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Time-Dependent Effects of Stress on Cocaine Conditioned Place Preference Using a Rat Model of Posttraumatic Stress Disorder

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Time-Dependent Effects of Stress on Cocaine Conditioned Place Preference Using a Rat Model of Posttraumatic Stress Disorder

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By

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Abstract

Posttraumatic Stress Disorder (PTSD) affects approximately 8% of the entire population within their lifetimes. A startling trend of co-occurring PTSD and cocaine use has surfaced among humans who express these disorders. The present study employed the rat model of PTSD, Single Prolonged Stress, to examine the effects of stress on the rewarding properties of cocaine. Place conditioning was used to specifically evaluate differences between animals that had undergone a post-stress delay of conditioning in comparison to animals that were not delayed. This delay before conditioning, or incubation period, is a time spent undisturbed in the home cage for 10-days post-stress and has been implicated as the phase in which many of the physiological, neurochemical and behavioral changes observed in humans who have experienced stress take place. Although cocaine conditioned place preference was observed, no significant differences were detected between animals that were not stressed, stressed but not incubated, or stressed and then incubated. These results suggest that it is possible that the changes that are understood to take place within this incubation period did not directly influence cocaine reward, or that they did not take place. Future work should focus on examining different drugs, in addition to including additional testing intervals, and varying drug dose.

Posttraumatic Stress Disorder

Posttraumatic Stress Disorder (PTSD) is a disorder that effects approximately 8% of the general population within their lifetimes (Back, Dansky, Saladin, Sonne, & Coffey, 2000, American Psychiatric Association [DSM-IV-TR], 2000). PTSD is marked by symptoms of avoidance of feared stimuli, intrusive thoughts or flashbacks, irritability, exaggerated startle response, poor concentration, and aggression (American Psychiatric Association [DSM-IV-TR], 2000). These symptoms result from an extremely traumatic experience for example combat, rape, or natural disaster, and may become more prominent with the passage of time. Although the term 'Posttraumatic Stress Disorder' may have only entered popular culture recently, being introduced as a disorder in 1980, it is far from a new phenomenon (Davidson, Stein, Shalev, & Yehuda, 2004). Among military veterans the condition has been called a number of things including; "shell shock", "combat fatigue", and "nervous exhaustion" (Gross, 2010).

Today, veterans of the wars in Iraq and Afghanistan present PTSD prevalence rates between 21% and 33% (Haskell et. al. 2010), and other groups have high rates of PTSD as well. Victims of violence applying for state compensation in 2010 showed PTSD prevalence rates as high as 1 in 2 (Kunst, Winkel, & Bogaerts, 2010). Among those who have experienced a natural disaster the rates are also extremely high. Wang et. al. found after the 2008 Sichuan earthquake between 13% and 37.8% of adults residing within 80 miles of the epicenter expressed PTSD. Even though 60.7% of men and 51.3% of women report experiencing extremely traumatic events within their lifetimes, the majority of these people do not go on to

develop PTSD (Davidson et. al., 2004). One week after a serious trauma 94% of individuals express symptoms of a serious stress response, but when those same individuals were examined again 3-9 months later, only 15-25% expressed any symptoms. These rates are proportional to the type and magnitude of the trauma but this indicates a serious difference between individuals who develop Acute Stress Disorder (a stress response lasting less than a month) and PTSD (a stress response lasting for a month or more) (Davidson et. al., 2004). However, as the number of patients diagnosed with PTSD remains staggeringly high, the mechanisms by which those symptoms come to be are still not well understood.

The Biology of Stress & PTSD

It is important to understand the neurological response to stress and how it may be different in those individuals who go on to express PTSD symptoms. In a traumatic situation, such as an imminent threat of death or serious injury to one's self or a loved one, it is normal to have a biological survival related response (Yehuda, 2009). In a healthy individual a normal response would be a startle reaction, activation of the sympathetic nervous system and release of adrenaline, the suppression of the parasympathetic nervous system, and the activation of the Hypothalamo-Pituitary-Adrenal Axis (HPA-Axis) (Davidson et. al., 2004; Yehuda, 2009).

The HPA-Axis has come to be implicated in the development of PTSD through possible abnormalities in the activation systems of those who express symptoms (Davidson et. al. 2004; Yehuda, 2009). In a healthy individual the HPA-Axis activation is a chain reaction. The hippocampus and amygdala activate the

hypothalamus, which releases corticotrophin releasing factor (CRF). The CRF prompts the activation of the pituitary gland, which releases adrenocorticotrophin hormone. This hormone then goes on to prompt the release of cortisol, which in turn suppresses the sympathetic nervous system response, and the HPA-Axis effectively returning the individual to normal functioning (Davidson et. al., 2004). A healthy individual's cortisol levels will return to normal within hours of trauma; however, many of those who go on to develop PTSD express lower than normal levels of cortisol and CRF. It is thought that these lower than baseline levels of cortisol are due to an over abundance of glucocorticoid receptors which lead to enhanced cortisol binding and may contribute to cortisol down-regulation (McNally, 2003). It is possible that PTSD is a result of an extremely prolonged stress response activation and later sensitization of the HPA-Axis due to these neurochemical and endocrinological abnormalities. In addition, a more sensitive sympathetic nervous system and a less active parasympathetic nervous system are also common abnormalities seen among PTSD sufferers (Davidson et. al., 2004).

While we know a good deal about some of the abnormalities related to the development of PTSD and prolonged stress activation, we are far from understanding all of the factors which mediate and moderate these abnormalities. Many suggest that this prolonged response could be due to lower levels of cortisol, which would normally serve to inhibit the initial stress responses (Davidson et. al., 2004; Yehuda, 2009). However, other hypotheses have been made about how these abnormalities come to be including abnormal circadian rhythm patterns, negative glucocorticoid feedback irregularities, tonic cortisol secretion, adrenal insufficiency

and more (McNally, 2003; Yehuda, 2009). In addition, certain brain structures have been found to be different within PTSD patients. Lindauer and colleagues showed in their 2005 study a 15% smaller left amygdala volume and significant reduction of hippocampal volume after combat and sexual abuse. These systems may impact the sensitization of the HPA-Axis in addition to influencing explicit memory systems, which are implicated in emotional memories such as those produced by trauma, and relived by those with PTSD. However, it should be noted that pre-stress structure volumes for these participants are not documented and thus directionality (PTSD causing decreased amygdala and hippocampal volumes, or smaller structures causing PTSD) cannot be definitively stated. It is important to work to develop an understanding of these mechanisms as many have found that the expression of PTSD is suggestive of a vulnerability to a variety of other disorder including; major depression, generalized anxiety disorder, phobias, sleep disorders (including night terrors and insomnia), and substance use (Davidson et. al., 2004; Yehuda, 2009).

PTSD & Cocaine Use

As researchers and clinicians work to better understand stress and PTSD, a alarming association with substance use has emerged. Not only has stress been highly implicated in the reinstatement of extinguished drug seeking behaviors, or relapse (Ahmed & Koob, 1997), but a startling trend of co-occurring substance use and PTSD has surfaced as well. Between 30%-50% of individuals seeking treatment for SUD (Substance Use Disorder) meet criteria for lifetime PTSD (i.e. experiencing PTSD at some point within their lifetime) and some studies report up to 20% meet criteria for PTSD at the time of the study (Back et. al., 2000; Parra et. al., 2009).

While many have found elevated levels of comorbid substance use and PTSD with drugs such as heroine, amphetamines, hallucinogens, and sedative/hypnotics, cocaine dependent individuals seem particularly prone to co-occurring PTSD and SUD (Driessen et. al. 2008; Simon, Gaher, Joacobs, Meyer, & Johnson-Jimenez, 2005). Cocaine is a psychomotor stimulant drug that stimulates transmission at the synapse of monoamines, specifically catecholamines, dopamine and norepinephrine (McKim, 2007). Cocaine is a reuptake-inhibiting drug, which activates the sympathetic nervous system causing vasodilatation and bronchodilation in addition to effects including positive mood, euphoria, insomnia, and analgesia (McKim, 2007). These affects are considered to be a result of stimulation of the mesolimbic dopamine system effecting processes such as pituitary gland secretions and dopamine release in the nucleus accumbens. Additional brain systems are also implicated including reduction of glucose metabolism in all areas of the neocortex, thalamus, midbrain, hippocampus, and the medial prefrontal cortex (McKim, 2007).

Both cocaine and PTSD have far reaching, albeit not always well understood, effects on the brain and there is an obvious relationship between the two. In one study of 91 cocaine dependent individuals, 42.9% met criteria for lifetime PTSD, and 22% met criteria for current PTSD (Back et al., 2000). Furthermore, cocaine is known to influence the secretion of HPA-Axis mediated hormones, and some have suggested that the presence of corticosterone (a stress related hormone released by the adrenal gland) may actually be necessary for the reinforcing effects of cocaine to take place (Goeders, 2001, 1997). However, all of the factors (hormonal, neurochemical, neuroanatomical, etc.) that mediate and moderate this link are still

poorly understood and warrant further research. Many recent studies have begun to look into the relationship between these two disorders with the intention of improving understanding of both PTSD and SUD.

Non-Human Animal Models

In the process of learning more about stress and substance use, it is important that researchers find ways to examine these behaviors in animal models. While the most opportune situation would be that humans could easily be used to experimentally examine PTSD and substance use, this is not normally the case. Numerous variables are simply too challenging to control for when using human participants, and thus the development of valid animals models and the use of non-human animals in research is extremely important (Carroll, & Overmeier, 2001). While humans may develop PTSD, the etiology, or the cause and course of the disorder, could drastically differ from person to person due to a variety of factors such as; past stress history, magnitude of stress experienced, baseline biological differences. In comparison, it is relatively easy to control the development and histories of animals.

In addition, animal models not only allow for the observation of behavior, but also for the collection of detailed data regarding brain and neurotransmitter systems. In the case of drugs, non-human animals allow for control of such things as; drug administration, and drug-environment, the collection of samples at all stages of addiction (Carroll, et. al., 2001). It would be ethically impossible to justify the administration of addictive narcotics to unknowing human participants, and exposing an individual to the levels of stress needed to produce PTSD symptoms

would be considered extremely unethical as well. However, with the proper safe guards (for example adhering closely to animal welfare regulations) it is appropriate and advantageous to use non-human animals in experimental procedures that can serve to promote understanding, health and wellbeing (Goodwin, 2008).

Place Conditioning

As we work specifically to create a better understanding of drug use, the development of paradigms that allow for the interpretation of drug effects on non-human animals is important. Place conditioning (CPP, or 'conditioned place preference') and self-administration are popular ways in which more can be learned about the rewarding, aversive, or reinforcing effects of a drug (Bardo & Bevins, 2000; Cunningham, Gremel, & Groblewski, 2006; Domjan, 2010). A self-administration paradigm is considered appropriate for studying drug reinforcement, as a drug would be considered reinforcing if it increased the probability that a certain operant behavior will be completed which has become associated with drug administration (Bardo, 2000). In addition, self-administration is considered to closely replicate human drug use as the subject administers the drug as they please similar to human addicts. In comparison, CPP is considered to be an effective model for studying drug reward or aversion in non-human animals. A drug that exhibits appetitive effects is considered to be rewarding in comparison to drugs that have aversive effects (Bardo, 2000; Domjan, 2010).

In place conditioning a distinctive location is paired with drug administration. After a number of drug/place pairings, the animal is given a choice

between the drug paired location and a non-drug, or saline paired location. Place conditioning is generally considered to be a form of Pavlovian conditioning in which an unconditional stimulus (US), for example food as in traditional Pavlovian conditioning, elicits an unconditional response (UR) such as salivation. If the US is paired with a second stimulus referred to as the conditional stimulus (CS), for example a tone, the CS can come to elicit a conditional response (CR). This CR is related to the initial UR but prompted not by the US, but instead by the CS (Cunningham, 2006; Domjan, 2010).

In place conditioning, the drug-paired location is identified as a CS, or CS+ while the non-drug paired location is a CS-. When the animal can differentiate between the drug state and the non-drug state then a conditioned response may be seen. If the subject can discriminate between the two locations and comes to associate one of them with the drug state (which is triggered by the drug or US) then responses in reference to either a appetitive or aversive drug effect (the UR) can be observed when the animal chooses one side over the other (the CR) (Cunningham, 2006). This is a unique and valuable aspect of place conditioning in that conditioned place preference or conditioned place aversion can be examined when an animal spends more or less time respectively in the drug-paired location (Bardo, 2000).

In the case of the drug cocaine, rewarding effects are generally noted and animals including rats, mice, and rhesus monkeys, all show an increase in the amount of time spent in the formally drug-paired location (Bardo, 2000). The assumption is made that this increase in time indicates that the animal shows a preference for the location that has come to be associated with the drug when tested

in a drug-free state. If an initial pre-test before the drug/place pairings indicates no preference for one side over the other it can be assumed that an increase in time indicates a preference for the drug-state over the non-drug state which was paired with the other environment. A decrease in time from pre-test to post-test would indicate the opposite and suggest that the drug is expressing aversive effects. Past work has examined the effects of stress on cocaine place preference and while some find stress to have an enhancing effect on CPP, others find that it may not be the stress, but the type of drug that influences conditioning.

Stress & Place Conditioning

The distinctive effects of different stressors on cocaine conditioned place preference was examined by Haile, GrandPre and Kosten in their 2001 study. Haile et. al. exposed rats to two different stress conditions. One group received chronic unpredictable stress, and the other group chronic predictable stress. The unpredictable stress consisted of a series of different stressors (including cage rotation, cold isolation, food and water deprivation, housing isolation) over a period of ten days, while the chronic predictable stress was simply 60 minutes of restraint every day for the same period of time. After the stress was completed, the animals went through cocaine place conditioning.

The two stressors were found to have differing effects on cocaine place conditioning. Exposure to prolonged unpredictable stress was shown to promote the behavioral effects of cocaine (locomotor activity in particular) and to increase place preference. In comparison, prolonged predictable stress failed to enhance locomotor activity in the subjects in addition to having no amplifying effect on

cocaine conditioned place preference. This suggests that after exposure to stress the effects of psychoactive drugs such as cocaine may differ depending on the type of stress. In particular, this experiment suggests that the lack of predictability of a stressor may play an important role in the possible later amplification of drug reward and later drug use. Haile and colleagues suggest that further research into types of stress, stress responses, and their relationships to factors that may contribute to vulnerability to cocaine addiction is warranted.

In comparison to Haile et. al. who examined the effects of unpredictable and predictable stress on cocaine conditioned place preference, Der-Avakian, and colleagues (2007) examined single sessions of uncontrollable stress. Single sessions of tail shock were administered to the rats within a inescapable shock group, and another comparison group had control over the duration of the stressor by completing an operant response (i.e. turning a wheel). Der-Avakian et. al. examined, through the use of place conditioning, the effects of inescapable stress on drugs outside of the opioid family including cocaine, and ethanol in comparison to oxycodone (an opiate drug). Der-Avakian and colleagues found that while the inescapable stress enhanced conditioned place preference for oxycodone there was no measurable effect on cocaine or ethanol. These findings suggest that the inescapable stress only showed enhanced rewarding properties on opioids, and not on the other drugs (i.e. cocaine and ethanol).

These results are not in complete agreement with the previous understanding of the possible relationship between unpredictable stress and the rewarding effects of cocaine. Haile et. al. reported this relationship when they

observed a enhancing effect of unpredictable stress on later cocaine conditioned place preference. However, while the Der-Avakian results do not completely support the data from the study done by Haile and colleagues regarding cocaine, they do support the notion that different types of stress may vary greatly from one another in their effects on different drugs (i.e. opiates such as morphine or oxycodone v. stimulants such as cocaine). These equivocal findings suggest that careful research into different types of stress, and their interaction with the rewarding properties of drugs needs to continue. In addition to different types of stress, an examination of different drugs such as opiates, stimulants, and even sedative-hypnotics (i.e. depressants) needs to be done to examine patterns of increased use. Der-Avakian and colleagues suggest that a detailed analysis of the neuronal, behavioral, and chemical mechanisms of stress and addiction are necessary in order to learn more about the relationship between stress, stress responses (such as PTSD) and cocaine use.

Animal Models of PTSD

While past research has examined different stress influences on cocaine using place conditioning (Der-Avakian et. al., 2007; Haile et. al., 2001), no work has used an animal model of Posttraumatic Stress Disorder (PTSD) to evaluate drug reward. As human studies have revealed higher than normal levels of comorbid PTSD and cocaine use, developing and examining an animal model could be extremely valuable due to the insights provided by non-human animal research (i.e. the impact of brain on behavior, neurochemical influences, and more). Liberzon, Krstov, and Young used the Single Prolonged Stress (SPS) paradigm, first introduced

by Antleman in 1988, in their 1997 experiment. The SPS paradigm has been supported as a potentially valid animal model of PTSD and uses a variety of stressors including forced swimming (20-minutes), restraint (2-hours), and exposure to ether (until the loss of consciousness) in close succession within the period of a single day. This stress is then followed by a period of 7 days in which the animal remains undisturbed in the home cage, a phase known as an incubation period. After the completion of the period, blood samples were taken from each animal directly after a short re-stress (30-minutes of restraint) (Liberzon, Krstov & Young, 1997).

Liberzon and colleagues found during the later stress test that the animals showed a sensitization of the HPA-Axis, which, as explained earlier, is implicated in stress responses and corresponding neurotransmitter and hormone release. Specifically, the animals showed enhanced negative glucocorticoid feedback, which, along with low levels of cortisol, has been strongly implicated in producing the maladaptive stress response associated with PTSD symptoms. Since the introduction of SPS a number of studies have supported the validity of the paradigm as a compelling animal model of PTSD.

Yamamoto et. al. supported SPS as an animal model of PTSD citing that after the incubation period, animals have shown many of the neurochemical, neuroanatomical, and behavioral changes seen in human PTSD sufferers. SPS replicates many of the phenomena seen by Liberzon specifically including pathophysiological abnormalities, which are changes in biochemical, and physical functioning after stress such as the sensitization of the HPA-Axis. In addition to

these changes, SPS also produces impairment of later fear extinction, decreases in exploration of a novel environment, exaggerated fear response, memory impairment (specifically spatial memory), and a number of other behavioral abnormalities associated with human PTSD (Yamamoto et. al. 2009).

Ding, Han & Shi (2010) also examined the SPS model to determine whether or not the paradigm produced effects that parallel PTSD symptoms in humans. Ding and colleagues explored the control of apoptic cell death (i.e. programed cell death) and apoptosis related genes in SPS rats to examine the possible cause of atrophy of the amygdala, which has been observed in human PTSD sufferers. The model was found to be successful in recreating abnormalities seen in humans with PTSD who show lower amygdala and hippocampal volumes (Ding, et. al., 2010, Lindauer, et. al., 2005). Ding and colleagues observed an increase in cell death after stress in addition to an imbalance of apoptosis regulating proteins, which promoted degeneration. In addition, the increase in apoptosis in the amygdala post SPS was noted to have begun 4-7 days after the initial stress. However, while the similarities of the effects of SPS to the human condition (i.e. structure volume deficiencies and hormonal abnormalities) are becoming well documented, unlike other previously mentioned stress paradigms (i.e., unpredictable, predictable, single-stress, and chronic stress), no work has examined the possible influence of SPS on drug reward using place conditioning.

Time Dependent Effects of PTSD

In the case of Posttraumatic Stress Disorder, studying factors that contribute to the relationship between the stress exposure and cocaine use is extremely

important and is still poorly understood. Typically, place conditioning occurs immediately after stress exposure, such as in the case of both Haile et. al. and Der-Avakian et. al. However, there is evidence to suggest that immediate testing may be overlooking some of the long-term effects of stress and may be discounting important aspects of the condition of PTSD. In comparison to these past studies, Harvey et. al. (2003) (who used a slightly modified Single Prolonged Stress paradigm) hypothesized that a time-dependent sensitization (TDS) was taking place during the incubation period in the days following stress. This delay after stress, or incubation period, is a time spent undisturbed in the home cage for 10-days post-stress and has been implicated as the phase in which many of the physiological, neurochemical and behavioral changes observed in humans who have experienced stress take place. Spatial memory post-stress was assessed using a Morris Water Maze, and corticosterone and hippocampal serotonin and protein concentrations were assayed. Harvey and colleagues observed significant effects of TDS and SPS on spatial memory showing an increase in memory deficits. In addition, a significant change in corticosterone, and serotonin receptor densities along with protein densities was reported post-incubation.

Harvey et. al. reported that this incubation period or time of sensitization may be the interval in which hippocampal and amygdala degeneration, endocrinological imbalances, leading to cognitive impairments (such as spatial memory deficits) associated with exposure to stress occur. These stress responses parallel physiological, neurochemical and behavioral changes observed in humans who have experienced stress, specifically sufferers of PTSD (Harvey et. al. 2003).

Harvey and colleagues suggest that an incubation period post-stress may be necessary for actual PTSD-like changes to take place. However, the majority of animal research today fails to account for the possibility of a significant influence of an incubation period when employing an animal model of PTSD. This potential oversight is particularly evident in the possible relation to enhancing drug reward.

Rationale

The present work aims to use a modified Single Prolonged Stress paradigm to examine the effects of stress, followed by an incubation period, on the rewarding properties of cocaine using place conditioning. As PTSD affects such a large portion of the population (approximately 8%), it remains imperative that more is learned about the mechanisms that mediate and moderate the disorder (American Psychiatric Association [DSM-IV-TR], 2000). In addition, the startlingly high rates of comorbid substance use, specifically cocaine use, suggest that a relationship between the disorders exists and warrant further research evaluating factors that may contribute to drug use, abuse, and PTSD comorbidity (Back et. al., 2000; Parra et. al., 2009). Additionally, while some have found that certain types of stress may enhance the behavioral effects of cocaine in animal models, others have reported no effect of stress on cocaine reward. These results suggest that continued investigations into different types of stress, such as SPS, and their effects on the rewarding properties of cocaine are important (Der-Avakian, et. al., 2007; Harvey et. al., 2003; Haile, et. al., 2001; Yamamoto et. al., 2009). Based on past equivocal findings and lack of work directly evaluating the effects of PTSD on the rewarding

effects of cocaine in animal models, more research using place conditioning paradigms to assess drug reward needs to be done.

An animal model of PTSD and its attendant effects on drug reward could help expose factors that contribute to human addiction. This work uses a SPS model, which has been modified to fit our laboratory situation, and a conditioned place preference paradigm to allow for the ability to measure drug reward. These findings may add to the literature by examining the differences between animals tested immediately post-stress, similar to traditional studies with equivocal findings, and animals that have had a post-stressor incubation period like those used in the Single Prolonged Stress model of PTSD.

It is hypothesized that stress (SPS) will strengthen subsequent cocaine place conditioning suggesting enhanced rewarding properties. In addition, it is proposed that subjects who are tested after an incubation period will show enhanced cocaine conditioned place preference due to time-dependent sensitization in comparison to animals that are tested immediately post-stressor.

Methods

Subjects

Forty-eight male Sprague-Dawley rats were used for this study. On day 1 of experimentation the rats were 51 days old having been individually housed in hanging wire-mesh cages for 11 days. Food and water were available ad libitum in the home cage and the colony room was maintained on a 12:12 light:dark cycle except for certain stress phases.

Apparatus/Materials

Stressors

During the stress phase of the experiment the rats in the experimental group received three stressors including a series of multiple uncontrollable stressors, swim stress, and restraint stress (see Table 1). This stress phase took place in a room separate from both the colony and the room containing the conditioning apparatus. For the series of initial multiple stressors, the subjects were placed in hanging wire mesh cages tilted at a 45-degree angle. Subsequently, animals underwent forced swim stress, and restraint stress as outlined by the SPS paradigm (Antleman et. al., 1988). However, due to the drug conditioning used in the present study, animals were not exposed to ether to the point of the loss of consciousness as is as was in the original SPS paradigm. Accordingly the 'multiple stressors' portion of the experiment was added and was adapted from stress manipulations used in previous stress research employed in our lab (Haile et. al., 2001). The swim stress took place in circular, 18-gallon tanks measuring 21.5 inches in height and 16.5 in diameter. The water was maintained at room temperature, 75 degrees Fahrenheit. Harvard Apparatus restraint cones were used for the restraint stress. The restraint cones were clear with the smaller end open to allow for the animal to breathe, and the larger end closed by the researcher for restraint.

Drugs

Cocaine HCl was obtained from Sigma Corporation for the present experiment. The cocaine was dissolved in sterile saline solution (0.9%) at a concentration of 15 mg/ml. Subjects received a cocaine dose of 15 mg/kg during the drug-paired phase of conditioning, and the animals received an equivalent

volume of sterile saline solution during the non-drug-paired portion of conditioning (i.e., 1 ml/kg). Cocaine and saline solution were administered via intraperitoneal (IP) injections.

Conditioned place preference

Place conditioning was used to measure preference for cocaine after the stress phase of the experiment. Four identical apparatuses housed in sound-attenuating chambers (80cm H x 94cm L x 61 cm W) were used for place conditioning. There was a single two-sided, Plexiglas place conditioning apparatus (21.6cm H x 44.5cm L x 26.7cm W) in each sound-attenuating chamber. An internal LED light was at the top of the sound attenuating chamber and two fans were placed on the Right and Left sides for the purpose of air circulation. In addition, the sound generated by the two 80x80x32mm Fulltech Fans, model UF-80A11, contributed to attenuating extraneous noises.

One side of the place conditioning apparatus consisted of black walls (laminated black construction paper, 21.6 cm H, 22.2 cm L) with a grid floor (29.2 cm L x 26.7 W, stainless steel bars: 2.38mm diameter, 12.7mm apart from one another). The opposite side had white walls (laminated white construction paper, 21.6 cm H, 22.2 cm L) with hole floor (29.2 cm L x 26.7 W, stainless steel plate with holes: 12.7mm diameter, 3.175mm apart from one another on staggered centers). A removable clear plastic partition was placed between the two sides allowing for confinement to one side of the apparatus during conditioning. During testing, a partition with a square opening (10.16 cm H x 10.16 cm W) in the center was used allowing for freedom of movement between the two compartments. Every chamber

was wiped-down with a damp sponge containing diluted soap after each subject's training or test trial. Performance during training and test were recorded using an EverFocus digital recording device and video camera mounted above the place conditioning apparatus. Accordingly, the location of each animal within the apparatus was visible during conditioning and testing on a television screen.

Procedures and Design

Stress

Every rat was handled for 5 minutes on two consecutive days to acclimate the subjects to being handled by researchers. On the night of day two of handling, the experimental groups began the stress phase with the 'multiple stressors' period, while the no-stress control animals remained undisturbed in the home cage. Subjects were transported and placed into the tilted individual hanging wire mesh cages. The animals were food and water restricted during this 12-hour period. The lights remained on during the 12-hour dark phase of each subject's light/dark cycle from 8 pm until 8 am. To ensure safety, the animals were weighed prior to and after the 12-hour period of food and water restriction. If a rat's weight droped below 10% of their free feeding weight, then food and water were returned for an hour prior to continuing the stress procedures outlined below.

Following the 'multiple stressors', four rats at a time received fifteen minutes of forced swimming in 18-gallon tanks. Each animal was monitored continuously and were removed from the tank if their head remained under water for 4 seconds. After the swim stress a 15-minute break occurred where the subjects returned to their home cages in accordance with the original SPS paradigm. After this

recuperation period, the animals underwent 30 minutes of restraint. Each animal was monitored continuously and restraint was terminated if a rat showed difficulty breathing, struggling, or excessive porphyrin staining. After the completion of this phase, the animals were returned to their home cages until cocaine place conditioning began. The no-stress control group remained in the home cage during this portion of the experiment.

Cocaine Place Conditioning

The animals in the stress group were separated into two stress sub-groups. The first group known as the 'stress/no-delay group' began place conditioning the day following the stress phase. The other group consisted of the remaining animals that had undergone the stress procedure. However, this second group, known as the 'stress/10-day delay group', was left undisturbed in their home cages for 10 days (i.e. the incubation period). At this time the animals were kept on a 12-hour light:dark cycle, and food and water was available ad libitum. The no-stress group (i.e. control group), which had not undergone the stress procedure, was separated into two no-stress sub-groups. One of these groups known as the 'no-stress/no-delay group' underwent place conditioning at the same time as the stress/no-delay group. The second no-stress sub-group known as the 'no-stress/10-day delay group' underwent place conditioning with the stress/10-day delay group.

Initially, all subjects received a 15-minute place preference test to evaluate time spent in either Black/Grid (B/G, black walls and grid floor) or White/Hole (W/H, white walls and hole floor) side of the place conditioning apparatus. A crossing from one side of the apparatus to the other was determined when the

animal's shoulders fully passed through the doorway of the apparatus. Time spent on both sides of the apparatus during the pre-test were measured and recorded.

Twenty-four hours later, one half of all of the animals in the no-delay groups (i.e. both no-stress/no-delay, and stress/no delay) received received a 15 mg/kg I.P. injection of cocaine and were placed on either the B/G or W/H side (drug-paired side, CS+) of the place conditioning apparatus for 15 minutes. The second half of the group received sterile saline solution (1 ml/kg, I.P.) and were placed on the non-drug paired side (CS-) of the apparatus for 15 minutes. On alternating days the animals were placed on one side of the apparatus, and then the other. All animals received 4 days of drug pairing and 4 days of saline pairing on alternating days. Additionally, side of drug pairing on either the left or right side of the apparatus was counterbalanced. Accordingly, one-half of all animals received cocaine on the W/H side of the apparatus (i.e W/H+) and one half received cocaine on the B/G side (i.e. W/H-). The same procedures were conducted with the 10-day delay groups (i.e. no-stress/10-day delay, and stress/10-day delay) except 10 days had passed after the initial stress procedure took place. Finally, one day after the last day of the conditioning phase, a place-conditioning test was conducted identical to the pre-test.

Research Design

The present study first uses a 2 x 2 x 2 mixed factorial design to specifically evaluate the two no-stress sub-groups at pre and post-test. The independent variables were a between groups factor of delay (no-delay, or 10-day delay), a between groups factor of drug pairing/cue (W/H+ or W/H-), and a within groups

factor of test (pre v. post). If no significant effect of delay is detected after place conditioning, then the animals in the two no-stress sub-groups will be combined into a single 'no-stress' group in the overall analysis.

Additionally, a 3 x 2 x 2 mixed factorial design was used in an overall analysis of outcomes. There were three independent variables that included: one between groups stress factor with three levels, a between groups drug cue factor with two levels, and a within groups test factor with two levels. The independent variables were stress/delay (i.e. no-stress, stress no-delay, or stress 10-day delay), drug pairing/cue (W/H+ or W/H-), and test (pre v. post). The dependent measure was the amount of time spent on the W/H side of the apparatus (see Table 2).

Statistical Analysis

Amount of time spent on the W/H side was evaluated in seconds for both the pre and post-tests. A single Mixed, Factorial Analysis of Variances (ANOVA) was used to determine differences between the no-stress control group tested immediately (i.e. no-stress/no-delay group) and the no-stress control group tested 10-days later (i.e. no-stress/10-day delay group). A second Factorial Analysis of Variance was used to assess possible main effects and interactions among the three factors of stress/delay (stress no-delay, stress/10-day delay, and no-stress), and cue at pre and post-test.

Results

No-Stress Sub-Groups

The 2 x 2 x 2 ANOVA revealed a main effect of test suggesting that there was a significant difference between the pre and post-tests for no-stress the groups (F

(1,12) = 5.89, $p < .05$). In addition, a significant main effect of cue was seen indicating that there may be a difference in time spent on the W/H side of the apparatus between animals that received cocaine on the W/H side (W/H+) and animals that received cocaine on the B/G side (W/H-) (F (1,12) = 41.56, $p < .05$). There was no main effect of delay which suggests that the animals within each of the delay groups did not differ from one another on time spent on the W/H side of the apparatus (F (1,12) = .001, $p > .05$).

There was a significant interaction between test and cue showing that the amount of time spent of the W/H side of the apparatus was different and may reveal that place conditioning did take place (F (1,12) = 116.998, $p < .05$). There was also a significant interaction observed for test and delay suggesting a difference between the delay groups from pre to post test (F (1,12) = 7.66, $p < .05$). However, there was no significant interaction between the factors of delay by cue (F (1,12) = 1.663, $p > .05$). This lack of interaction between delay and cue indicates no unique effect of delay on strength of place conditioning. In addition there was no significant three-way interaction between test, delay and cue indicating no unique effect of any of the factors across the levels of the remaining two (F (1,12) = .862, $p > .05$). Due to the fact that there were no statistically significant differences between the two no-stress sub-groups (no-stress/no-delay and no-stress/delay) on place conditioning they were combined into a single no-stress group in all later analyses (see Figure 1)

No-Stress, Stress/No-Delay, and Stress/10-Day Delay

A second overall analysis was conducted for all three levels of stress/delay conditions (i.e. no-stress, stress/no-delay, and stress/10-day delay), the two levels

of drug pairing conditions (W/H+ or W/H-) at both pre and post-test(see Figure 2). This ANOVA revealed a main effect of test suggesting a significant difference between pre and post-test ($F(1,42) = 8.72$, $p < .05$). In addition, the ANOVA displayed a main effect of cue showing a significant difference between times on the W/H+ sides of the apparatus between the W/H cocaine paired group (W/H+) and the B/G cocaine paired group (W/H-) ($F(1, 42) = 78.94$, $p < .05$). However, no main effect was seen for the stress manipulation, suggesting no difference between no-stress, stress/no-delay, and stress/10-day delay groups ($F(2,42) = .121$, $p > .05$).

Notably, there was a significant interaction between test and cue suggesting a difference in the amount of time spent on the W/H side of the apparatus as a function of side of drug pairing (either W/H+ or W/H-) from pre-test to post-test ($F(1,42) = 86.65$, $p < .05$). As Figure 2 shows, there was no difference between the time spent on the W/H+ or W/H- sides of the apparatus at pre-test. However, a significant cocaine place preference is observed as these times differ significantly from one another at post-test. There were no significant interactions between test and stress/delay ($F(2,42) = 1.38$, $p > .05$), nor was there an interaction between stress/delay and cue ($F(2,42) = .36$, $p > .05$). In addition, no 3-way interaction was observed between test, cue, and stress/delay ($F(2,42) = .28$, $p > .05$) suggesting no unique effect of any single factor across the levels of the other two variables.

Discussion

The present findings indicate that, although there was strong place conditioning, no significant difference between any of the stress/delay groups as measured by CPP was observed. Figure 1 demonstrates that neither of the no-stress

sub-groups differed significantly from one another in strength of CPP. Because there was no difference in strength of place conditioning between the two groups, they were combined into a single 'no-stress' group. As Figure 2 shows, there was no significant difference at pre-test between animals that were going to receive cocaine on the W/H side of the apparatus (W/H+) and those that were going to received cocaine on the B/G side (W/H-). In addition, there was no difference in initial preference due to the stress exposure. However, after conditioning, the post-test revealed that W/H+ paired animals (i.e. W/H+, B/G- animals) spend significantly more time on the W/H side of the apparatus than those who received cocaine on the W/H- (non-cocaine paired) side (i.e. the W/H-, B/G+ animals).

In the second analysis of all three stress/delay groups (i.e. no-stress, stress/no-delay, and stress/10-day delay) the main effects of test and of cue suggest that there was a difference between pre and post-test and that there was a difference between the W/H+ groups and the W/H- groups across the levels of the other variables. However while the main effects of these variables are important, it is necessary to note that the significant interaction between these two factors of test and cue is what indicates that place conditioning has occurred. Additionally, at pre-test there was no significant difference between any of the stress/delay groups (see Figure 2). The interaction observed highlights that while no initial differences were observed at pre-test, there was a significant difference among the drug pairing groups (either W/H+ or W/H-) at post-test. This means that after conditioning the animals that received cocaine on the W/H side of the apparatus spent significantly more time on the W/H side than those who had received cocaine on the B/G side of

the apparatus. This outcome shows robust cocaine conditioned place conditioning among all three stress/delay groups (no-stress, no-delay, and 10-day delay).

While all three groups showed conditioned place preference after cocaine conditioning there were no significant differences between any of the stress groups as measured by CPP. We had hypothesized that stress would enhance subsequent cocaine place conditioning. In addition, it was proposed that in addition to stress enhancing CPP, the 10-day delay group that had received the stress procedure would show significantly more cocaine conditioned place preference in comparison to animals in the stress/no-delay group. A three-way interaction between test, cue, and stress/delay would have indicated that there was a unique effect of stress across the levels of test and cue. In other words, the three stress groups would have shown significant differences from each other in time spent on the W/H side at post-test. An enhancement due to stress, however, was not observed as neither of the groups that had received the stress procedure showed enhanced place conditioning in comparison with the no-stress group. In addition, an enhancement due to delay or incubation was not observed in relation to the no-delay group or the no-stress group.

In the past, some have shown that different types of stress express different effects on cocaine conditioned place preference (Der-Avakian et. al., 2007; Haile et. al., 2001). Haile and colleagues reported that prolonged unpredictable stress enhanced place preference while prolonged predictable stress had no observable amplifying effect on CPP. However, Der-Avakian and colleagues reported no enhancing effect of a single session of uncontrollable stress on cocaine conditioned

place preference. This experiment supports past results that suggest that certain types of stress may not strengthen the rewarding properties of cocaine and thus show no enhancing effect on conditioned place preference.

In addition to the factor of stress showing no enhancing effect in this experiment, the incubation period seems not to have had a significant influence on CPP in comparison to non-stressed and stressed then non-incubated groups. Many in the past have suggested that this incubation period is vital for the neurochemical, neuroanatomical, behavioral, and cognitive changes associated with stress to take place (Liberzon et. al., 1997, Yamamoto et. al., 2009, Ding et. al., 2010, Harvey et. al., 2003). Specifically, Harvey et. al. argued that the incubation period post-stressor was in fact a time of sensitization in which many of the changes associated with PTSD take place. These changes included hippocampal, and amygdala degeneration, endocrinological imbalances, and cognitive changes which have not only been implicated in PTSD (Harvey et. al., 2003, Ding et. al., 2010), but also in possible drug reward systems (Goeders, 2001; Goeders, 1997). However, the current study observed no measureable effect of a post-stressor incubation period on CPP.

No past work has been done examining the specific effects of the SPS paradigm, (specifically stress followed by the incubation period) on cocaine place conditioning. There is significant past work, however, to support the notion that there is a relationship between PTSD and cocaine use in humans. The biochemical and neurological changes, which have been reported in past work that used SPS, may not have directly influenced cocaine place conditioning in the present paradigm. In addition, it is possible that as an animal model the SPS paradigm may

not be reproducing all aspects of the human condition of PTSD, specifically those responsible for the enhanced rewarding effects of some drugs.

However, the stressors previously employed by researchers studying time-dependent sensitization were slightly different than those used in the present study. While we chose to replace exposure to ether with a series of multiple stressors first used by Haile and colleagues, it is possible that these procedural differences could have influenced the production of PTSD-like changes both on stress over-all and as a result of incubation. Furthermore, some of the experiments that employed SPS also used a short re-stress procedure before behavioral testing. Although the animals in this experiment were exposed to many situational reminders of the stress (i.e. being exposed to the same researcher, being handled, and residing in hanging wire-mesh cages similar to those used in the stress) a specific re-stress phase was excluded in the present study. It is possible that this exposure to a stress reminder situation could enhance the sensitization of the HPA-Axis and could possibly influence the development of the maladaptive stress response itself (i.e. PTSD).

As Der-Avakian suggested, the effects of stress may be more evident in studies of different drugs, specifically opioids. Past research with human PTSD sufferers has revealed increased levels of cocaine use, but there have also been reports of increased opioid use (Driessen et. al., 2008). It is possible that these time-dependent changes that have been reported would exert their effects differently on other classes of drugs. It may be advantageous for future work using the SPS paradigm to examine the time-dependent effects of stress on other drugs such as morphine, or heroin.

In addition to examining the possible influences of the time-dependent effects of SPS on other drugs, there are many other possible future directions for this research. Acquisition of an association between the drug state and the drug-paired environment may be more rapid among stressed animals and/or among incubated animals. Future research may consider including probe-tests, or tests at different intervals throughout conditioning to determine rate of acquisition. Additionally, the use of a self-administration paradigm may yield different results as the animals would be given the freedom to administer cocaine as they like. However, due to the more costly and invasive nature of self-administration CPP, was used to measure the rewarding effects of cocaine in this experiment.

In addition, the present study only examines the time-dependent effects of stress on a dosage of 15 mg/kg. It may be interesting to look at higher and lower doses of cocaine as well. Perhaps the effects of stress or delay would present themselves in a situation where the stress/delay animals develop an observable place preference with a lower dose as compared to non-stressed animals. Conversely, a higher dose may produce place aversion in stressed animals or enhanced place conditioning over non-stressed or non-incubated animals.

PTSD may be a major risk factor for developing a substance use problem or Substance Use Disorder (SUD) and increasing our knowledge about the factors that may mediate and moderate this interaction is important. Developing a better understanding of PTSD and SUD, specifically cocaine use, through the use of animal models such as SPS could be extremely influential. The knowledge that is gained through developing and implementing these models helps us to employ effective

prevention programs, in addition to guiding possible treatments. While the results of this particular study may not replicate the relationship seen between the human conditions of PTSD and cocaine use, these results do have important implications for furthering the development of effective animal models which in turn can be used to improve understanding of these two disorders.

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Table 1
Sequences of stressors for subjects in stress group

All stress induction manipulations occurred in a laboratory room adjacent to the animal colony. Each animal was returned to the home cage in colony room at the end of the procedure.

Phase	*Procedure*	*Duration*
1	Multiple Stressors	12 hours
2	Swim Stress	20 minutes
3	Break	15 minutes
4	Restraint stress	30 minute

Table 2
Treatment conditions

Group	*Sub-groups*	*Total Number of Rats*	*Cue (# of rats)*
No-Stress: No stress treatment received			
	'No-Stress/No-Delay'	8	W/H+(4), W/H-(4)
	'No-Stress/ 10-day delay'	8	W/H+(4), W/H-(4)
Stress: Stress treatment received			
	'Stress/No-delay'	16	W/H+(8), W/H-(8)
	'Stress/10-day delay'	16	W/H+(8), W/H-(8)

Figure 1.

Mean time spent in seconds in the W/H side of the apparatus for the CS+ (W/H+) or CS- (W/H-) groups for the initial no-stress groups (No-Stress/ No-delay, and No-Stress/10 Day Delay) prior to (pre-test) and after conditioning (post-test). Standard error of the mean is indicated.

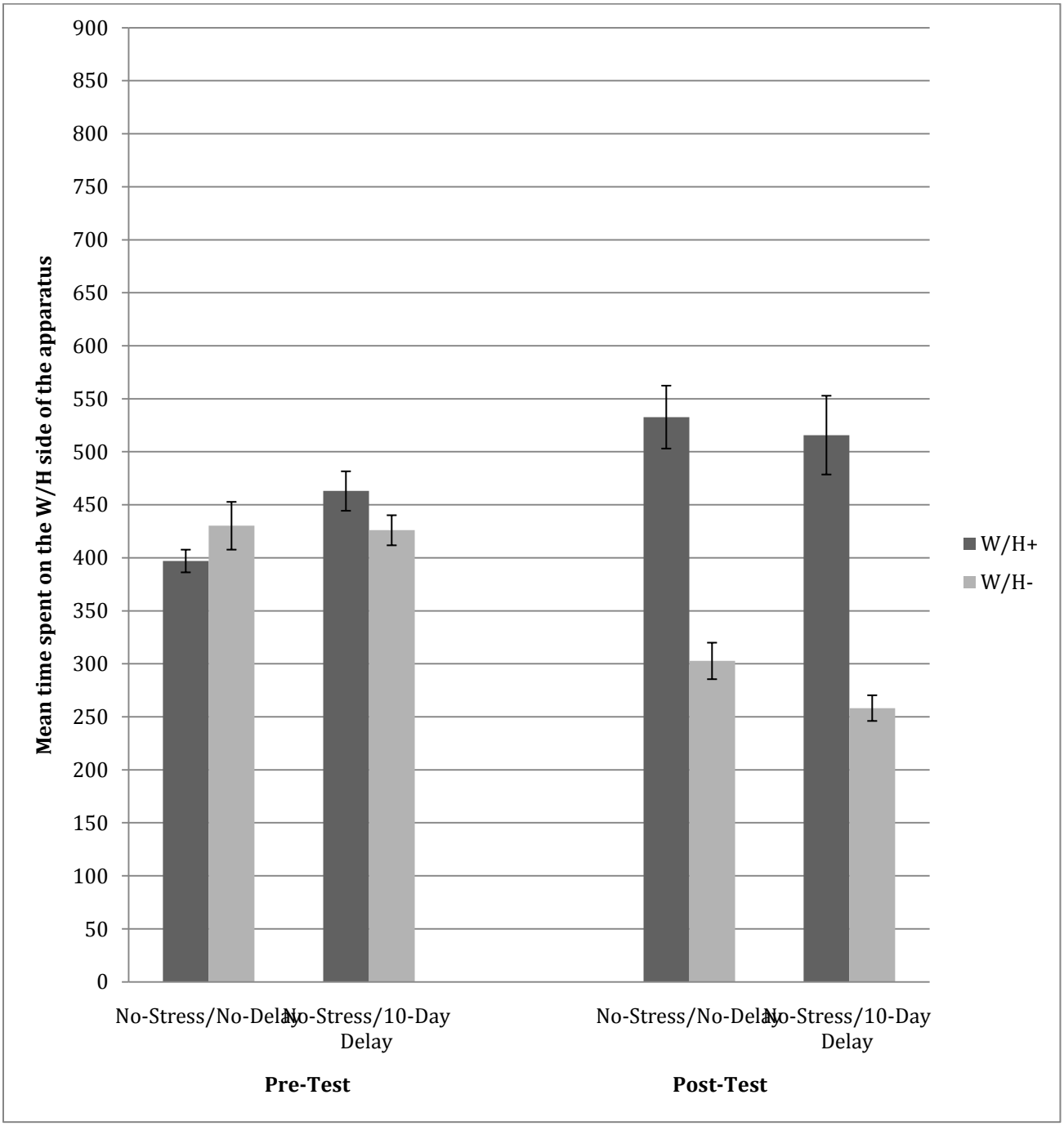

Figure 2.

Mean time spent in seconds on the W/H side of the apparatus for the CS+ (W/H+) or CS- (W/H-) groups for the three stress/delay groups (No-Stress/ Control, No-Delay post-stress, and 10 Day Delay post-stress) prior to (pre-test) and after conditioning (post-test). Standard error of the mean is indicated.