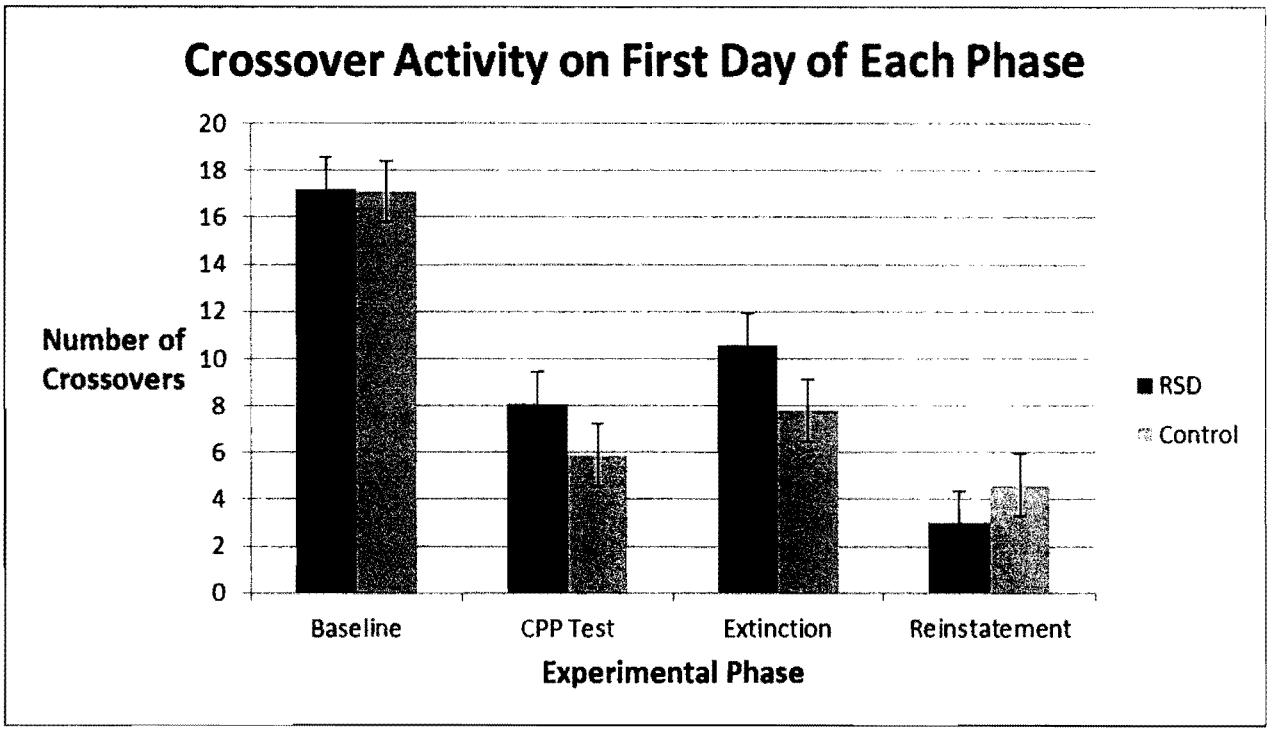


**Figure 10.** Locomotor activity of subjects on the first day of each phase. Note: The CPP test and reinstatement test consist of one day each.

The second 4 X 2 ANOVA conducted on the last day of each phase revealed that there was also a main effect of experimental phase on locomotor activity [$F(3,13) = 4.00, p = 0.01, \eta^2 = 0.22$; Figure 11]. The eta-squared value ($\eta^2 = 0.22$) also indicates a large effect size (Cohen, 1988). Follow-up paired-samples *t*-tests revealed that there was a significant decrease in locomotor activity from the final day of extinction to the reinstatement test [$t(14) = 3.63, p = 0.003, d = 1.22$; $M_{Extinction} = 7.93$, $SD = 3.53$; $M_{Reinstatement} = 3.87$, $SD = 3.09$; Figure 11], but no other pairwise comparisons were significant ($p > 0.05$ for all analyses). Sleep condition did not have a main effect [$F < 1, p = 0.62, \eta^2 = 0.02$; Figure 11], and the interaction between experimental phase and sleep condition was also not significant [$F(3,13) = 1.08, p = 0.37, \eta^2 =$

0.02; Figure 11]. These results indicate that there was a significant change in locomotor activity at the end of the reinstatement test, but this change was not influenced by sleep condition.

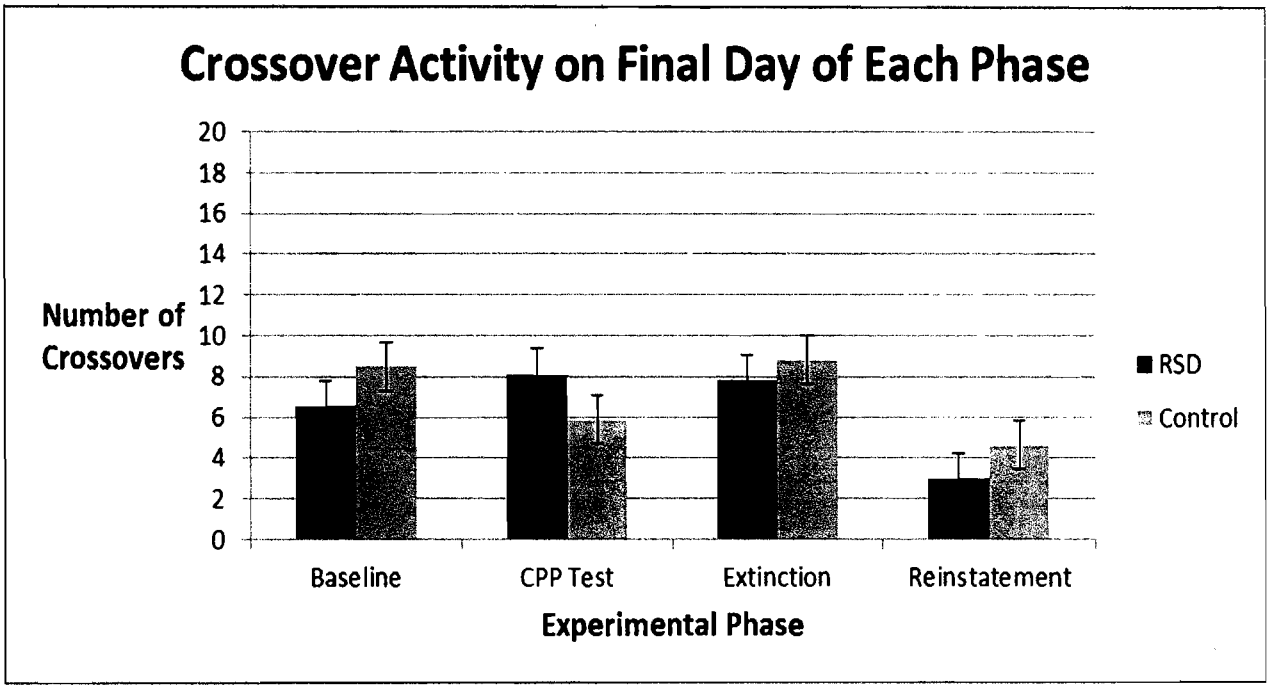


**Figure 11.** Locomotor activity of subjects on the final day of each phase. Note: The CPP test and reinstatement test only consist of one day each.

Collectively, the results of the two ANOVAS conducted during the locomotor activity analysis demonstrate that there were significant changes in locomotor activity throughout the course of the experiment, but these changes were not influenced by sleep condition.

A 2 X 2 (sleep condition; shock condition) between-groups ANOVA was conducted in order to assess whether sleep condition interacted with shock condition to produce any changes within locomotor activity during the reinstatement test. The analysis revealed that neither shock condition [$F < 1, p = 0.95, \eta^2 < 0.001$] nor prior sleep condition [$F < 1, p = 0.38, \eta^2 = 0.02$] had a

main effect on crossover activity during reinstatement. The interaction between the two factors was also not significant [$F < 1, p = 0.36, \eta^2 = 0.03$; Figure 12]. This was also reflected in the similar numbers of crossovers for each group [$M_{RSD/Shock} = 3.75$, $SE = 1.01$; $M_{Control/Shock} = 3.67$, $SE = 1.27$; $M_{RSD/NoShock} = 4.00$, $SE = 1.01$; $M_{Control/NoShock} = 4.00$; 1.27; Figure 12].

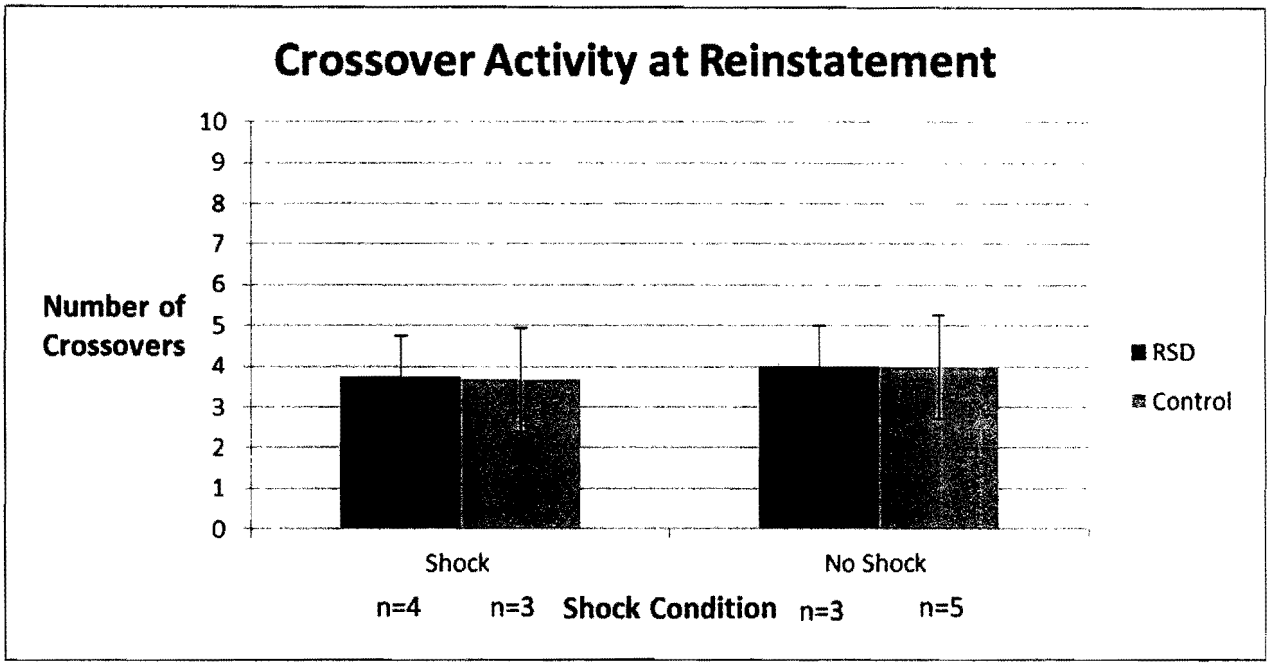


**Figure 12.** Locomotor activity during reinstatement test

Bivariate correlational analyses were conducted to examine possible associations between locomotor activity during various phases of the CPP and rate of extinction. Pearson's product-moment correlational analysis revealed that there was no significant association between the number of crossovers made during reinstatement and days to extinction [$r = 0.24, p = 0.40$; Figure 13]. The same was revealed for crossovers made during acquisition and days to extinction; however, this association was trending towards significance [$r = 0.43, p = 0.07$; Figure 14]. This suggests that increased locomotor activity during CPP acquisition may be associated with resistance to extinction.

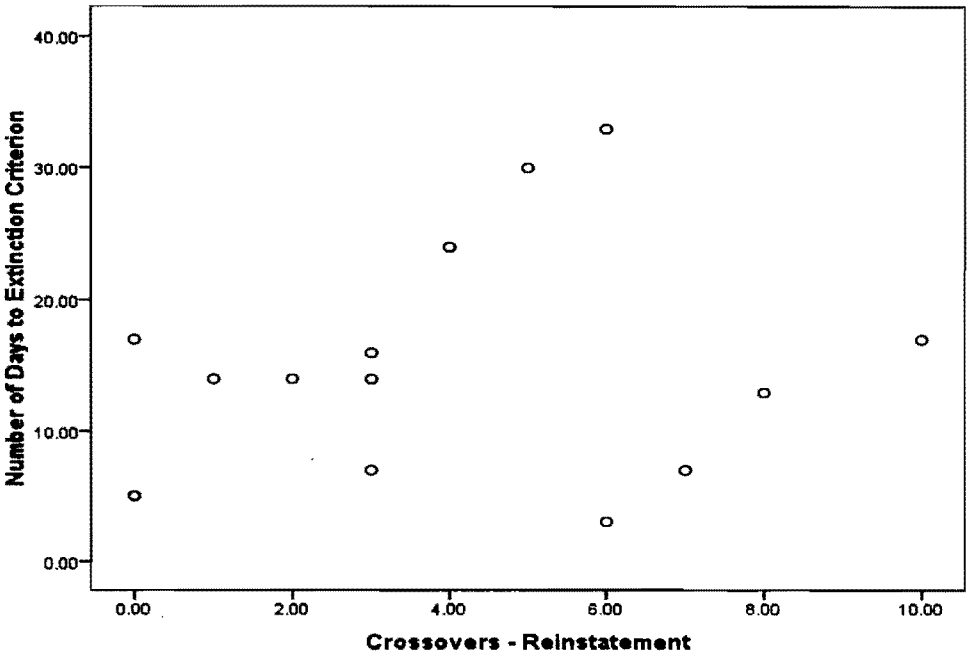


**Figure 13.** Scatterplot of the relationship between crossover activity during the reinstatement test and number of days needed for extinction.

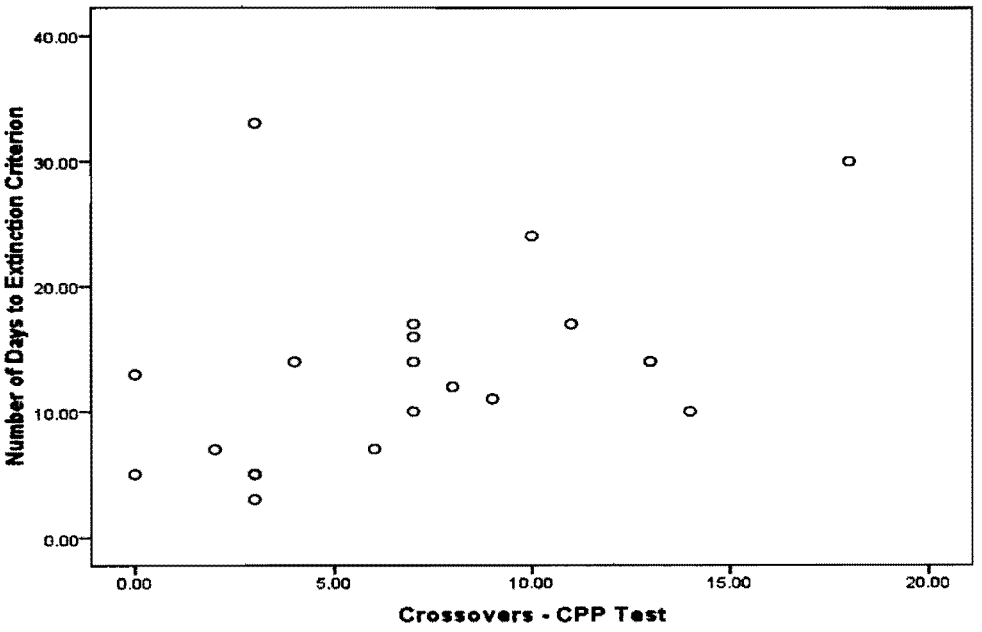


**Figure 14.** Scatterplot of the relationship between crossover activity during the CPP test and number of days needed for extinction.

**Body Weight**

A 5 (phase: baseline, acquisition, CPP test, extinction, reinstatement) X 2 (sleep condition: RSD, control) repeated-measures ANOVA was used to assess whether there were significant changes in mean body weight at each of the five phases of the experiment and whether sleep condition had any influence on these changes. The purpose of this analysis was to determine whether there were any changes in the rats' physiological health throughout the experiment and whether these changes were influenced by the sleep conditions they were subjected to. Results revealed that there was a main effect of experimental phase [$F(4, 17) = 134.58$, $p < 0.001$, $\eta^2 = 0.89$; Figure 15]. In addition, the eta-squared value ($\eta^2 = 0.89$) indicates a very large effect size (Cohen, 1988). Follow-up paired-samples *t*-tests revealed that there was a significant increase in mean body weight from the CPP test that occurred after acquisition to extinction [$t(18) = -9.63$, $p < 0.001$, $d = -1.08$; $M_{CPP} = 428.00$, $SD = 31.65$; $M_{Extinction} = 461.62$, $SD = 30.68$; Figure 15] and a significant increase in mean body weight from extinction to the reinstatement test [$t(18) = -9.13$, $p < 0.001$, $d = -0.71$; $M_{Extinction} = 461.62$, $SD = 30.68$; $M_{Reinstatement} = 486.58$, $SD = 38.82$; Figure 15]. In addition, there was no significant difference in mean body weight between baseline and acquisition, suggesting that METH had an effect on weight during acquisition by suppressing weight gain – an effect typically associated with METH use [$t(18) = -0.66$, $p = 0.52$, $d = -0.04$; $M_{Baseline} = 428.79$, $SD = 29.32$; $M_{Acquisition} = 430.00$, $SD = 30.94$; Figure 15]. There was no effect of sleep condition [$F(1, 17) = 1.09$, $p = 0.31$, $\eta^2 = 0.06$; Figure 15], indicating that the change in weight was not influenced by RSD. In addition, there was no significant interaction between experimental phase and sleep condition [$F < 1$, $p = 0.78$, $\eta^2 = 0.002$; Figure 15].

Due to the different number of days in each phase, two additional 5 X 2 repeated-measures ANOVAs were conducted in order to more clearly understand the nature of the differences occurring across study phases. In a similar fashion to the locomotor activity analyses, the first 5 X 2 ANOVA assessed the weights of all the rats on the first day of each phase. Results of this analysis revealed that there was still a main effect of experimental phase with a very large effect size [$F(4, 17) = 107.78$, $p < 0.001$, $\eta^2 = 0.86$]. In addition, there was neither a main effect of sleep [$F < 1$, $p = 0.34$, $\eta^2 = 0.05$] nor a significant interaction [$F < 1$, $p = 0.74$, $\eta^2 = 0.002$] between experimental phase and sleep condition, indicating that sleep condition did not influence body weight at the beginning of each experimental phase and that the effect of phase on body weight was not dependent on sleep condition. The second 5 X 2 ANOVA assessed the weights of all the rats on the final day of each phase. Results of this analysis revealed that there was still a main effect of experimental phase with a very large effect size [$F(4, 17) = 128.264$, $p < 0.001$, $\eta^2 = 0.88$]. In addition, there was neither a main effect of sleep [$F(1, 17) = 1.14$, $p = 0.30$, $\eta^2 = 0.06$] nor a significant interaction between experimental phase and sleep condition [$F < 1$, $p = 0.72$, $\eta^2 = 0.002$], indicating that sleep condition also did not influence body weight at the end of each phase and that the effect of phase on body weight was not dependent on sleep condition. Therefore, these analyses collectively demonstrate that body weight changed significantly throughout the experiment but that sleep condition did not have an effect on these changes.

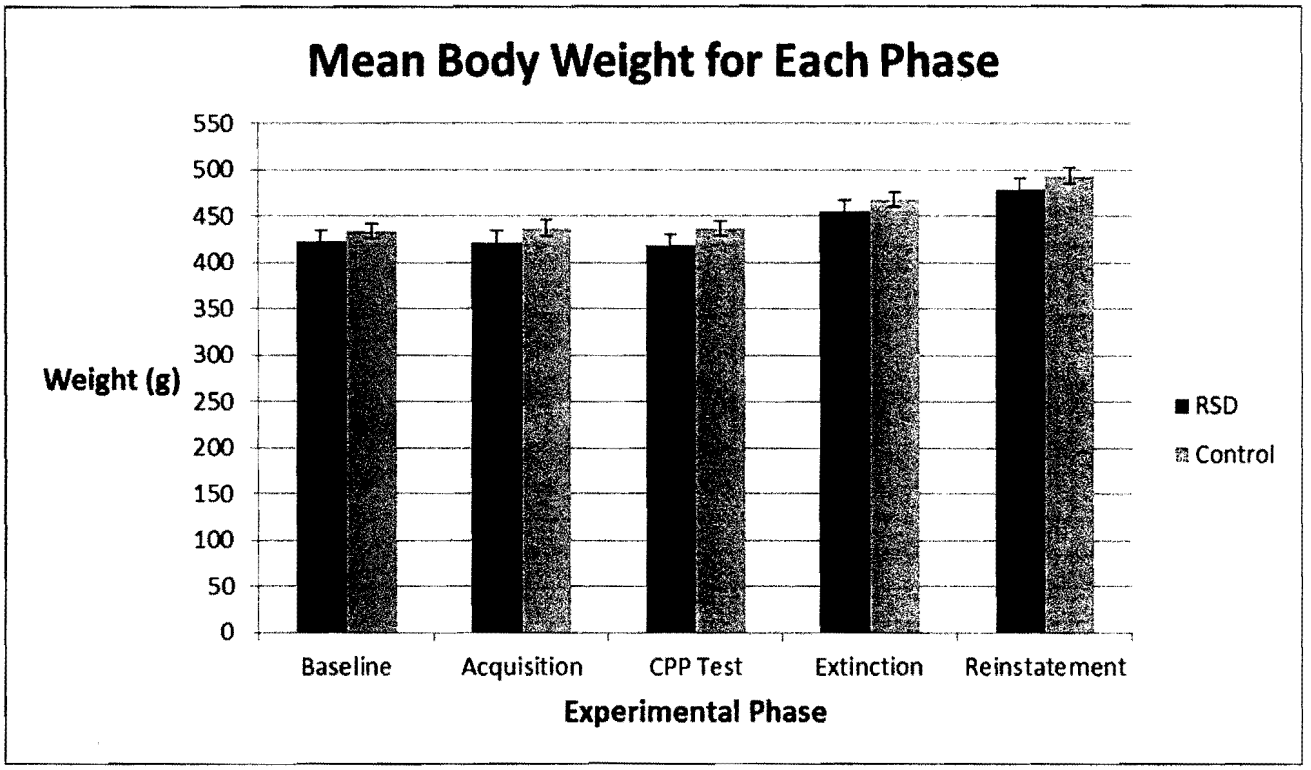


**Figure 15.** Mean body weight during each phase of the experiment

## Discussion

The results of the present study demonstrated that short-term RSD that occurred every four days during the acquisition phase of a METH-induced CPP did not affect its acquisition, extinction, or reinstatement. Neither chamber preference nor locomotor activity significantly differed between sleep conditions during either acquisition or reinstatement, as demonstrated by the similar outcomes for each measure in each sleep condition. Additionally, there was no effect of sleep condition on rate of extinction, with both RSD subjects and control subjects taking an average of two weeks to extinguish.

Despite these RSD parameters not having an effect on the acquisition phase, a preference for the METH-paired chamber was successfully established among all subjects, as demonstrated by the significantly higher PPS scores during the preference test at the end of acquisition compared to baseline. In addition, all subjects successfully extinguished their acquired preferences during the extinction phase, despite the unanticipated variability in number of days taken to do this. However, the results of this study also suggest that the unsignaled footshocks that were administered immediately prior to reinstatement testing were ineffective in producing stress-induced reinstatement for the METH-paired chamber.

The results of the present study suggest that RSD does not impair the acquisition of a learning task, which contradicts previous literature (Alvarenga et al., 2008; Ishikawa et al., 2006; Silva et al., 2004; Smith & Rose, 1995; Smith et al., 1998). Despite previous studies demonstrating that RSD duration of just four hours was sufficient for impairing the acquisition of and subsequent performance on other hippocampal-dependent tasks, such as the Morris water maze (Smith & Rose, 1995) and the radial arm maze (Smith et al., 1998), six hours of RSD had no effect on the CPP paradigm, another hippocampal-dependent task, in this study. The results

of the present study suggest that while spatial memory and contextual conditioning are both hippocampal-dependent, it seems that other brain structures may play a more integral role in the latter. McDonald and colleagues (2010) demonstrated this possibility in a study that examined the roles that the amygdala and dorsal hippocampus played in the CPP task and in the Morris water maze task. The authors concluded that the amygdala played a critical role in CPP expression, but played little to no role in the Morris water maze, while the opposite was true for the dorsal hippocampus. Thus, it would seem that the amygdala is the structure more closely associated with contextual conditioning, or at least as assessed by the CPP, while the hippocampus is more closely associated with spatial learning. The results of McDonald and colleagues (2010) therefore suggest that the amygdala, rather than the hippocampus, may play a critical role in the acquisition of a CPP and possibly in contextual reward conditioning in general. Future research should focus on understanding the relationship between the amygdala and drug-induced CPP as well as how RSD does or does not affect this relationship.

Other factors of the RSD procedure, such as RSD duration and timing, however, should also be considered. For example, Alvarenga and colleagues (2008) found that RSD impaired the acquisition of a discriminative avoidance task. However, the RSD duration was 96 hours – 16 times longer than that which was used for the present study. Other studies that have demonstrated RSD-induced impairment of acquisition have also used longer periods of RSD, including 72 hours (Silva et al., 2004) and 24 hours (Ishikawa et al., 2006). Thus, although previous research has demonstrated that shorter periods of RSD can affect memory for previously learned information, it is possible that the RSD period used for this study was too short and was, therefore, not sufficiently long enough to impair the acquisition of the CPP for the METH-paired chamber.

The current findings are also in contrast to those of Shi and colleagues (2011) who demonstrated that six hours of total sleep deprivation (TSD) impaired the acquisition of a morphine-induced CPP. However, it is important to note that the rats used in their study were exposed to six hours of TSD immediately after a CPP test, whereas the rats in the current study were deprived of REM sleep during the acquisition phase. In addition, Shi and colleagues' (2011) acquisition phase lasted eight consecutive days with both morphine and saline injections occurring each day, resulting in eight episodes of TSD. The current study's acquisition phase also contained eight treatment days, but rats did not receive both injections on the same day, making a total of four METH treatments and four saline treatments and a total of four RSD episodes. Finally, the drug used in Shi and colleagues' (2011) study, morphine, differs from the one used in the current study, METH; the former is an opiate, while the latter is a psychostimulant. Although there are some distinct differences between the two studies, a comparison of the results from Shi and colleagues with the present results seems to indicate acquisition of a CPP is not dependent specifically on REM sleep but on non-REM sleep. Alternatively, perhaps the TSD manipulation used by Shi and colleagues was perceived as stressful by the animals, and this stress impaired CPP acquisition.

In addition to the length of the RSD period, the timing of the RSD in relation to acquisition is also an important factor. In the present study, RSD occurred immediately after each METH treatment during the acquisition phase. Previous research, however, has found that acquisition impairment occurred when RSD was implemented after a *delay* of about four to five hours after the end of training in the Morris water maze (Smith & Rose, 1995). However, the same authors also found in a separate study that acquisition of the radial arm maze was impaired only when RSD occurred *immediately* after the training for the task. RSD had no effect when it

occurred after a delay at the end of training (Smith et al., 1998). Other studies have also demonstrated significant impairments when RSD occurred immediately after acquisition of the task (Alvarenga et al., 2008; Hanlon et al., 2010; Ishikawa et al., 2006). It is possible that depriving subjects of REM sleep after a delay of a few hours after METH administration could have significantly impacted the acquisition of the METH-induced CPP in this study, which is consistent with the findings of Smith and Rose (1995). In addition, the fact that the same authors (Smith & Rose, 1995; Smith et al., 1998) found conflicting effects of RSD across different hippocampal-dependent tasks suggests that the nature of the particular spatial task being implemented may also be a factor as well. This suggestion is consistent with the findings of McDonald and colleagues (2010) who do make such a distinction between the CPP task and the Morris water maze by demonstrating that different brain structures play prominent roles in each task (the amygdala and hippocampus, respectively).

In addition to RSD duration and its timing in relation to acquisition, RSD's timing in relation to testing is also another possible contributing factor to consider. For example, the two previously mentioned studies (Smith & Rose, 1995; Smith et al., 1998) conducted training and testing on consecutive days. After depriving a rat of RSD after training, it was tested the next day. Therefore, it is possible the rats in these studies were sleep-deprived on testing days, which could have affected their subsequent test performance and would have more clearly demonstrated the effects of RSD. The design of the acquisition protocol in the current study enabled rats to recover from RSD for three days before being REM-deprived again. Therefore, it is possible that the REM rebound that RSD rats likely experienced between RSD sessions could have reduced the effectiveness of RSD during acquisition.

Drug reward memory involves the action of several different brain structures, including the PFC, orbitofrontal cortex, cingulate cortex, mediodorsal nucleus of the thalamus, and the hippocampus (Everitt & Robbins, 2005; Kuo et al., 2011; Rhodes et al., 2005; Robbins et al., 2008). The hippocampus, in particular, is a brain structure that is closely associated with contextual conditioning (Holland & Bouton, 1999). In addition, RSD has been shown to affect hippocampal function (Hagewoud et al., 2010), which is part of the rationale of the current study's examination of the effects of RSD on the contextual conditioning underlying the CPP task.

With respect to candidate neurotransmitters, research suggests that glutamate transmission may play a contributing role. In a recent study, Herrold and colleagues (2013) utilized a METH-induced CPP to demonstrate that the fifth subtype of the metabotropic glutamate receptor (mGluR5) was critical in the maintenance of CPP memory as well as CPP expression. After a METH-induced CPP was established, glutamate antagonists that targeted distinct mGluRs were administered to the rats. The mGluR5 antagonist inhibited the expression of the CPP at mGluR5, implicating the role of glutamatergic transmission in mediating METH-induced CPP (Herrold et al., 2013). Similarly, Gass and colleagues (2009) found that MTEP (3-((2-methyl-l,3-thiazol-4-yl)ethynyl)pyridine, an mGluR5 antagonist, significantly reduced the reinforcing effects of METH.

RSD has been shown to reduce glutamatergic transmission, particularly at the GluR1 receptor (Lopez et al., 2008; Ravassard et al., 2009), an AMPA receptor. The pyramidal neurons of the CA1 section of the hippocampus, in particular, have been implicated as a potential target area affected during RSD (Ravassard et al., 2009; Yang et al., 2008a). Based on these findings, it was predicted that RSD rats would have exhibited reduced CPP expression due to reduced

glutamate transmission; however, this did not occur. It could be argued, though, that the length of RSD was not sufficient for exerting a complete antagonistic effect on the increased glutamate activity that occurred as a result of METH administration during acquisition. Previous research has shown a positive association between mGluR5 expression and GluR1 expression in the hippocampus (Uslaner et al., 2009), which suggests that RSD may indirectly antagonize mGluR5 activity through its direct antagonism of GluR1 activity. As a result, the shorter duration of RSD in the present study may have produced a "weaker" antagonism of both mGluR5 and GluR1 receptors that was not strong enough to inhibit the glutamate transmission in a way that would produce a measurable effect on the acquisition and subsequent expression of the CPP. Therefore, instead of counteracting the agonistic effects of METH and inhibiting the CPP, this weaker form of antagonism only partially inhibited METH's agonistic effects. As a result, changes in glutamatergic transmission are "cancelled out" by the conflicting forces of both METH and RSD; neither one is strong enough to override the effects of the other. Therefore, the glutamate transmission would then return to baseline levels. Behaviorally, this could manifest itself as a lack of a difference in PPS between the RSD and control groups, which is what occurred in the present study. This suggestion could also potentially explain why longer periods of RSD seem to impair acquisition of a task (Alvarenga et al., 2008; Ishikawa et al., 2006; Silva et al., 2004). Thus, perhaps longer periods of RSD provide stronger AMPA antagonistic effects that are capable of counteracting the glutamate agonistic effects of METH. However, such a suggestion will only be more readily testable when research discovers a causal relationship between mGluR5 expression and GluR1 expression. Only then will future studies be able to more directly observe this potential relationship.

In addition to demonstrating that short-term RSD occurring intermittently during acquisition had no effect on subsequent rate of extinction, the results of the present study also revealed a large amount of unanticipated variability – a range of one month – in number of days to extinction. While it is quite possible that individual differences within the rats themselves could have contributed to this variability, the extinction criteria used for this study may have been a factor as well. In the present study, the extinction phase ended when every rat displayed a PPS of 0.53 or less for three consecutive days. These criteria differed from those used in other studies (Voigt et al., 2011; Yang et al., 2008b), which did not require each individual rat to extinguish its preference; rather, mean preference scores for each group were calculated to determine if extinction occurred. In other words, as long as the average drug-paired chamber preference for the *group* indicated that there was no longer a significant preference for the drug-paired chamber, then extinction was said to have occurred; thus, this did not necessarily require extinction to occur in each individual rat. Therefore, it could be argued that the extinction to criterion protocol used in the present study produced a "truer" form of extinction in that *each* rat had to extinguish before progressing to the reinstatement test. Rather than monitoring extinction over the course of the phase by periodically calculating mean PPS for each sleep condition, extinction was monitored for each rat, regardless of sleep condition in order to ensure a 100% successful extinction in this CPP protocol. However, despite the extinction to criterion protocol ensuring successful extinction in all subjects in the present study, stress-induced reinstatement did not successfully occur in either sleep condition. Perhaps this lack of a reinstatement effect was somehow attributed to the amount of time it took for successful extinction to occur as a result of the protocol used. If this were the case, this would suggest that perhaps reinstatement is more likely to occur when extinction does not occur within every subject, which is often the case

in studies that determine the occurrence of extinction based on group averages. Future research should explore this possibility in order to determine whether the decision of *when* to end extinction can actually affect subsequent reinstatement.

Extinction resistance has also been reported in other METH-induced CPP studies that did not use the extinction to criterion protocol, however. Voigt and colleagues (2011), for example, found that rats were still displaying a preference for the METH-paired chamber 24 days after extinction training had begun. The authors attributed this to the inherently robust effects of METH and hypothesized that those effects intensified the learned association between the drug and the context with which it was paired. METH has been reported to be frequently abused due to its strong potency and robust, sensitization-inducing effects (Cruickshank & Dyer, 2009; Gehrke et al., 2003; Lan et al., 2009; Sulzer et al., 2005), so perhaps the nature of the drug itself contributed to the large variability in extinction rate. In addition, since extinction learning is said to involve the "overlaying" of the extinction memory onto the previous acquisition memory (Rescorla, 2002) , it is possible that the robust effects produced by METH were strong enough to make the acquired CPP memory highly resistant to extinction. However, this explanation does not take into account the rats that extinguished relatively quickly. Voigt and colleagues (2011), however, also found that baclofen, a $GABA_B$ receptor agonist, facilitated extinction training. Rats that received baclofen (2 mg/kg) immediately after each extinction session during the first four cycles were able to extinguish their CPPs, suggesting that increased $GABA_B$ receptor activity could be a potential physiological mechanism for the extinction of a METH-induced CPP (Voigt et al., 2011). Therefore, the individual differences between rats mentioned earlier could be due to differences in $GABA_B$ergic transmission. While the findings of Voigt and colleagues (2011) provide a possible mechanism for extinction learning in a METH-induced

CPP, more research is needed to determine whether there are interactions between the potency of the drug used, the extinction paradigm used in a CPP design, and $GABA_B$ receptor activity that may influence the rate at which an animal extinguishes a chamber preference.

Six hours of RSD that occurred every four days during acquisition also did not have an effect on subsequent reinstatement, but shock used in the stress-induced paradigm did not reinstate preference for the previously METH-paired chamber. Although the shock parameters used in the present study (10 shocks, 0.5 msec duration, 1.0 mA) have effectively produced shock-induced reinstatement previously in this laboratory, it remains a possibility that these parameters may have been too low to successfully reinstate a preference in these particular animals. Stress-induced reinstatements of METH-induced CPPs have been demonstrated with a shock of lower magnitude (0.63 mA) and similar duration time (0.5 msec; Beardsley et al., 2010). In addition, prior research has demonstrated successful reinstatement of other drugs, such as heroin (Shaham & Stewart, 1995) and cocaine (McFarland et al., 2004), with shock intensities that ranged from 0.75 mA – 1mA (0.5 msec durations) administered on a variable-interval schedule. Thus, the lack of an effect of shock condition on reinstatement in the current study contradicts results using the same protocol with other drugs. In addition to parameters that might have been too low for the rats used in the current study, the small groups for each reinstatement condition (which ranged from three to five rats) could have contributed to a lack of an observed effect of the shock condition by reducing statistical power, especially since visual display of the data (Figure 9) indicate a possible interaction between sleep condition and shock condition such that rats in the RSD/Shock group exhibited a lower PPS than rats in the RSD/No Shock group, whereas the rats in the Control/Shock group exhibited a higher PPS than rats in the Control/No Shock group. Another possible explanation is that the extinction memory formed

during the extinction phase was strong enough to resist the stress caused by the shocks. Future studies could attempt to determine whether it is the actual magnitude of the shock, the number of shock administrations, the non-contingent nature of the shock administrations, or a combination of these factors that influences the required amount of stress to reactivate drug-seeking behavior within an organism. Within a neurological context, stress-induced reinstatement has been linked to activation of the hypothalamic-adrenal-pituitary (HPA) axis and the release of the stress hormone, corticotropin-releasing factor (CRF; Aguilar et al., 2009). Studies have shown that CRF antagonists have been successful in attenuating or completely blocking the reinstatement of morphine-induced CPPs (Lu et al., 2000; Lu et al., 2005), especially when administered to the bed nucleus of the stria terminalis (BNST; Wang et al., 2006). In the current study, both RSD and control rats in the shock condition had a relatively similar level of preference for the METH-paired chamber during the final CPP test – with the RSD group's PPS being slightly higher.

In addition to not affecting chamber preference, the present study's RSD parameters did not significantly affect locomotor activity during the CPP test, extinction, or reinstatement. This contradicts previous work that has demonstrated measurable increases in locomotor activity after RSD (Albert et al., 1970; Van Hulzen & Coenen, 1981). In addition to RSD not having an effect, the effect of METH on locomotor activity appears differently depending on how the data were analyzed. The current study revealed that when looking at the first day of each experimental phase, METH seemed to decrease locomotor activity, save for an increase in activity from the CPP test to extinction (Figure 10). However, when looking at the final day of each experimental phase, it appears that METH did not have any effect on locomotor activity throughout the experiment (Figure 11). This difference in results could possibly be attributed to novelty detection. The first day of a new phase of an experiment presents a new, different set of

circumstances to the rats. By detecting that something new is occurring, their locomotor activity in response to the METH could potentially increase, which could possibly account for the increased locomotor activity displayed by both groups on the first day of baseline than on the final day of baseline and that the RSD rats displayed on the first day of extinction compared to the final day of extinction, when habituation to these phases could have possibly occurred. This idea has been suggested in other studies, such as that conducted by Hooks and colleagues (1991), which found a positive correlation between responses to novel contexts and AMPH-induced changes in locomotor activity. However, this does not account for the control rats' slight increase in locomotor activity on the final day of extinction (Figure 11) compared to the first day of extinction (Figure 10) in the present study. Hooks and colleagues (1991) do acknowledge, though, that this relationship is a correlational one and that individual differences can affect the extent to which novelty responsiveness serves as a predictor for AMPH-induced changes in locomotor activity.

The lack of an effect of METH that is apparent when examining locomotor activity on the final day of each phase contradicts much previous literature, which has demonstrated that 1 mg/kg of METH administered even fewer times than in the current study caused a measurable change in locomotor activity in both the elevated-plus maze paradigm (Pometlova et al., 2012) and in the open field paradigm (Good & Radcliffe, 2011). Other studies have demonstrated that even 0.75 mg/kg of METH administered once per day over the course of five days and then once per week for five consecutive weeks afterwards was enough to cause changes in locomotor activity (Lan et al., 2009), suggesting that it may not have been just the dose itself, but also the number of drug administrations that played a role in locomotor effects. Therefore, given the results of the previous literature indicating otherwise, the results of this study seem to indicate

that 1 mg/kg of METH administered four times over the course of sixteen days is not sufficient for causing changes in locomotor activity. It is also possible that the timing of drug administration could have also played a critical role in these results. As previously mentioned, the rats in the current study only received four METH administrations, but they were each separated by an interval of three days. In the studies mentioned earlier (Good & Radcliffe, 2011; Lan et al., 2009; Zakharova et al., 2009) in which a METH-induced effect on locomotor activity was found, METH administration occurred daily. Therefore, it is possible that the interval of rest between each METH administration in the current study could have prevented the effects of the drug on locomotor activity

It should also be emphasized, however, that crossover activity was not recorded during the acquisition phase, since the rats were isolated to chambers during each acquisition training session. Therefore, locomotor activity was not recorded during the acquisition phase. In addition, because the rats' movements inside the chambers during acquisition were not recorded, the locomotor activity that was being recorded during subsequent phases of the experiment was really in response to the chamber itself. Thus, locomotor activity in response to METH was not recorded, but locomotor activity in response to the METH-paired chamber was. Therefore, the locomotor activity measured in this experiment was really *conditioned-cue-induced* locomotor activity. Thus, it is also possible that the dose of METH used for the present study was sufficient for producing changes in locomotor activity that were not recorded, but the association made between this particular dose of METH and the chamber with which it was paired was not sufficient to produce changes in conditioned locomotor activity. However, this cannot be confirmed without a non-METH-treated control group during acquisition. Future studies could monitor locomotor activity during isolation to a chamber during acquisition to observe any

METH-induced changes in locomotor activity relative to baseline levels and also include a control group during the acquisition phase in order to note any changes in conditioned locomotor activity elicited by the METH-paired chamber during subsequent phases of the procedure.

In regards to the influence of RSD, previous literature has demonstrated that RSD has a sensitizing effect on locomotor activity. For example, Albert and colleagues (1970) found that three, six, and nine days of RSD (interval unspecified) using the flowerpot technique caused significant increases in locomotor activity in rats as demonstrated by increased numbers of home cage crossings. However, the findings of the current study contradict those of Albert and colleagues (1970) and suggest that RSD did not have a sensitizing effect on conditioned locomotor activity even when occurring after the administration of a psychostimulant. However, the RSD technique used in Albert and colleagues' (1970) study involved RSD that occurred on nine consecutive days, whereas RSD occurred every four days in the current study. Future research could investigate whether the frequency of RSD occurrence has any effect on locomotor activity alone or whether it interacts with the dose of METH to produce changes.

Although the results of the present study demonstrated changes in body weight across phases of the experiment, it is likely that these changes reflect the natural weight gain that occurs with development. RSD during acquisition did not affect body weight throughout the experiment, which suggests that six hours of RSD that occur every four days do not produce an amount of stress that is strong enough to manifest itself in the form of adverse physiological consequences, such as weight loss. These findings support those made by Van Luijtelaar and Coenen (1985), who examined the stress levels induced by three methods of RSD, including the inverted flowerpot method (Mendelson et al., 1974), and found that RSD only induces mild

stress when produced by each of the three methods. Therefore, the results of the current study suggest that if any stress was produced by the RSD protocol, it was mild in nature.

The fact that METH is a stimulant must also be considered when interpreting these results. Like most stimulants, METH produces wakefulness-promoting effects that can potentially disrupt the sleep cycle. In acute (5-30 mg; Cruickshank & Dyer, 2009) doses, METH has been shown to heighten arousal (Cruickshank & Dyer, 2009). However, studies that reported sleep deprivation caused by METH often used higher doses (e.g. Kuczenski et al., 2009) and administered METH via means other than intravenous injection (e.g. Perez et al., 2008). In addition, it has also been reported that 1 mg/kg of dextroamphetamine administered to humans did not produce the sleep difficulties that 10 mg/kg of the drug caused (Bonnet et al., 2005). While the literature search conducted prior to the present study did not yield any studies examining the specific effects of 1 mg/kg of METH on sleep, the current state of the literature would seem to suggest that the wakefulness-promoting effects of METH do not seem to take an effect until administered at a dose of around 5 mg/kg. Each rat in the present study only received 1 mg/kg a total of four times. In addition, the administrations did not occur on consecutive days, but rather every four days. Despite these parameters, however, the possibility that METH's stimulant properties affected subsequent RSD after each METH treatment still remains. As a result, the drug itself could have potentially contributed to the lack of an effect of RSD in the present study.

Several limitations to this study must also be considered. First, due to the unanticipated loss of five subjects, the sample size of this experiment was reduced, thereby reducing statistical power. Reinstatement testing, in particular, contained four groups with very small sizes due to technical error that lost the data of four additional subjects for that particular portion of the

experiment. Second, all subjects within this study received the same dose of METH on all days of acquisition. While this study demonstrates that six hours of RSD after a 1 mg/kg METH administration has no effect on the CPP, future studies could implement a similar paradigm with different groups of rats that each receives a different dose in order to determine whether the effects of RSD are dependent on the dose of METH being used. For example, Gehrke and colleagues (2003) and Zakharova and colleagues (2009) both found differences in CPP that were dose-dependent; however, the results of both of these studies also showed that rats actually had a stronger CPP for smaller doses of METH (0.3 mg/kg in Gehrke et al., 2003; 0.5 mg/kg in Zakharova et al., 2009), suggesting that while the effects of METH on CPP may be dose-dependent, the nature of this relationship is not necessarily unidirectional. In addition, Gehrke and colleagues (2003) also found that pre-treating rats with a neurotoxic dose of METH (10 mg/kg) resulted in a stronger CPP as compared to rats not pre-treated with METH. Therefore, future research could investigate the possible interactions that RSD could have with these dose-dependent effects. Third, the literature concerning whether or not a significant amount of stress is produced during RSD is inconsistent, with some studies claiming that it does by elevating CRF levels (Koban et al., 2006) with others claiming that it does not (Van Luijtelaar & Coenen, 1985). Mendelson and colleagues (1974) assessed weight of adrenal glands, food intake, and body weight after 96 hours of RSD using the flowerpot technique in order to detect any potential stress caused by the technique and found no significant changes in any of those measures, suggesting that this particular RSD protocol does not produce a measurable amount of stress. Due to this inconsistency in the literature, though, it is difficult to totally discount the possibility that stress caused by RSD was a potential factor in these results. Finally, the CPP paradigm has its own respective limitations in serving as an effective model for drug addiction – many of

which were mentioned in the introduction of this study. However, as mentioned in a review of the paradigm by Bardo and Bevins (2000), one limitation in particular that should be emphasized is that the CPP does not totally model human drug consumption behavior since it is lacking an element of self-administration. While it is true that many people consume drugs in a particular setting and eventually learn to associate the properties of the drug with the contextual stimuli within the environment in which the drug is consumed, they are also administering the drug to themselves in some manner. In the CPP paradigm, an experimenter administers the drug to the animal before placing it into the drug-paired chamber. Therefore, the animal also does not actually consume the drug within the presence of the environment; it is exposed to it after receiving the drug. Future research should aim to create a paradigm that combines elements of both self-administration and CPP in order to create a more holistic model that more effectively captures drug-taking behavior and its relationship to environmental context.

Within the broader context of learning, the results of the present study suggest that short-term RSD occurring every four days does not have an effect on the acquisition, extinction, or reinstatement of a contextual conditioning task. However, the lack of an effect of the RSD parameters on stress-induced reinstatement specifically should be interpreted cautiously, since the shock procedure in the current study did not produce reinstatement in the non-REM-deprived control rats. The lack of an effect of RSD on acquisition contradicts previous literature that has found a measurable aversive effect of RSD on the acquisition of a discriminative task (Alvarenga et al., 2008; Silva et al., 2004) as well as aversive impacts on physiological mechanisms, such as LTP induction in the dentate gyrus (Ishikawa et al., 2006). Also, the results of this study support previous research in regards to extinction of contextual conditioning. Silvestri (2005) found that six hours of RSD affected the extinction of cued, but not contextual, fear. While the CPP in the

present study assessed reward learning via classical conditioning, rather than fear conditioning, it still utilized contextual conditioning. Therefore, when combined with the results of the present study, this suggests that the effects of RSD on contextual learning are unclear and may depend on variables that have yet to be identified. Finally, RSD does not affect the reinstatement of a conditioned reward memory after it has been extinguished. Collectively, the results of the present study demonstrate that six hours of RSD that occur every four days do not exert a significant effect on contextual memory as assessed by the CPP.

The CPP paradigm utilizes classical conditioning to assess reward learning and its relationship to environmental context. The results of the present study suggest that short-term, intermittent RSD has no effect on this relationship, suggesting that the acquisition, extinction, or reinstatement of a drug reward memory is resistant to the effects of these RSD parameters. This also implies that RSD may not have any impact – therapeutic or aversive – on the formation of tolerance to and dependence on METH, abstinence from METH, and relapse to METH caused by environmental stress. However, it is important to note that the present study suffered from a lack of power, particularly during the reinstatement test, which limited the ability to see significant differences between groups. In addition, it should be further emphasized that the results of the present study only demonstrate that six hours of RSD that occur every four days do not influence reward memory. This does not necessarily mean that RSD has absolutely no effect on reward memory. Future studies that alter such RSD parameters as duration and timing could potentially reveal relationships between RSD and reward memory that the parameters of the present study were unable to assess.

In conclusion, the present study demonstrates that six hours of RSD that occur every four days during acquisition have no effect on chamber preference during the acquisition, extinction,

or reinstatement phase of a METH-induced conditioned place preference. These same parameters also did not affect conditioned locomotor activity after the acquisition phase or that during the extinction and reinstatement phase. Continued research is necessary for determining whether alteration of experimental variables such as RSD duration or dose of METH would produce a measurable effect of RSD on CPP parameters. The specific relationship between extinction criteria (i.e. when to end the extinction phase) and subsequent performance during stress-induced reinstatement should be further studied as well, especially since this can aid in developing more effective and accurate CPP experimental designs in the future. In addition, future research is necessary for determining the exact roles that glutamate, $GABA_B$, and CRF expression play in the acquisition, extinction, and reinstatement, respectively, of a METH-induced CPP, particularly in brain structures such as the hippocampus and the BNST. The results of such continued research could provide further insight into the relationship between RSD and METH-induced associative learning and aid in the development of future treatment options for METH abuse, or even psychostimulant abuse in general.

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