MECHANISMS UNDERLYING THE EFFECTS OF EXTINCTION ON REINSTATEMENT BEHAVIOR IN THE RAT MODEL OF DRUG RELAPSE

by

LAKSHMI KELAMANGALATH

(**Under the Direction of John J.Wagner**)

ABSTRACT

Cocaine addiction is defined as a process that generally starts with recreational use of cocaine and deteriorates over time into a compulsive and chronically relapsing drug taking disorder. The occurrence of relapse is one of the major challenges in the treatment of drug addiction. The rodent self-administration and reinstatement model is accepted as having good predictive validity to study relapse preclinically. An extinction protocol is usually incorporated in this model before the animals are tested for reinstatement after the self-administration training. The lever presses are not reinforced during extinction and thus the animals extinguish their lever pressing behavior. Extinction involves a new learning process and the new learning process requires the recruitment of N-methyl D-aspartate receptor (NMDAR) mediated synaptic plasticity mechanisms. Alteration of the NMDAR activity during extinction is supposed to affect the extinction learning process and/or the effects of extinction on reinstatement. We altered the NMDAR activity during extinction pharmacologically by either blocking the NMDAR activity during extinction using a competitive antagonist of NMDAR, 3 (-2 carboxipiperazin-4-yl) propyl-1-phosphonic acid ((±) CPP) at a dose of 5 mg/kg i.p. or facilitating the NMDAR activity during extinction using a full agonist of NMDAR at the glycine site, D-serine at a dose of 100 mg/kg i.p. We found that

activation of NMDAR mediated mechanisms during extinction is necessary for the effects of extinction on drug induced reinstatement. Facilitating the NMDAR activity during extinction is shown to enhance the effectiveness of extinction on reducing the drug induced reinstatement in animals trained to self-administer cocaine in a short and a long access protocol. The investigation on the requirement of NMDAR mediated mechanisms in the ventral hippocampus for extinction to be effective did not yield conclusive results. We also showed that abstinent animals incubate their drug seeking behavior only to the contextual drug stimuli and the extinguished animals show the effect of spontaneous recovery in response to the contextual drug stimuli and the drug prime when tested after 3 weeks of extinction. However extinction is always shown to be effective in reducing the reinstatement and thus preventing relapse even if there is a delay in the initiation of extinction training.

INDEX WORDS: Addiction, Relapse, Self-administration, Extinction, NMDAR, Reinstatement, Abstinent, Incubation.

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DEDICATION

To

My Beloved Family

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CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

Cocaine addiction is a process that generally starts with recreational use and deteriorates over time into a compulsive and chronically relapsing drug taking disorder (Dackis and O'Brien, 2001). The National Institute on Drug abuse (NIDA) describes addiction as a brain disease that needs to be understood through a coordinated analysis of brain function and behavior. This concept implies that the brain is changed as a result of repetitive drug taking and when the drug taking is stopped or when the drug is no longer present; there is a strong drive towards the resumption of drug taking. This resumption of drug taking is clinically referred to as relapse and this could happen months or even after years of abstinence. Relapse is one of the major challenges in the treatment of drug addiction. According to the information provided by NIDA in 2006, at least 6 million Americans of age 12 and older had abused cocaine in the year prior to being surveyed. The NIDA funded 2007 'Monitoring the future' study showed that 2% of 8^{th} graders, 3.4% of 10^{th} graders and 5.2% of 12^{th} graders had abused cocaine in 2006 (Source- 'NIDA'- National institute of Drug abuse). These numbers indicate that drug addiction is a major socio economic problem in United States. Numerous studies are funded by NIDA in the clinical and pre-clinical fields in the effort to understand different aspects of addiction.

There are several useful animal models to study the addiction process and the relapse in human beings. Among these, self-administration procedures are now a standard tool for studying addiction. Laboratory animals (ranging across mammalian species, from laboratory mice to primates) self administer many classes of addictive drugs. The reinforcing property of

intravenously delivered stimulant drugs has been well documented (reviewed by Spealman and Goldberg, 1978).

Animal models of drug addiction:

Drug self-administration:

In experimental psychology, drug self-administration is described as an instrumental conditioning paradigm where the drug is voluntarily administered in a response-contingent manner. A wide range of routes of self-administration have been used in animal research on addiction including oral, intragastric, intraperitoneal, intravenous and intracerebral (reviewed by Gardner, 2005). The oral and intra venous routes are more analogous to the human situation, while the intra cerebral route is extremely useful for identifying the neural substrates underlying the addiction process. The actual response required to receive the drug may vary among the laboratories. With the laboratory rodents, lever pressing or nose poking is commonly used and in non-human primates, lever pressing is widely used. In my study, I have used a lever pressing model with rats. The reinforcement contingencies required to deliver a drug infusion may vary considerably. It can be a fixed ratio schedule, variable ratio, fixed interval, or a variable interval schedule (reviewed by O'Brien and Gardner, 2005).

Under the fixed ratio (FR) of reinforcement in intravenous drug self-administration, the animal receives the drug according to a fixed ratio of responses. Thus under the FR-1 schedule, the animal needs to make only one required response to get the drug. This is also called as the continuous method of reinforcement and this is most commonly used form of drug self-administration in animal studies of addiction. In a FR-3 schedule, three responses will be required to get a single drug infusion. These low response-low cost FR reinforcement conditions are ambiguous measures of reinforcing efficacy of the drug (Roberts, 1989; Arnold and Roberts,

1997). For measuring the degree of reinforcing efficacy, the variable cost or variable pay off fixed ratio of reinforcement is used. Here, the work cost imposed upon the animal (number of lever presses) is randomly increased from 1 to 10 (FR1 to FR10). Another strategy for measuring the degree of reinforcing efficacy is drug self-administration under progressive-ratio (PR) reinforcement. In this variant of self-administration, the work cost imposed upon the animal to receive a single drug infusion is progressively increased. In every PR drug self-administration session, a point is reached at which the animal's work effort falls below a certain criterion level which is called the break point. This break point is taken as a measure of the reinforcing efficacy of the drug or a measure of the degree of addiction for a particular animal.

Conditioned reinforcement:

Drug self-administration under any of the FR-,PR-, and interval reinforcement schedules utilize a conditioned reinforcement paradigm. Such paradigms are hybrid paradigms combining the instrumental and Pavlovian (classical) conditioning reflecting stimulus learning rather than response learning. The drug is given in a response independent manner in the presence of specific environmental stimulus or a set of stimuli, so that the stimulus or set of stimuli become associated with the subjective state engendered by the drug and acquire the ability to provoke a response in the absence of the drug. In our model, the animals self-administer in an operant chamber which is environmentally very distinct and the animals associate the actual infusion of cocaine with the cue light and auditory tone presented simultaneously with the drug infusion. Through this Pavlovian learning, the animals acquire the self-administration behavior and the discrete environmental stimuli (light and tone) presented along with the drug acquire the properties of a conditioned reinforcer.

Emergence of the long access model of addiction:

Most of the laboratories utilizing animal self-administration procedure for studying addiction have employed a limited access self-administration procedure. The validity of such a protocol is in question now because this will only result in a stable pattern of self-administration and hence this is insufficient to show the binge pattern of drug taking in human addicts (reviewed by Roberts et al., 2007). The stable pattern of self-administration achieved using the limited access model has resulted in behavioral assays that are ideally suited for studying the receptor pharmacology, neurotoxic lesions or hormonal effects (Roberts et al., 1994; Brebner et al., 2000) though it has failed to model the fundamental characteristics of addiction as a progressively deteriorating disease state.

Over time, the Diagnostic and Statistical Manual IV (DSM IV) has provided guidance for self-administration laboratories attempting to address specific aspects of addiction process (Deroche-Gamonet et al., 2004). Now, there is a growing literature on self-administration concentrating on the increase in the rate of drug intake, otherwise described as the escalation of drug intake by subjecting the animals to a longer daily self-administration session (usually 6-7 hours a day). This kind of long access model could show a progressive increase in the rate of drug intake (Ahmed and Koob, 1998; Ahmed et al., 2002) and is now considered as a better model for addiction (Deroche-Gamonet et al., 2004; Vanderschuren and Everitt, 2004).

Brain reward substrates underlying addiction:

Addictive drugs are both rewarding and reinforcing. A reward is a stimulus that brain interprets as intrinsically positive, or as something to be approached. A reinforcing stimulus is one that increases the probability that the behavior paired with it will be repeated. But all the reinforcers are not rewarding. For example a negative or punishing stimulus will reinforce

avoidance behaviors. It appears that drug-induced reward is both rewarding and reinforcing. The neural substrates that underlie the perception of the reward and the phenomenon of positive reinforcement are a set of interconnected forebrain structures commonly referred to as the reward pathway. The discovery of the brain reward pathway comes from the novel study by Olds and Milner (Olds and Milner, 1954) showing that animals volitionally self-administer electrical stimulation delivered through electrodes surgically implanted to specific loci in the brain. Later it was reported that only a limited number of brain sites support this brain stimulation reward (Olds and Olds, 1963). Their finding strongly suggested that there are anatomically specific circuits in the brain dedicated to neural mediation of the reward or pleasure. Later it was also found that electrical stimulation of these circuits can also evoke natural consummatory behaviors such as eating and drinking (Hoebel and Teitelbaum, 1962; Margules and Olds, 1962). This implied that such electrical stimulation activates neural systems involved in natural reward and motivation. Three years after the discovery of this electrical self stimulation phenomenon, it was found that addictive drugs derive their rewarding properties by activating such brain reward circuits (Killam et al., 1957) and this observation has since been amply confirmed.

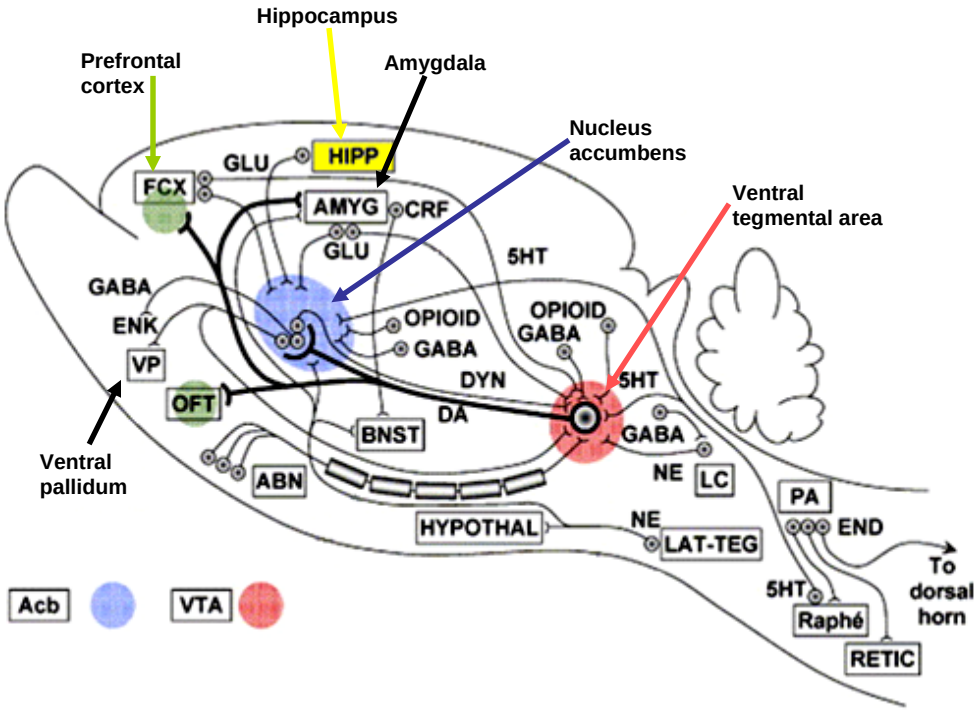
This acute enhancement of brain reward mechanisms appears to be the single essential pharmacological commonality of addictive drugs and is the most compelling hypothesis available on the neurobiology of addiction. The electrical brain stimulation reward is so powerful that the hungry animals ignore food and water to get it, just like the cocaine taking animals ignore these during a drug binge (Routtenberg and Lindy, 1965).

Anatomy and Neurochemistry of the Brain reward Pathway:

Although electrical stimulation of several brain structures is reinforcing in the intra-cranial self stimulation experiments, it was found that stimulation of the median forebrain bundle

and closely associated areas results in the strongest reinforcement of paired behavior. The medial forebrain bundle consists of ascending and descending fiber tracts that connect rostral basal forebrain and midbrain structures and includes dopaminergic, noradrenergic and serotonergic fibers derived from monoamine nuclei of the brain stem. All addictive drugs enhance the brain stimulation reward, including amphetamines, cocaine, opiates, nicotine, phencyclidine (PCP) and ketamine, cannabinoids, benzodiazepines, barbiturates and ethanol. Reinforcement produced by stimulation of median forebrain bundle is caused by the activation of the mesocorticolimbic dopamine system which is also the critical substrate for the reinforcing effects of the drugs and natural rewards.

Mesocorticolimbic dopaminergic projections originate in the ventral tegmental area of the ventral midbrain and project through the medial forebrain bundle to limbic and forebrain structures. The dopaminergic projections that extend from the VTA to NAc is the best established neural substrate for reinforcement.



Adapted from O'Brien CP & Gardner EL, Pharmacol Ther. (2005)

Figure 1.1 Anatomy of the brain reward pathway

Mid-sagittal diagram of the mammalian (laboratory rat) brain, showing brain-reward loci and reward-related circuitry involved in drug-seeking behavior, drug-taking behavior, drug craving, and relapse to drug-seeking behavior. Sites at which various addictive drugs act to enhance brain-reward substrates are indicated. The principal circuit mediating drug-induced reward appears to be the dopaminergic circuit originating in the **ventral tegmental area** and projecting to the **nucleus accumbens** and olfactory tubercle. The principal circuit mediating drug-induced stimulus-response and stimulus-reward learning appears to involve the **ventral tegmental area, nucleus accumbens, prefrontal cortex, and amygdala**. The principal circuit mediating drug-triggered relapse to drug-seeking behavior appears to involve the nucleus accumbens. The principal circuitry mediating stress-triggered relapse to drug-seeking behavior appears to be 2-fold: 1 involving the lateral tegmental noradrenergic cell groups and their projections to

hypothalamus, bed nucleus of stria terminalis, nucleus accumbens, and amygdala; and 1 involving corticotropin-releasing factor (CRF) cell groups in the amygdala and their projections to the bed nucleus of the stria terminalis. The principal circuitry mediating cue-triggered relapse to drug-seeking behavior appears to involve glutamatergic cell groups in the amygdala and hippocampus and their projections to the meso-accumbens dopaminergic pathway. Abbreviations of brain loci: ABN, anterior bed nuclei of the medial forebrain bundle; Acb, nucleus accumbens; AMYG, amygdala; BNST, bed nucleus of the stria terminalis; FCX, prefrontal cortex; HIPP, hippocampus; HYPOTHAL, hypothalamus; LAT-TEG, lateral tegmental noradrenergic cell groups; LC, locus coeruleus; OFT, olfactory tubercle; PAG, periaqueductal grey matter; Raphe´, Raphe´ nuclei of the brain stem; RETIC, reticular formation of the brain stem; VP, ventral pallidum; VTA, ventral tegmental area. Abbreviations of neurotransmitter systems and projections: CRF, corticotropin-releasing factor; DA, opamine;DYN, dynorphin; END, endorphin; ENK, enkephalin; GABA, gamma-amino-butyric acid; GLU, glutamate; 5HT, 5-hydroxytrypamine (serotonin); NE, norepinephrine (noradrenaline); OPIOID, endogenous opioid. Miscellaneous abbreviation: BSR, brain-stimulation reward (electrical brain-stimulation reward).

Ventral Tegmental area:

This is located in the midbrain and appears as a ventromedial extension of the substantia nigra pars compacta. VTA has a large population of dopamine neurons arranged as a dorsal tier and a ventral tier. The dorsal tier is linked to the limbic system and the ventral tier is closely linked with the areas of the striatum. Descending projections to the VTA include indirect connections from the hippocampus by way of septal nuclei and hypothalamus. The VTA sends projections through the medial forebrain bundle to limbic areas (mesolimbic system) and to

cortical areas (mesocortical system). Targets of the dopaminergic fibers from VTA include dorsolateral and medial prefrontal cortex (PFC), the anterior cingulated gyrus (mesocortical system) and nucleus accumbens, the hippocampus and amygdala (mesolimbic system). VTA dopaminergic system is postulated to be involved in reward associated learning of new behaviors in contrast to maintenance of previously learned behaviors. This system responds to the novelty of an unexpected stimulus, to primary rewards and to the conditioned stimulus associated with that reward.

Nucleus accumbens:

This is a small nucleus near the midline just rostral to the diencephalon. Nucleus-accumbens (NAc) is continuous with the caudate or putamen and extends ventrally as the olfactory tubercle. Two major divisions of the NAc are the core and the shell. The core is the ventromedial extension of the caudate or putamen. The shell of the NAc surrounds the core on its medial and ventral borders. The shell extends caudomedially to blend with the central division of amygdala, providing evidence for close relationship between NAc and the limbic system. The shell is different from the core in that it has fibers that project into the central nucleus of extended amygdala and to the lateral hypothalamus. The extended amygdala is said to comprise several basal forebrain structures that share similar morphology, immunocytochemical features, and connectivity that are well suited for mediating the aspects of reward function; these include the bed nucleus of stria terminalis, the central medial amygdala, the shell of the NAc, and the sublenticular substantia innominata.

The NAc and VTA are the central components of the reward circuitry underlying reward and memory of reward. The activity of the dopaminergic neurons in the VTA is linked to reward prediction and the NAc is involved in learning associated with reinforcement. Addictive drugs

appear to have a greater effect on dopamine release in the shell than in the core of the NAc. The anatomic connections among GABAergic medium spiny neurons of the NAc are believed to be a critical component of the limbic-extrapyramidal interface involved in reward and reinforcement. These neurons integrate the glutamatergic inputs from the cerebral cortex with dopamine inputs from different parts of the brain. In both NAc and dorsal striatum, the interactions between glutamate and dopamine may underlie learning and presumably involve plasticity at synapses formed between cortical pyramidal neurons and neurons of the NAc and dorsal striatum. One subtype of GABAergic medium spiny neurons forms the striatopallidal pathway and this subtype might be the one strongly linked to reward related behaviors. These neurons coexpress enkephalin and to some extent D_2 receptors, project from the NAc to the ventral pallidum. Inactivation of this pathway using a cocktail of $GABA_A$ and $GABA_B$ receptor agonists blocks both food and drug induced reinforcement (McFarland and Kalivas, 2001).

Amygdala:

Amygdala is a nuclear complex located inside the temporal lobe, deep within the uncus. There are 3 main nuclear areas within this; lateral (basolateral), central and medial nuclei. Sensory information about the external environment reaches the amygdala through the fibers of the visual association cortex of the temporal lobe. The fibers from the insular cortex provide sensory information about the internal environment.

Projections from the basolateral amygdala go to cortical areas like the orbital cortex, temporal lobe, and hippocampus and cingulate gyrus. All these areas are related to the perception of fear and anxiety. Mediodorsal portion projects fibers to all areas of the prefrontal cortex. Hence this portion is also attached with emotional significance. A large portion of the amygdala efferents terminate in the ventral striatum including the NAc. These projections are believed to

be important for the formation of the stimulus-reward associations. Neurons of the amygdala fire in response to food related stimuli also. Lesions of the amygdala disrupt the ability of the experimental stimulus to remember the pairing of a stimulus with a reward and can lessen the response to a conditioned reinforcer previously paired with a natural reward. Medial nucleus of amygdala is more associated with the emotional aspects of smell, taste and pain. Central nucleus is more predominant output channel that end the fibers in the dorsal nucleus of vagus and brainstem. The central nucleus of amygdala has also been implicated in aversive effects of drug withdrawal also.

Overall amygdala has access to integrated sensory information from higher cortical areas. Dopaminergic fibers arrive from the VTA and there exists a special relation between amygdala and hippocampus by the direct and indirect link through fibers of the entorhinal cortex.

Hippocampal formation:

The hippocampus is located in the medial temporal lobe of each side of the brain. It occupies a central position in the limbic system. Interest in the hippocampus increased when a patient known as H.M. had his medial temporal lobe bilaterally removed surgically as a treatment for epilepsy. From that point, this person was not able to form any new long-term declarative memories (Scoville and Milner, 1957). He could remember his past (his name, child hood, how to speak English, etc.), but could not form any new declarative memory after surgery. Everyday H.M was reintroduced to his doctors, as he did not remember their names from the day before. The discovery that the loss of areas of the medial temporal lobe could affect memory without disturbing other cognitive functions had an important significance and it became apparent that to encode memory we need a distinct area of the brain that includes the hippocampus.

The cells of the hippocampus are organized into layers, and transmit information in a predictable manner. The pyramidal and granule cells of the hippocampus are glutamatergic, and make up 90% of hippocampal neurons, the other 10% of cells are GABA-ergic interneurons (Freund and Buzsaki, 1996). The hippocampus is comprised of several distinct regions, including: the entorhinal cortex, the dentate gyrus, the hippocampus proper (which is divided into three regions, CA3, CA2 and CA1), and the subiculum. These areas are linked, in respective order, from one to next by one way projections primarily (Burwell et al., 1995). The entorhinal cortex supplies the main input into the dentate gyrus via the perforant pathway. The dentate gyrus sends axons from the granule cells, called mossy fibers to the CA3 (Amaral and Witter, 1995). CA2 and CA3 share the same general projections. CA3 pyramidal cells send axons called the Schaffer collaterals to the CA1, and send a projection to the contralateral hippocampus by the associate commissural pathway. The pyramidal cells of the CA1 send projections to the subiculum, and the subiculum sends axons back to entorhinal cortex forming a loop. Unlike other cortical regions of the brain where reciprocal connections are the norm, in the hippocampus mostly unidirectional connections exist (Amaral and Witter, 1995). The subiculum is considered to be the main output of the hippocampus, although it receives some afferent connections (Witter et al., 1989). The subiculum is made up of pyramidal cells and interneurons.

Hippocampus and drug relapse:

Attempts to develop new drugs for treatment of addiction usually focus on the dopamine rich reward circuitry of the brain. But in recent years, scientists have found that the reward function operates independently of craving for a drug. In a study it was observed that stimulating the ventral subuiculum of the hippocampus (VSUB) with a brief (8 seconds) theta burst stimulation could evoke drug seeking behavior in rats (Vorel et al., 2001). It was also shown that

VSUB stimulation enhances VTA dopamine neuron firing via an indirect pathway (Legault et al., 2000; Todd and Grace, 1999). NAc dopamine increase after VSUB stimulation depends on VTA glutamate because it has been reported that the NAc dopamine release is blocked by glutamate receptor antagonist kynurenic acid applied into the VTA (Legault et al., 2000). In this particular study to elucidate the role of hippocampus in relapse, they found that microinjection of kynurenic acid (50nm), not the vehicle to the VTA, blocked the reinstatement after VSUB stimulation (Vorel et al., 2001). This suggests the involvement of AMPA and/or N-methyl D-aspartate (NMDA) ionotropic glutamate receptors in reinstatement. Based on this report, we decided to investigate extensively the role of NMDA receptors in extinction and reinstatement behavior. A major part of the work for my dissertation is focused on modulating the NMDAR function by different pharmacological means and how this modulation affects the reinstatement behavior in animals trained to self administer cocaine.

The hippocampus subserves contextual learning and reinstatement after VSUB stimulation may reflect the read-out of an encoded association between the context of cocaine experience (i.e operant chamber) and the previously available cocaine (Hirsh R, 1974; Holland and Bouton, 1999 and Eldridge et al., 2000). Vorel et al proposed that VSUB stimulation has predictive or incentive properties that facilitate the initiation of lever responding. Though pharmacological stimulation of the mesolimbic dopamine transmission triggers reinstatement, the stimulation of the VSUB results in longer lasting release of dopamine (30 seconds) as opposed to the brief (less than 5 seconds) release of dopamine after the stimulation of the median forebrain bundle (Vorel et al,2001). Because the VSUB contains glutamate, and the reinstatement could be blocked by kynurenate and elicited by NMDA in the VTA, the glutamate system is proposed as a potential target for the treatment of drug addiction. In my study, based

on this hypothesis, I tried to target the glutamate system (especially the NMDA receptor-dependent synaptic plasticity mechanisms) in extinction as a potential target for addiction therapy.

Challenges to the treatment of drug addiction:

In my study, I have used the cocaine self-administration and the reinstatement model to investigate different aspects of addiction and relapse. The main characteristics of cocaine addiction are compulsive drug use and high rates of relapse during periods of abstinence (Mendelson and Mello, 1996). Despite decades of research, there are no effective medications to treat drug addiction and the treatments usually fail due to the high rates of relapse. The current influential hypothesis is that, cocaine addiction is due to drug induced neuroadapatations in reward related learning and memory processes in the mesocorticolimbic dopamine system and glutamatergic coricolimbic circuitry in which the dopamine projections are embedded (Nestler, 2002; Wolf et al., 2004; Everitt and Robbins, 2005; Kalivas and O'Brien, 2008). These neuroadaptations have been hypothesized to cause hypersensitivity to cocaine-associated cues (Di Chiara, 1998; Everitt and Wolf, 2002), impulsive decision making and abnormal habit like learned behaviors that are insensitive to adverse consequences. Hence the addiction process involves a learning component that is normal to all kinds of habit learning, but the so called drug induced neuroadaptations affect it adversely and force the addicts to make decisions without thinking about the adverse consequences. The 'drug induced neuroadaptation' hypothesis focuses on the role of the cellular events and signaling cascades that underlie synaptic plasticity.

Neuroadaptations relevant to relapse in cocaine addiction:

Chronic administration of either cocaine or alcohol upregulates the cAMP pathway in the NAc and these drugs also activate CREB in the same region (reviewed by Nestler, 2004). This

impairs the reward pathway and represents a mechanism of motivational tolerance and dependence. Many of the adaptations reported in the VTA-NAc pathway are common to most of the drugs of abuse which include alterations in G-protein subunits, tyrosine hydroxylase (the rate limiting enzyme in the synthesis of dopamine), neurofilament proteins, glutamate receptors and neuropeptide systems (reviewed by Nestler, 2004). Self-administration of cocaine or palatable food increases the AMPA to NMDA ratio in the ventrolateral bed nucleus of stria terminalis (BNST) and this is responsible for the increase in magnitude of the excitatory synaptic transmission in this region. In contrast, the passive administration of cocaine or natural reward was not found to increase the AMPA to NMDA ratio (Dumont et al., 2005). Acute administration of cocaine is reported to induce c-Fos and several other proteins of the Fos family in the NAc and dorsal striatum (Graybiel et al., 1990; Young et al., 1991). But the chronic administration of cocaine was found to reduce the levels of c-Fos and other Fos proteins and was found to increase the levels of an activator protein (AP-1 complex) which are the transcriptionally active dimers of Fos and related Jun family proteins. Later it was confirmed that this long lived AP-1 protein complexes are induced by a novel Fos protein which are the modified isoforms of ΔFos B (Hope et al., 1991). ΔFos B is stable, long lived for months and is both necessary and sufficient to sensitize animals to both drug and non-drug rewards and might even increase the drive for such rewards. Hence, ΔFos B could be a sustained molecular switch that helps to initiate and maintain a state of addiction (Nestler et al., 2001). However, ΔFos B is not stable or long lived enough to explain the permanent changes in the brain brought about by drug addiction. Chronic administration of cocaine is reported to cause an expansion in the dendritic arborization of NAc neurons that can persist over months after the last drug exposure (Robinson and Kolb, 1997) and some short lived molecular adaptations might cause long lived

structural changes in the brain. In the recent years, it is proposed that the molecular and cellular basis of near permanent behavioral changes that accompany addiction is analogous to the changes in learning and memory (Hyman and Malenka, 2001; Nestler, 2002). Learning and memory and drug addiction are modulated by the same neurotrophic factors (e.g. brain derived neurotrophic factor) and both share several intracellular signaling cascades and depend on activation of CREB. Both are accompanied by similar adaptations in neuronal morphology (e.g. dendritic spine density) and alterations in synaptic plasticity (e.g. long term potentiation and long term depression) at particular glutamatergic synapses in the brain (reviewed by Nestler, 2004).

Animal models of relapse:

The two major aims of preclinical research in the treatment of addiction are 1) to elucidate the behavioral, environmental, and neural mechanisms underlying drug relapse and 2) to discover medications that will prevent relapse. One basic animal model that has been developed to study relapse preclinically is the reinstatement model based on the self-administration model.

Reinstatement model of relapse:

Reinstatement refers to the resumption of a previously extinguished conditioned response after acute non-contingent exposure to the unconditioned stimulus. In a reinstatement model, the animal is first surgically implanted with a catheter in the jugular vein and allowed to acquire the self-administration behavior (responding on the lever for the drug and drug associated cues) to a satisfactory stable level of degree and stability. An extinction protocol is usually incorporated in the reinstatement model. Extinction inhibits conditioned responses by learning new contextual relationships (Bouton, 2002). After meeting the operational criteria for self-administration behavior, the animals are then subjected to behavioral extinction of drug taking habit by with-

holding the drug reinforcer. In animal reinstatement models usually both the primary and the conditioned reinforcers (cue light and tone) are with-held. Once the animals learn the new contextual relationships, the level of responding on the active lever will fall below a certain level. After a satisfactory degree of extinction is achieved, the animal is then tested to see whether a triggering stimulus or a priming stimulus such as drug (de Wit and Stewart, 1981), drug associated environmental cues (Crombag and Shaham, 2002, Meil and See, 1996) or stressors (Shalev et al., 2001) can evoke nonreinforced responding on the drug paired lever. Researchers working in this area seek to understand how environmental or interoceptive events cause drug seeking. The dependent variable in these studies is the number of responses on a lever previously associated with the drug delivery (reviewed by Roberts DCS et al., 2007). The effect of drug pretreatments or other stimuli such as drug associated cues or foot shock is assessed on the lever responding. The animal reinstatement model has a better face validity in modeling relapse in human addicts because it has been demonstrated that clinically effective anticraving medications such as Des-methyl imipramine attenuate the drug seeking behavior as measured in this model (Markou et al., 1992). Throughout my studies, I have utilized the reinstatement model to investigate various behavioral, molecular and neurobiological aspects of relapse.

The nonreinforced lever pressing behavior evoked by the various priming stimuli following extinction is described as the drug seeking behavior, not drug taking behavior. The resumption of drug seeking behavior is said to be the result of craving. And at times, the reinstatement model has been suggested as a model for drug induced craving (Grimm et al., 2001).

There are different variants of the reinstatement protocol and the priming method. In the between session variant of the reinstatement protocol (e.g. Stretch et al., 1971), the acquisition of

drug self-administration, extinction of self-administration, and reinstatement testing are carried out in defined sessions on different days. In the within session variant (e.g. de Wit and Stewart, 1981) all the three steps are carried out sequentially in defined short sessions within a day. In the between-within session variant (e.g. Tran-Nguyen et al., 1998) the acquisition of self-administration takes place on first specific days and then, the extinction and reinstatement sessions are carried out in the same session sequentially during a subsequent day. If the animal's behavior is directed towards the drug paired lever during reinstatement testing, the animal is said to be effectively reinstated. There exist two types of priming method, non-contingent and contingent. In the non-contingent method, the experimenter primes the animal and in the contingent method the primes are response dependent from the subject. Some studies have reported that the response independent presentation of the stimuli are ineffective (Grimm et al., 2000) and some other studies indicate that response independent presentations of the stimuli can evoke reinstatement (McFarland and Ettenberg, 1997). Much of the reported research has utilized the non-contingent method for the drug priming experiments and the contingent method for the cue priming experiments. In the clinical studies of craving, the drug related stimuli are presented independently of behavior (i.e. noncontingently). Hence the contingent method of cue induced assessment of drug seeking behavior in the animal reinstatement model is considered as an important point of departure between the clinical studies of craving and its animal model (Katz and Higgins, 2003). In our studies with cued reinstatement, we have taken every effort to model the clinical situation by presenting the cue in a non-contingent manner.

Neural substrates underlying relapse/reinstatement:

Lesioning and inactivation studies of the different brain loci have provided a deep insight to the neurobiological substrates subserving different forms of reinstatement. Basolateral

amygdala was found to be the important neural substrate mediating the cue-induced relapse (See RE, 2005). Similarly inactivation of ventral subiculum (an extension of the ventral hippocampus) was found to attenuate cue induced and drug induced reinstatement responding (Sun and Rebec, 2003). Similar results were observed by selective inhibition of ventral hippocampus using baclofen and muscimol (Rogers and See, 2007). Inactivation of dorso medial prefrontal cortex (dmPFC), but not BLA abolishes cocaine primed reinstatement of extinguished cocaine seeking behavior (McFarland and Kalivas, 2001). Tetrodotoxin (TTX)-induced inactivation of the dmPFC, BLA or dorsal hippocampus (DH) inhibited the contextual reinstatement of cocaine seeking, while inactivation of the DH failed to alter explict CS induced and cocaine primed reinstatement (Fuchs et al., 2005).

Extinction of the Pavlovian learning:

As explained in the reinstatement paradigm, an extinction training protocol is usually included in this model. The idea of extinction was actually presented in Pavlovian writing (Pavlov, 1927). Extinction is a well-known and important behavioral phenomenon that allows the organism to adapt its behavior to a changing environment and is widely used in many behavioral paradigms such as conditioned fear extinction, conditioned taste aversion and conditioned place preference. Extinction of the reinforced lever pressing behavior learned in the operant chamber environment through the associative learning paradigm is just one example among these. In general, extinction is aimed at inhibiting the conditioned response by discontinuing the reinforcement in all the cases. But, there is a slight difference in the extinction training methods employed in other behavioral studies compared to self-administration, extinction and reinstatement model for drug relapse. In almost all the other cases, the associative learning is extinguished in the presence of CS (conditioned stimulus, e.g., a tone and/or light). So

here the resulting reaction to the tone (e.g. fear if it is conditioned fear model) is extinguished or eliminated by presenting the CS alone repeatedly without the unconditioned stimulus (foot-shock). Similar principles apply to extinction in operant learning in which an instrumental action (lever pressing behavior) which was first reinforced by a positive event (e.g., delivery of a drug infusion or delivery of a sucrose pellet) is then extinguished by removing the reinforcing event (Bouton and Swartzentruber, 1991). Here usually the CS is not presented during the extinction training, hence the CS is considered to be not extinguished. During the extinction training, the animals will be placed in the operant chamber and the cannula will be connected to the tether as on the self-administration days, but the responses on the active lever will have no programmed consequences. Neither CS nor US (unconditioned stimulus, here cocaine or sucrose pellet) will be delivered as a result of the responses on the active lever. Thus the animals extinguish their lever pressing behavior as they get extinguished to the self-administration context (operant chamber environment). Once a stable, low level of extinction (less than 20% of the total number of responses on the active lever on the first extinction day) is achieved, the animals are usually tested for the reinstatement of drug seeking behavior. In our experiments, depending on the experimental aims to be achieved, we have utilized maximal (5 days of 90 minute extinction training) or sub-maximal (1 day of 90 minute extinction session) extinction training protocols.

Extinction learning:

There is now ample evidence that extinction, the loss of learned performance that occurs when a Pavlovian signal or instrumental action is repeatedly presented without its reinforcer, does not reflect a destruction or erasure of original learning (Bouton, 2002; Rescorla, 2001). Instead it is believed that extinction reflects a new learning process as a result of which the signal or instrumental action acquires a second meaning that is available along with the first. According

to this, the current meaning of the signal or action is ambiguous. The current meaning of the signal or action is determined by the current context (context of extinction) and hence an extinguished Pavolvian signal will evoke different reactions in different contexts (reviewed by Bouton, 2002).

Research on extinction has uncovered at least four different phenomena indicating that it does not destroy the original learning and all of those are described as potential mechanisms of relapse. They are reinstatement, renewal, spontaneous recovery and reacquisition (reviewed by Bouton, 2002). Reinstatement is the recovery of the behavior that occurs when the subject is exposed to the significant event (US) after extinction (Pavolv, 1927; Rescrola and Heth, 1975). This is strongly controlled by the contextual conditioning produced when the US is presented. Hence the phenomenon is the strongest when CS is tested in the context in which the US had occurred. So here the testing is done in the original learning environment itself.

The second phenomenon to support extinction as a new learning rather than unlearning is renewal. In the renewal effect, the subject may receive conditioning in context “A” , undergo extinction in context “B” and will be subjected to testing in context “C” or context “A”. That is the testing occurs in a different context as compared to extinction. So here it can be either an ABC renewal or ABA renewal. The return to either the original context or the introduction to a new context renews the extinguished response (Bouton and Bolles, 1979a; Bouton, 2002). The ABA form of renewal is the most widely studied one and is the strongest one though ABC form also exists.ABC renewal is important because it indicates that mere removal from the extinction context can renew the extinguished response. Similarly a form of AAB renewal also exists in which the testing occurs in a different context after conditioning and extinction happening in the same context. The renewal effect seems general and robust and can occur after extensive

extinction training (Bouton, 2002). The studies report that both operant and Pavlovian learning remain after extinction, ready to be retrieved by the manipulation of the context.

The most robust recovery effect noted by Pavlov (Pavlov, 1927) was spontaneous recovery. He noted that if time elapses after extinction, the extinguished response can recover spontaneously when tested again. This effect has been noticed in every conditioning method. The idea is that both renewal and spontaneous recovery come about because the organism fails to retrieve extinction outside the extinction context. Memory theorists suggest that the passage of time may naturally provide a gradually changing context. Just as extinction is context specific to its physical context, it might be equally specific to the context of time. Thinking in this way, spontaneous recovery is the renewal effect that happens when the CS or action is tested in a new temporal context (Bouton, 1988). In one of my experimental aims, I have tried to analyze the persistence of the effect of extinction and the influence of spontaneous recovery in recovering the extinguished response by testing the extinguished animals after a period of non-extinction or simply enforced abstinence.

Reacquisition can cause the return of extinguished responding if the extinguished signal or action is paired again with the reinforcer. The idea that reacquisition is rapid after extinction is consistent with the evidence that extinction is not unlearning. However, reacquisition is not always fast and it can be significantly slower also after an extensive extinction training (Bouton, 2002).

Neurobiology of extinction learning:

A growing literature on the neurobiology of extinction learning supports the idea of extinction learning as a new learning rather than unlearning. The synaptic plasticity mechanism known as long term potentiation (LTP) is the cellular analogue of learning and memory.

N-methyl D-aspartate receptor (NMDAR) is the most important molecule implicated in synaptic plasticity mechanisms. Hence it is the first molecule implicated in extinction learning also. In the absence of a defined anatomical locus of extinction, many investigators resort to the use of systemic manipulations (reviewed by Myers and Davis, 2002). For some of the behavioral paradigms like fear conditioning, investigators have focused on intra-amygdalar infusion studies. In our studies, we have utilized a systemic approach to pharmacologically manipulate the extinction learning using NMDAR drugs. Hence, from here onwards, I will try to limit the discussion to NMDAR dependent synaptic plasticity and extinction learning. Before going into the details of the synaptic plasticity mechanisms, an overview of the NMDAR will be useful.

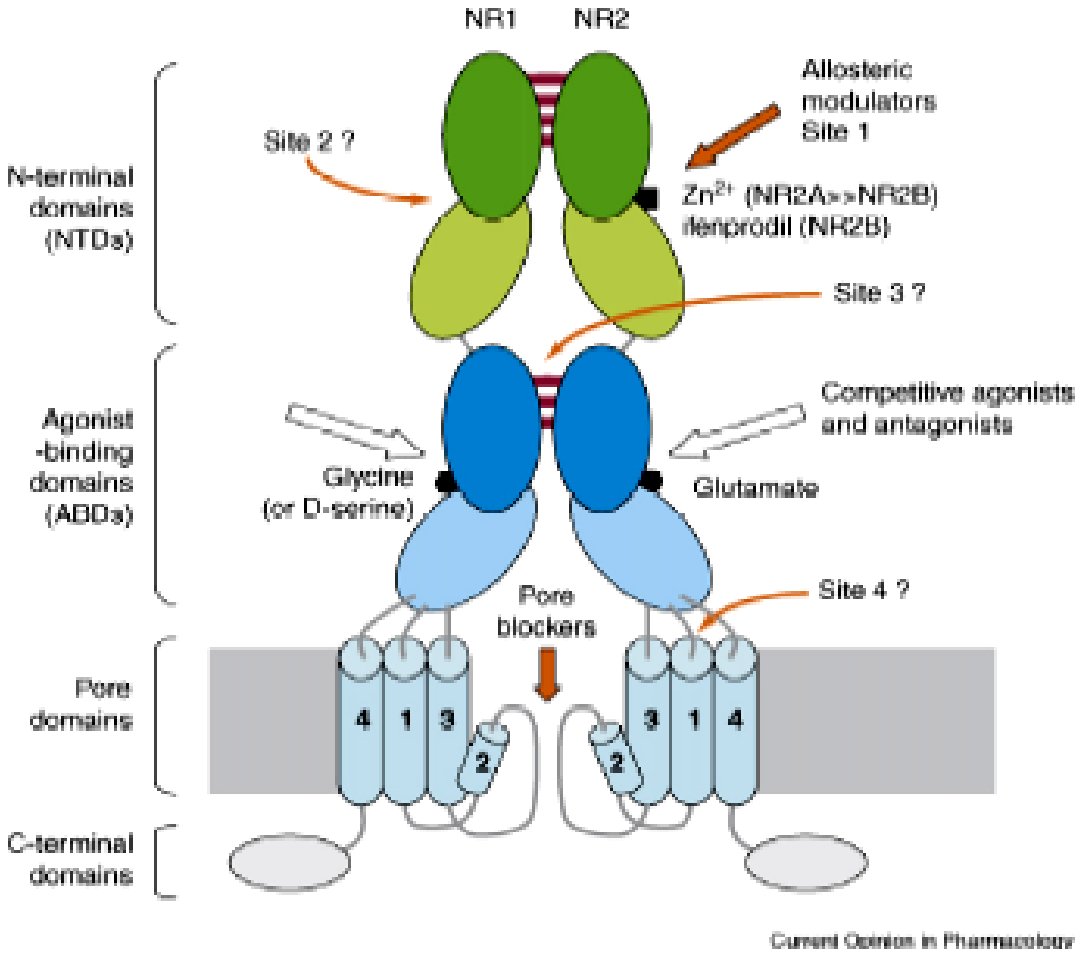
NMDAR and Synaptic plasticity mechanisms:

Within the large family of excitatory ionotropic glutamate receptors, NMDARs constitute a subfamily identified by specific molecular composition and unique pharmacological and functional properties (Dingledine et al., 1999; Cull-Candy and Leszkiewicz, 2004). It is well accepted that triggering of LTP requires activation of NMDARs. NMDARS are unique ligand gated ion channels because their activation requires the binding of two agonists, glycine and glutamate, but also demands the relief of voltage dependent Mg^{2+} block (Mayer et al., 1984). NMDARs are often called as coincidence detectors since they can sense the simultaneous, repetitive activity at a number of adjacent synapses. These adjacent synapses are activated by synaptically released glutamate and this activity depolarizes the post synaptic cell sufficient enough to relieve the blockade of NMDAR channels by $Mg^{2+.}$ Activation of NMDAR produces a nonspecific increase in the permeability of Na^{+} and K^{+} like other glutamate receptors. Unlike other glutamate receptors, the NMDAR channels are extremely permeable to Ca^{2+}. The

permeation of Ca^{2+} through the NMDAR channels initiates signal transduction cascades that in turn modulates synaptic strength (MacDermott et al., 1986).

Structure and molecular composition of NMDAR:

NMDARs are heteromeric complexes incorporating different subunits within a repertoire of three subtypes: NR1, NR2 and NR3. Most NMDAR are believed to assemble as tetramers, associating two NR1 and two NR2 subunits in a 'dimer of dimers' quarternary structure (Paoletti and Neyton, 2007). NMDAR subunits all share a common membrane topology. It is characterized by a large extracellular N-terminus, a membrane region comprising 3 transmembrane segments (TM1, 3 and 4) and a re-entrant pore loop (M2). There is an extracellular loop between TM3 and TM4, and a cytoplasmic 'C' terminus which varies in size depending upon the subunit and provides multiple sites of interaction with numerous intracellular proteins (Dingledine et al., 1999; Cull- Candy and Leszkiewicz, 2004). The N-terminal domain (NTD) contains binding sites for allosteric inhibitors such as Zn^{2+} and ifenprodil. The agonist binding domain (ABD) binds glycine in NR1 and NR3, whereas NR2 ABDs bind glutamate (Furukawa et al., 2005). The glycine site on NMDAR are distinct from the inhibitory strychnine sensitive glycine receptor which mediates the neurotransmitter functions of glycine. The sequences of the regions lining the pore are highly conserved in NR2 subunits and accordingly, permeation properties (e.g. ionic selectivity), as well as the affinity for the pore blocking Mg^{2+} vary little among the different NR1/NR2 receptor subtypes. In contrast, inclusion of the NR3 subunit markedly decreases single channel conductance, Ca^{2+} permeability and Mg^{2+} block because of the presence of positively charged amino acid Arginine at the pore region (Cull-Candy and Leszkiewicz, 2004). pH is another important allosteric modulator of this receptor and the receptor activity is almost completely suppressed at pH less than 6.



(Adapted from Pierre Paoletti and Jacques Neyton, 2007)

Figure 1.2 Binding sites on the N-methyl D-aspartate receptor.

NMDAR dependent long term potentiation:

Once the postsynaptic cell is depolarized (usually by the activation of the alpha-amino-3 hydroxy-5-methyl 4-isoxazole propionic acid (AMPA) receptors, the Mg^{2+} block of the NMDAR channels will be relieved. Under these conditions, if glutamate and glycine (or D-serine) binds to the respective agonist binding domains, the NMDAR will be activated allowing Na^{+} as well as Ca^{2+} to enter the dendritic spine. The consequent rise of intracellular Ca^{2+} is the critical trigger for LTP. Antagonists of NMDAR and the presence of calcium chelators preventing the rise in post synaptic Ca^{2+} can block LTP (Teylor and DiScenna, 1987). Direct increases in Ca^{2+} brought

about by photolysis of caged calcium in the postsynaptic cell can mimic LTP. Thus, any manipulation that influences the magnitude or dynamics of Ca^{2+} increases within the postsynaptic cell may influence the synaptic plasticity through a vast array of intracellular signaling pathways initiated by this high Ca^{2+} level (e.g. Activation of Calcium-Calmodulin kinase, Protein kinase C, protein kinase A etc). With this background information on NMDAR dependent synaptic plasticity mechanisms, we tried to manipulate the activity of the NMDAR by systemically administering the NMDAR drugs during extinction training, to study whether the actual learning of extinction and/ or the recall of extinction is influenced by these treatments.

NMDAR dependent synaptic plasticity in extinction learning:

The major part of the information for the neural mechanisms of extinction comes from the classical fear conditioning which provided an appropriate model system to study extinction. There has been successful use of extinction based exposure therapies for the treatment of anxiety disorders (Rothbaum and Schwartz, 2002). However there remained an effective possibility of improving the effectiveness and shortening the duration of treatments if we could facilitate the extinction learning process by pharmacological manipulations or other methods (Davis et al., 2006). To achieve this, a clear insight to the neural mechanisms of extinction is necessary. In our study we focused on the NMDAR dependent synaptic plasticity mechanisms in extinction learning and the recall of extinction.

Extinction memory is more likely distributed across a network of neural structures and the extinction related plasticity in different structures may not serve identical roles. Plasticity in the hippocampus or prefrontal cortex may allow for contextual modulation of the fear extinction and plasticity in amygdala may serve to inhibit the fear expression (Bruchey et al., 2007). Like any other learning, extinction learning occurs in 3 different phases: acquisition, consolidation

and retrieval. Acquisition of extinction is the initial phase when the conditioned responses decline within an extinction training session. This is followed by consolidation phase lasting for several hours during which physiological and molecular processes stabilize a long-term memory for extinction. Subsequent to this, presentation of the extinguished CS triggers the retrieval of extinction. Poor retrieval of extinction is characterized by high levels of responding reflecting the memory of the original conditioning event (Quirk and Muller, 2008).

The first molecule implicated in acquisition of extinction learning was N-methyl D-aspartate receptor (NMDAR). Systemic administration of NMDAR antagonist MK801 prevented extinction (Baker and Azorlosa, 1996; Cox and Westbrook, 1994). Since extinction was carried out over many days, it was not possible to distinguish the impairments in acquisition vs. consolidation. But, when a massed extinction trial was used, it was observed that systemic administration of another NMDAR antagonist, (±) CPP before extinction training did not prevent the acquisition of extinction, but impaired the retrieval of extinction memory the following day (Santini et al., 2001; Suzuki et al., 2004), suggesting a role for NMDARs in consolidation. Recently it was shown that Ifenprodil, a selective antagonist of NR2B subunit of NMDAR, blocked the acquisition of extinction within a session (Stores-Bayton et al., 2007). The discrepancy of findings between ifenprodil and (±) CPP is likely due to the higher affinity for ifenprodil to the NR2B subunit in contrast to higher affinity of (±) CPP to NR2A subunit (Lozovaya et al., 2004). Basolateral amygdala (BLA) was the first structure implicated in fear extinction because local infusion of NMDAR antagonists and kinase inhibitors prevented extinction (Falls et al., 1992; Lu et al., 2001; Lin et al., 2003b). Thus, it appears that NMDARs are required for acquisition of extinction.

Studies with systemic administration of voltage gated calcium channel (VGCC) inhibitor nifedipine (Cain et al., 2002; Barad et al., 2004) blocked the acquisition of extinction. This together with the NMDA findings suggests that calcium currents are required for the initial learning process. As explained earlier, high levels of intracellular calcium is the critical trigger for LTP.

In my study, I pre- treated the animals with NMDAR drugs to investigate the synaptic plasticity mechanisms underlying the extinction learning. If the pharmacological agents administered before the extinction training do not impair the actual acquisition of extinction, but results in a poor retrieval of extinction memory, the agents are proposed to have affected the consolidation of the memory of extinction. A complementary approach to study the mechanisms involved in consolidation, is by administering the agents shortly after the extinction training. In my study I have utilized both these protocols to investigate the neural mechanisms underlying the effectiveness of extinction training in affecting the reinstatement response in extinguished animals.

Drugs-Pharmacology:

1. **Cocaine**

Cocaine produces its psychoactive effects by potentiating monoaminergic transmission through the actions on dopamine, serotonin and norepinephrine transporters. These transporter proteins are responsible for the clearance of the synaptically released monoamine neurotransmitters back into the presynaptic terminal and thereby terminating their action. Blocking the function of these transporters will result in the elevation of the extracellular levels of the monoamines which is responsible for the reinforcing effects. Actions on the dopamine transporter (DAT) are believed to be more important for the reinforcing effects of these psychostimulants. Cocaine binds to and

competitively inhibits the transporter function, thereby increasing the duration of action of the synaptically released dopamine. Cocaine similarly affects serotonin and norepinephrine transporters. Thus cocaine acts as an indirect dopamine agonist. At higher doses cocaine inhibits the function of the voltage gated sodium channels and acts as a local anesthetic.

2. (±) CPP

This is a competitive antagonist of NMDAR and prevents the binding of the glutamate at the agonist binding domain of the NR2 ligand binding core of NMDAR complex. This blocks the activation of the NMDAR and the subsequent development of NMDAR dependent synaptic plasticity.

3. D-serine

D-serine is a full agonist at the strychnine insensitive glycine site of the NMDAR in the agonist binding domain of NR1 ligand binding core. D-serine facilitates the activation of NMDAR by glutamate and thus the induction of NMDAR dependent synaptic plasticity.

Summary:

The aim of this chapter is to familiarize the reader with the drug addiction literature and about the animal models of addiction and drug relapse. Animal reinstatement model of drug relapse is well accepted to model relapse in clinical situations. Since this paradigm is considered to be of good predictive validity, the findings from these kinds of studies are important in translational medicine. From the background information provided in this chapter, it is apparent that certain areas in the animal self-administration and reinstatement model are relatively understudied and hence needs to be explored in greater detail. There are relatively few studies addressing the mechanisms underlying the effects of extinction on reinstatement. Since the NMDAR is one of the first molecules implicated in extinction learning, I have focused my study

on pharmacological manipulation of the NMDAR dependent synaptic plasticity mechanisms in extinction learning to investigate how this affects reinstatement responses. With the available background information, the following hypotheses were formulated and these hypotheses were investigated using systematically designed experiments.

Specific aims/hypotheses:

1. In animals trained to self-administer cocaine, extinction training will reduce the level of reinstatement responding as compared to enforced abstinence.

   Here, the aim was to determine the effects of extinction training in comparison to enforced abstinence on the reinstatement of drug seeking behavior. For this purpose, we studied the reinstatement response to the contextual drug stimuli, response to the non-contingent cue prime and the response to the non-contingent drug prime.

2. Since the extinction process involves new learning rather than unlearning, factors that disrupt the learning process during extinction will also alter the subsequent reinstatement behavior in animals trained to self-administer cocaine.

   We determined the extent to which NMDAR mediated synaptic plasticity was involved in extinction learning and studied whether the reinstatement was affected by NMDAR drugs during extinction. For this purpose, we studied the reinstatement response to the contextual drug stimuli, response to the non-contingent cue prime and the response to the non-contingent drug prime.

The findings for the first and second hypotheses are summarized in chapter1. Cocaine is an addictive drug reward. We wanted to continue this study with a natural reward like sucrose pellets and investigate whether we get similar results for extinction vs. abstinence and

whether similar mechanism of NMDAR dependent plasticity was involved in extinction and subsequent reinstatement behavior. Hence our third hypothesis is based on this objective.

3. Since the natural reinforcers also target the same brain reward pathway as the drugs of abuse, extinction training will reduce the level of reinstatement in animals trained to self-administer a natural reward (sucrose pellets) as previously observed in cocaine self-administered and extinguished animals.

The aim was to investigate the effects of extinction training in comparison to enforced abstinence on the reinstatement behavior in animals trained to self-administer a natural reward. For this purpose, we studied the reinstatement response to the contextual drug stimuli, response to the non-contingent cue prime and the response to the non-contingent sucrose pellet prime.

4. Since the extinction process involves new learning, factors which disrupt learning mechanisms (e.g. NMDAR-dependent synaptic plasticity) during extinction will also alter the subsequent reinstatement behavior in animals trained to self-administer a natural reward (sucrose pellets).

We determined the extent to which N-methyl D-aspartate receptor (NMDAR) mediated plasticity was involved in extinction learning in animals trained to self-administer a natural reward and evaluated whether the reinstatement was affected by treatments with NMDAR drugs during extinction. For this purpose, we studied the reinstatement response to the contextual drug stimuli, response to the non-contingent cue prime and the response to the non-contingent sucrose pellet prime.

Findings from the study with the natural reward are summarized as an appendix to chapter 1.

During the course of the first study to investigate the NMDAR mediated synaptic plasticity in extinction learning and subsequent reinstatement behavior, we noticed that treatment with D-serine, (the full agonist of NMDAR at the glycine site) significantly facilitated the extinction learning on day 1 and day3 of extinction training as compared to the control group. But we did not see any difference in their reinstatement behavior, probably because of the overtraining effect due to the 5 day extinction protocol. We could see that, by the day 5 of extinction training, all the groups reached a floor level of extinction. My next hypothesis originated from this finding. If D-serine actually facilitates the extinction learning, we should be able to see a difference in the reinstatement behavior if we provide only a sub-maximal level of extinction training. This will avoid the overtraining effect and we should be able to delineate the effect of D-serine on extinction and subsequent behavior.

5. Since extinction learning was facilitated by D-serine treatment in our earlier study, D-serine treatment in animals subjected to a sub-maximal level of extinction learning will facilitate NMDAR-mediated synaptic plasticity during extinction learning and influence subsequent reinstatement behavior.

Here, the aim was to determine the extent to which D-serine treatment during extinction training can enhance the NMDAR mediated synaptic plasticity involved in extinction learning in animals trained to self-administer cocaine and to study whether the reinstatement behavior was affected by this pharmacological manipulation. For this purpose, we studied the reinstatement response to the contextual drug stimuli, response to the non-contingent cue prime and the response to the non-contingent drug prime.

6. D-serine treatment during extinction in "long access" cocaine animals (suggested to be a better model for addiction) will enhance the effectiveness of extinction training on reinstatement behavior.

We evaluated the extent to which D-serine treatment during extinction training enhanced the NMDAR mediated synaptic plasticity involved in extinction learning in animals trained to self-administer cocaine in a long access model and studied whether the reinstatement behavior was affected. For this purpose, we studied the reinstatement response to the contextual drug stimuli, response to the non-contingent cue prime and the response to the non-contingent drug prime.

We have conducted a thorough investigation of the effects of D-serine during extinction in cocaine self-administered animals utilizing the two well accepted models of self-administration. The ultimate aim of my study was to see whether D-serine administration during extinction can be used as an effective treatment measure to enhance the extinction learning process and/ or reduce the level of reinstatement in cocaine self-administered animals. This strategy can be considered as a conjunct to psychotherapy of extinction in human addicts so as to prevent the chances of relapse.

7. Since our studies with pharmacological manipulation of NMDAR dependent synaptic plasticity during extinction yielded better results with drug prime reinstatement and since ventral hippocampus is known to be involved in cocaine induced reinstatement, inhibiting NMDAR dependent synaptic plasticity mechanisms in the ventral hippocampus during extinction will influence the extinction and/ or reinstatement behavior.

We investigated the extent to which NMDAR mediated synaptic plasticity in the ventral hippocampus was involved in extinction learning and studied whether the reinstatement was affected by the intra-cranial infusion of NMDAR drug, (±) CPP during extinction. For this purpose, we evaluated the reinstatement response to the contextual drug stimuli, response to the non-contingent cue prime and the response to the non-contingent drug prime.

8. Since spontaneous recovery is proposed as one of the mechanisms responsible for relapse after extinction therapy, the effectiveness and persistence of the effects of extinction on reinstatement in cocaine animals will vary when extinguished and tested at different time points after self-administration.

Here, the aim was to determine the effectiveness of extinction therapy in reducing the reinstatement response in animals trained for extinction either immediately after self-administration or after waiting 3 weeks since the last self-administration session. The persistence of the effects of extinction in reducing the reinstatement response was determined in animals trained for extinction immediately after the self-administration and tested for reinstatement at different time points after undergoing the extinction training. This is an important consideration currently in the field of psychotherapy in human drug addicts and our findings from this study helps to point out some facts about the persistence of the effectiveness of extinction.

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CHAPTER-2

THE EFFECTS OF EXTINCTION TRAINING IN REDUCING THE REINSTATEMENT OF DRUG-SEEKING BEHAVIOR: INVOLVEMENT OF NMDA RECEPTORS.

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Abstract

Although the process of extinction has been well documented for various forms of behavioral responses, the effects of extinction on the reinstatement of drug seeking behavior are relatively understudied. In this report, the effectiveness of an extinction training protocol to reduce primed reinstatement responses was compared with the effectiveness of an equivalent period of enforced abstinence. We found that extinction training performed in the drug taking environment significantly reduced reinstatement behavior subsequently primed by either contextual cues, conditioned cues, or cocaine infusion. The ability of extinction to reduce cocaine primed reinstatement was blocked by the systemic administration of the competitive NMDAR antagonist ((±) CPP, 5 mg/kg i.p.) administered prior to each extinction training session. Interestingly, this pharmacological intervention had no impact on the effectiveness of extinction to reduce drug-seeking behavior primed by either contextual cues or conditioned cues. These results suggest that an extinction training experience involves multiple mechanisms that can be dissociated into nonNMDAR and NMDAR dependent components with respect to the type of reinstatement (i.e. context-, CS-, or drug-induced) being assessed.

Keywords: self-administration, cocaine, extinction, reinstatement, NMDA receptor, (±) CPP, D-serine

1. Introduction

The reinstatement of drug seeking behavior in rodents as a model for relapse in humans has taken a prominent position in the preclinical field of addiction research [26]. In this model, exposure to various modes of priming stimuli (e.g. environmental context, conditioned cues, addictive drugs, stressors) following either abstinence or extinction can evoke, or reinstate, instrumental behaviors previously associated with the self-administration of an addictive substance [27]. An extinction training phase is typically incorporated into such protocols, in order to reduce instrumental responding to a low, stable baseline level from which the effectiveness of primed reinstatement can be assessed, although the resumption of drug-seeking behavior evoked by priming can also be assessed following periods of prolonged abstinence [28]. Much effort has been directed toward determining the neural substrates involved in the mechanisms of priming itself induced by various stimuli [24], however surprisingly little information is available concerning the mechanisms underlying the effectiveness of an extinction training experience to reduce instrumental responding following a priming event.

Using other behavioral models, evidence indicates that new learning is occurring during the extinction training experience [4, 20, 21]. This new learning may be dependent upon the activation of n-methyl-d-aspartate receptors (NMDARs), and either blocking NMDARs with antagonists or enhancing NMDAR activity with coagonists would be expected to affect the ability of an extinction training experience to alter the response to primed reinstatement. For example, conditioned fear has been used to demonstrate that NMDAR antagonists administered prior to extinction sessions can significantly inhibit extinction [2, 10, 22], and recent reports have indicated that treatment with D-cycloserine (an NMDAR coagonist) can facilitate extinction of

conditioned fear [17, 35]. Together, these findings indicate an involvement of NMDARs in the learning process that occurs during an extinction training experience.

With respect to the various methods employed in previous studies to prime reinstatement of drug seeking behavior, both diffuse (environmental context; [6]) and discrete (conditioned stimuli; [7,27]) cues have been evaluated, as well as the administration of the unconditioned stimulus (drug itself; [7,27]). In addition, stress and anxiety have been suggested to be effective inducers of reinstatement behavior [8, 29]. Interestingly, different neural mechanisms appear to underlie the reinstatement induced by these priming stimuli, and several specific brain regions are involved in some of these events. For example, one early report identified that the basolateral region of the amygdala as being critical for the reinstatement response to conditioned stimuli (CS) priming [19]. Subsequently, Grimm & See [13] demonstrated that inactivation of the nucleus accumbens was effective in preventing drug (cocaine) induced drug seeking, but had no effect on CS induced priming of reinstatement. The opposite relationship was found to exist for inactivation of the basolateral amygdala, as tetrodotoxin infusion did not affect drug primed reinstatement. This study established a basis for multiple, discrete neuronal mechanisms in mediating the primed reinstatement of drug-seeking behavior. Additional work has found that the dorsomedial prefrontal cortex is also involved in CS induced reinstatement [18], and that inactivation of the dorsomedial prefrontal cortex, the basolateral amygdala or the dorsal hippocampus can inhibit context induced reinstatement [11].

In this report we have tested the hypothesis that NMDARs may also be involved in the extinction of drug-seeking behavior. The efficacy of extinction was directly compared with the responses measured in another group of abstinent rats that remained in their home cage environments for an equivalent amount of time. The results demonstrate that extinction training

is effective in reducing the reinstatement of drug seeking behavior elicited by noncontingent exposure to contextual cues, conditioned stimuli, or cocaine infusion. Facilitating the activation of NMDARs during extinction training did not significantly affect the subsequent reinstatement, but inhibiting NMDAR activation resulted in the selective blockade of the extinction effects on drug primed reinstatement. These results indicate that diverse mechanisms participate in mediating the effects of extinction training on the expression of reinstatement of drug seeking behavior.

2. Materials and Methods

2.1. Animals:

Male Sprauge-Dawley rats (Harlan) weighed approximately 300 g at the beginning of the experiment and were housed individually in a temperature and humidity controlled vivarium having a 12 hour light/dark cycle (lights off at 7:00 P.M.). They were given access to food and water *ad libitum* and were handled daily for 5 days prior to the surgery in order to diminish stress associated with handling. The housing and experimental procedures followed the *Guide for the Care and Use of Laboratory Animals* and were approved by the local ACUC at the University of Georgia.

2.2. Jugular catheterization protocol:

The animals were anesthetized using a combination of ketamine (70 mg/kg), xylazine (10 mg/kg) and acepromazine (1 mg/kg) administered i.p. Depth of anaesthesia was assessed by monitoring respiration rate and palpebral and pedal withdrawal reflexes. Under anesthesia, the right jugular vein was isolated. The catheter was exteriorized by passing it subcutaneously to the base of the skull, where it was connected to a modified 22 gauge cannula. A silastic catheter (Dow Corning) was then inserted into the vein (4-5 cm) and secured in position with silk sutures

(6/0). The animal was then placed in a stereotaxic frame (Stoelting), where the right-angled cannula (Plastics One) was mounted to the top of the skull using dental cement and 4 screws. Immediately after surgery and once daily for 5 days, the animals were treated with gentamicin at a dose of 5 mg/kg, i.v. The catheters were flushed every day with saline prior to each self-administration session and with heparin (10 USP/ml) after the session to maintain the patency of the catheter. Catheter patency was verified daily by drawing blood from the catheter.

2.3. Self-administration environment:

The operant chambers (Med associates) were equipped with 2 levers, one "active" and another "inactive" with lights positioned above each lever. The chambers had a rod grid floor, a house light, a speaker/tone generator (2.9 kHz, 10 dB above ambient) and were housed inside enclosures equipped with ventilation fans. A syringe pump was located outside the enclosure. The method for delivering a cocaine infusion was as follows: The modified 22 gauge cannula mounted on the rat's skull was connected to a liquid swivel with PE-50 tubing protected by a metal spring. The swivel was connected with tygon tubing to the syringe mounted in the infusion pump. Infusion volumes were calculated according to the animal's weight. For cocaine animals, the syringes mounted in the infusion pump contained cocaine hydrochloride (NIDA) dissolved in normal saline at 4 mg cocaine/ml of solution. Each infusion delivered an infusion volume of 0.125 ml/kg body weight, hence the dose of cocaine self-administered was 0.5 mg/kg/infusion. The MED-PC software program recorded the number of active lever presses, inactive lever presses and the number of infusions.

2.4. Self-administration protocol (days 1-15):

The animals having patent indwelling catheters were subjected to self-administration training for a period of 15 days with one session each day. Self-administration training sessions

were 90 minutes in duration. Upon entry into the self-administration environment, the house light and the ventilation fan were on. In addition to triggering an infusion, active lever presses had the following programmed consequences: the house light was turned off, and the active lever light/tone (i.e. the CS) was turned on for a period of 30 seconds. Additional responses on the active lever during this 30 second period had no programmed consequences, although the program continued to count the number of active/inactive lever presses and infusions. This "timeout" period protected the animals from cocaine overdose. After this 30 second period the lever light and tone were terminated and the house light came back on. Rats were initially trained for 12 days on an FR-1 (fixed ratio schedule-1) schedule in which each active lever press outside the timeout period triggered the programmed consequences. For the last 3 days of self-administration training, an FR-3 schedule was imposed where 3 active lever presses outside the time out period were required to trigger an infusion and the CS. Each rat was placed in the same operant conditioning chamber throughout the course of the experiment.

2.5. Extinction protocol (days 16-20):

After the 15 days of self-administration training, the animals were divided into 4 groups (balanced for cocaine intake): 1) extinguished (saline), 2) extinguished ((±) CPP), 3) extinguished (D-serine), 4) abstinent (saline). All groups received i.p. injections of their respective treatments in their home cage environment. Both the extinguished group 1 and the abstinent group 4 received injections of saline (1ml/kg). Group 2 received an injection of (±) CPP (5 mg/kg) and group 3 received an injection of D-serine (100 mg/kg). Groups 1-3 underwent extinction training 4-5 hours following their respective daily pharmacological treatments, whereas group 4 rats remained in their home cages. During their extinction training sessions, the animals in the operant chambers were attached to the drug tether but exposed only

to the environment stimuli (i.e. diffuse, contextual cues). Responses on the active lever had no programmed consequences during the extinction training phase. For protocol days 16-20, responses on both active and inactive levers, as well as the equivalent "number of infusions" were counted by the software (although as stated above, syringe pumps were not activated during this phase of training). Extinction proceeded for a period of 5 days, with one 90 minute session each day during which the animals in the extinction training groups 1-3 were taken to the operant chambers. Under these conditions, the animals extinguished their lever pressing behavior to less than 20% of their former activity during self-administration. As previously mentioned, group 4 abstinent animals remained in their home cages throughout days 16-20.

2.6. Reinstatement tests (protocol days 21-24):

On days 21-24, all the animals (including the home cage abstinent animals), were placed back in the operant chambers for reinstatement tests. The reinstatement test session conditions were similar to an extinction session in that the animals were exposed only to the contextual cues of the operant chamber environment and the active lever responding were not reinforced by the contingent availability of either CS or US**.**

Reinstatement to the contextual drug stimuli:

On test day 21, response to the contextual prime was assessed from active lever presses during the first 10 minutes in which the animals were exposed only to the contextual cues of the drug environment.

Reinstatement to the CS cues:

Later during the same test session on day 21, lever presses evoked in response to a CS presentation were then assessed. A single, noncontingent presentation of the CS was delivered at the 40th minute of the 90 minute test session. Thus the initial 40 minutes of the 90 minute

session served as an extinction period to allow lever presses initiated by exposure to contextual stimuli to subside before the CS reinstatement test. As the CS was expected to evoke an immediate response from animals, the noncontingent CS was quantified as the number of lever presses during the subsequent 10 minutes following the priming event (t=40-50 min).

Reinstatement to the drug prime:

Response to the drug prime stimulus was assessed on days 22-24. We tested the reinstatement of drug seeking behavior using 3 different doses (0.25 mg/kg, 0.5 mg/kg and 1 mg/kg) of cocaine on three consecutive days (22^{nd}, 23^{rd} and 24^{th} day), respectively. A single, noncontingent drug prime was programmed to be infused intravenously by the syringe pump at the 40^{th} minute of the 90 minute session on these 3 US reinstatement test days. Again, the initial 40 minutes of the 90 minute session served as an extinction period which allowed lever presses initiated by exposure to contextual stimuli to subside before the US reinstatement test. Drug seeking behavior elicited by the different doses of cocaine was quantified from the number of responses on the active lever following the drug prime for 30 minutes immediately after the priming event (t=40-70 min).

2.7. Drugs & Dosage Justification:

Cocaine hydrochloride was a gift from NIDA (RTI). The NMDA receptor antagonist 3(-2 carboxipiperazin-4-yl)propyl-1-phosphonic acid ((±)CPP) and the coagonist D-serine were obtained from Sigma (St. Louis). D-serine and (±)CPP were administered in the home cage environment approximately 4 hours prior to the extinction sessions on protocol days 16-20. These compounds have long-lived effects when administered i.p. at the indicated doses [14,15], and therefore any state-dependent or acute locomotor effects were minimized by this advance treatment in the home cage environment. The D-serine dose of 100 mg/kg was chosen in order to avoid possible nephrotoxic effects at higher doses [37], while still affecting learning [31]. The

(±)CPP dose of 5mg/kg has been shown to disrupt synaptic plasticity processes in the hippocampal formation 12-24 hours following i.p. administration [34].

2.8. Statistics:

The number of active lever presses, infusions and inactive lever presses were recorded for each session. These data were used to calculate the responses during each experimental session. A repeated measures ANOVA was applied to the data in figure 1B, and a 2-way repeated measures ANOVA was applied to figure 4. A 2-way ANOVA was used for the other data analysis. The two factors taken into consideration for the 2- way ANOVA were: 1) either trial (pre vs. post priming responses) or days and 2) either condition (extinction vs. abstinence) or treatment, as the case may be. A value of $p<.05$ was taken as significant, being determined from the Holm-Sidak post hoc test method. All the statistics were done using SigmaStat software.

3. Results

3.1. Cocaine self-administration and extinction of the drug seeking behavior:

Animals having indwelling jugular catheters were trained to self-administer cocaine in an operant chamber environment for 15 consecutive days. During the daily 90-minute sessions, rats were initially trained on an FR-1 schedule for first 12 days and switched to an FR-3 schedule for the last 3 days of self-administration training. The transition to the FR-3 schedule was done to increase the number of active lever pressing responses. Animals typically achieved stable self-administration by day 10 of training, and the FR-3 schedule did not significantly alter the number of earned infusions per session (Figure 2.1). Over the entire fifteen day self-administration training phase the average total number of infusions earned was 334 ± 12, or the equivalent of approximately 11 mg/kg/day of cocaine per animal. There was no significant difference in the

average number of infusions earned per animal among the four different groups of self-administration rats utilized for the reinstatement studies described in this report (data not shown).

After 15 days of SA, the animals were subjected to either an extinction training phase (extinction groups) in the same operant chamber environment for 90 minute sessions in the absence of both CS and US (i.e. the active lever had no programmed consequences) for a period of 5 days (Figure 2.1B, protocol days 16-20), or they were kept forcibly abstinent in their home cage environment (abstinent group). Active and inactive lever presses were monitored during the extinction sessions and it was found that the animals extinguish their drug seeking behavior under these conditions. Evaluation of drug seeking behavior between extinction sessions demonstrates that lever pressing activity observed on second-fifth days of extinction is significantly decreased as compared with the first day of extinction (Figure2.1 B). Conversely, the second-fifth days' activities were not significantly different from each other.

3.2. Reinstatement of drug seeking behavior:

Evaluation of drug seeking behavior within an extinction session illustrates that the majority of lever pressing activity occurs during the initial ten minutes in the operant chamber environment, suggesting that environmental contextual cues are priming this response. Following this initial burst of activity, active lever pressing diminishes rapidly. As illustrated during the fifth extinction session on protocol day 20 (Figure 2.2A), active lever responses are minimal (< 1) by 20-30 minutes and remain low for the remainder of the 90 minute session. This within session response pattern was observed during all extinction days (data not shown). Therefore, during the reinstatement experiments involving the noncontingent presentation of either CS or US stimuli, the priming event was delivered at time=40 min of the test session. A temporal distinction can thus be made between the drug-seeking activity induced by introduction

to the operant chamber environment (i.e. activity during the first ten minutes) versus the subsequent activity induced via noncontingent presentation of priming events delivered later within the same test session.

3.3. The effects of extinction training versus enforced abstinence on drug-seeking behavior.

The resumption of lever-pressing activity was induced using three forms of priming stimuli: contextual cues, conditioned cues, and drug prime. Once the animals underwent either extinction training or enforced abstinence for a period of 5 days (days 16-20), they were tested for the resumption of drug seeking behavior following exposure to the contextual stimuli and the conditioned stimuli on day 21, and to cocaine on days 22-24.

The resumption of drug seeking induced by diffuse environmental cues was assessed during the first ten minutes of the test session conducted on day 21 ((Figure 2.2B). The level of responding on the active lever in the extinguished (saline) group of rats during this period was significantly decreased ($p<0.01$, unpaired t-test) compared with responding during the same period in the abstinent (saline) group kept in the home cage environment for 5 days (6.4 ± 0.9 vs. 41 ± 5.3, respectively). These results suggest that the 5-day extinction training experience decreased the efficacy of the contextual cues present in the operant chamber environment to provoke drug seeking behavior.

Until time=40 min of the day 21 reinstatement test session, the animals experienced extinction conditions. At this point, a single, noncontingent presentation of the discrete CS complex was delivered for a period of 30 seconds, and active lever responding for the next 10 min was measured as an indication of the reinstatement of drug seeking behavior evoked by the CS (Figure 2.3A). As previously described for extinction conditions, active lever presses had no programmed consequences at any time during these test sessions. In addition to the active lever

presses, the inactive lever presses were also monitored, so as to ensure that the noncontingent CS prime was in fact inducing activity previously associated with cocaine infusion. In order to confirm that any responding due to contextual cues had subsided by the time of the CS reinstatement test, active lever pressing during a "pre-prime" ten minute period of time (t=30-40) was also assessed and compared with the cue-induced "post-prime" level of reinstatement (t=40-50. Results for both the extinguished (saline) and abstinent (saline) groups of rats tested in this manner are illustrated for 10 min bins of time before and following the CS priming event. A 2-way ANOVA on the data of figure 2.3A confirmed an effect of trial, $F_{trial}(1,68)=15.398$ and condition, $F_{condition}(1,68)=7.452$. The post hoc analysis showed that the 10 minute post priming response was significantly greater than the 10 minute pre priming response for both the extinguished (saline), ($t=2.276$, $p=.026$) and the abstinent (saline), ($t=3.204$, $p=.002$) group. The magnitude of post priming response of the abstinent group was significantly greater than that of the extinguished group, ($t=2.698$, $p=.009$), indicating that an extinction training experience decreases the efficacy of noncontingent CS cues to trigger responses on the active lever. Importantly, the level of responding during the same period of time (i.e. 40-50 min) during the final day of self-administration (day 15) in these animals was 9.3 ± 0.8 and 8.1 ± 0.8 for the extinguished (saline) and the abstinent (saline) groups, respectively. By comparison, the close agreement in terms of the magnitude of the response in the abstinent animals (i.e. 8.1 ± 2.2) demonstrates the effectiveness of this noncontingent priming protocol to evoking drug seeking behavior and further indicates that a genuine reinstatement of drug seeking had occurred in response to the CS event.

Cocaine-induced reinstatement was tested on the next 3 consecutive days (protocol days 22-24) at 3 different priming doses (0.25 mg/kg, 0.5 mg/kg, 1 mg/kg) with a single,

noncontingent intravenous infusion of drug delivered at time=40 minute of the session at the respective dose on each day. The active lever responding for the next 30 minutes was then measured as an indication of the reinstatement of drug seeking behavior evoked by the US stimuli. The dose-response results are plotted to demonstrate the shift in the sensitivity to cocaine-induced drug seeking activity (Figure 2.3B). At the 0.25mg/kg dose of cocaine, 2-way ANOVA confirmed an effect of trial, $F_{trial}(1,66)=4.046$ and condition, $F_{condition}(1,66)=5.864$. The post hoc analysis showed an increase in the 30 minute post priming response for the extinguished group, ($t=2.486$, $p=0.015$)as compared to the 30 minute pre prime response (this comparison was not significant for the abstinent group due to their high baseline response on day 22). At the 0.5mg/kg priming dose of cocaine the 2-way ANOVA showed an effect of trial, $F_{trial}(1,63)=20.336$ and condition, $F_{condition}(1,63)=10.89$. The post hoc analysis showed that this priming dose of cocaine significantly reinstated the drug seeking behavior among the abstinent group, ($t=4.324$, $p<0.001$) but not among the extinguished group. The comparison of the 30 minute post priming response levels between the extinguished and the abstinent group showed that the reinstatement in the abstinent group was significantly greater than that of the extinguished group, ($t=3.828$,$p=0.000$). A similar distinction was observed at the priming dose of 1mg/kg for which $F_{trial}(1,61)=35.548$ and $F_{condition}(1,61)=5.816$ was obtained. Pair-wise comparisons showed a significant effect of priming for both the extinguished ($t=3.444$, $p=.001$) and the abstinent group ($t=4.869$, $p<0.001$). The post prime response of the abstinent group was significantly higher than the extinguished group at this priming dose ($t=2.984$, $p=.004$). Also, in smaller groups of these extinguished (saline) and the abstinent (saline) conditions, the animals were tested on day 25 with a saline prime to test for any conditioned effects of the noncontingent cocaine primes that might accumulate over days 22-24. No evidence for such an effect was

observed, as there was no change in active lever pressing following the saline prime, as responding was 2.3 ± 1.0 (n=8) and 1.6 ± 0.8 (n=4) for the extinguished (saline) and the abstinent (saline) groups, respectively).

These drug prime reinstatement results illustrate the effectiveness of extinction training in reducing sensitivity to cocaine-induced drug seeking as compared to an equivalent period of enforced abstinence. The level of responding during the same period of time (i.e. 40-70 min) during the final day of self-administration (day 15) in these animals was 27.6 ± 1.9 and 24.0 ± 1.0 for the extinguished (saline) and the abstinent (saline) groups, respectively. In comparison, at the 0.5mg/kg priming dose, the extinguished (saline) group response on the active lever was 8.7 ± 2.4, whereas abstinent (saline) group responses were significantly greater (26.5 ± 6.9). A similar distinction was observed for the 1mg/kg priming dose, as the responses from the extinguished (saline) and abstinent (saline) groups were 12.9 ± 2.5 and 25.5 ± 6.6, respectively. As mentioned previously with the CS priming test, the close agreement in the magnitude of the response in the abstinent animals indicates that a genuine reinstatement of drug seeking had occurred during this period of time in response to the noncontingent US priming events.

3.4. NMDAR involvement in the effects of extinction training on reinstatement behavior.

The role of NMDARs in the extinction process was also evaluated in two additional groups of extinguished rats treated with either D-serine (a coagonist of NMDAR at the glycine site), or (±) CPP (a competitive antagonist of NMDAR). These compounds were administered (D-serine: 100 mg/kg i.p., (±)CPP: 5 mg/kg i.p.) in the home cage environment prior to the extinction sessions (see Methods). Over the course of the 5-day extinction phase carried out on days 16-20, it was observed that the D-serine treatment significantly facilitated extinction as compared to the saline group. A 2-way RM ANOVA ($F_{treatment}(2,204)=3.106$ and

$F_{day}(4,204)=110.912$) showed this facilitation of extinction to be statistically significant on days 1($t=2.956$, $p=.004$) and 3($t=2.138$, $p=.034$). Treatment with (±) CPP had no discernable effect on the progression of extinction, as active lever responding was not significantly affected on any day of the extinction protocol. By protocol day 20, the final extinction day, all groups exhibited similar levels of drug-seeking behavior during the 90 minute session.

The resumption of lever-pressing activity induced by diffuse environmental cues is illustrated in Figure 2.5A, and was again assessed during the first ten minutes of the test session conducted on protocol day 21 (as described previously for Figure 2.2B). The level of responding on the active lever in the saline group of rats during this period was 6.4 ± 0.9 and both the D-serine and (±) CPP treated animals showed comparable levels of responding (6.4 ± 1.3 and 8.7 ± 1.8, respectively). Thus, neither positive modulation (w/D-serine) nor competitive antagonism (w/(±)CPP) of NMDAR activity during extinction training had significant impact on the subsequently measured contextual reinstatement as compared with the saline treated control group following a five-day extinction protocol.

In order to investigate the effects of NMDAR activity during extinction on the discrete CS-induced reinstatement, active lever pressing was again measured during a "pre-prime" ten minute period of time as well as the "post prime" period (Figure 2.5B). Analysis via 2-way ANOVA confirmed an effect of trial, $F_{trial}(1,100)=27.003$, but not an effect of treatment. Pairwise comparisons showed that the post priming activity following the CS prime was significantly greater than the pre priming activity for the control ($t=3.328$, $p=.001$), the D-serine ($t=2.816$, $p=.006$) and the (±) CPP ($t=2.940$, $p=.004$) groups. Thus, although active lever pressing activity was significantly increased in the ten minute period immediately following the CS prime in both the D-serine (0.0 ± 0.0, 3.4 ± 1.2, n= 15) and (±) CPP (0.2 ± 0.1, 3.6 ± 1.1, n=

17) treatment groups, since the magnitude of post prime responding was not significantly different from that of the saline treated extinguished group (3.7 ± 1.0, n= 22), no role for NMDAR activity during the extinction training experience is indicated for the CS-induced reinstatement outcome.

Finally, cocaine-induced reinstatement was tested on the next 3 consecutive days (protocol days 22-24) at 3 different priming doses (0.25 mg/kg, 0.5 mg/kg, 1 mg/kg) with a single, noncontingent intravenous infusion of drug delivered at time=40 min of the session. The active lever responding during the next 30 minutes was measured as an indication of the reinstatement of drug seeking behavior evoked by the US stimuli. The dose-response results are plotted to demonstrate the shift in the sensitivity to cocaine-induced drug seeking activity (Figure 2.5C). Treatment with D-serine during extinction did not alter reinstatement at any of the priming doses tested as compared with the saline treated group. At the 0.25mg/kg priming dose of cocaine, the 2-way ANOVA confirmed an effect of trial, $F_{trial}(1,93)=24.037$ but not an effect of treatment. The post hoc analysis of this data showed that the 30 minute post priming response was significantly greater than the pre priming response for the saline control (t=3.383, p=.001) and the (±)CPP (t=3.409, p=.001) groups. The analysis of the data at the priming dose of 0.5mg/kg showed an effect of trial, (F_{trial} (1, 84) = 35.075), treatment, ($F_{treatment}(2,84)=4.204$) and an effect of interaction between these two factors($F_{interaction}(2,84)=4.749$). The post priming responses were significantly greater than the pre priming responses for both the control (t=2.738, p=.008) and the (±) CPP group (t=5.720, p=0.000). Pairwise comparison between the post prime responses of the different treatments showed that the (±) CPP treated group responded significantly greater than the saline group (t=3.753, p=0.000). At the 1mg/kg priming dose of cocaine, the 2- way ANOVA showed an effect of trial, $F_{trial}(1,86)=37.21$, but did not show an

effect of the treatment and interaction. The post hoc analysis showed that within the treatment groups, the post priming responses were significantly greater than the pre priming responses (control(t=3.150,p=.002), D-serine(t=2.756,p=.007) and (±) CPP(t=4.681,p=0.000)). Even though the main effect of treatment was not observed at this dose, planned comparisons of the post priming responses of the treatment groups showed a significantly greater response for the (±)CPP group compared to the control group (t=2.439, p=.017). These results indicate that the ability of extinction to reduce drug primed reinstatement (c.f. Fig.2.3C) is dependent upon the activation of NMDARs during the training protocol.

In sum, altering NMDAR activity during extinction training did not affect the resumption of lever pressing activity induced by diffuse contextual or discrete CS cues. However, the blockade of NMDARs during the extinction protocol did affect the US (cocaine) primed reinstatement, and the magnitude of responding in this group was similar to that of the abstinent group. This suggests that the effectiveness of a prior extinction training experience to reduce the reinstatement response to drug priming involves an NMDAR dependent mechanism, and is therefore distinguishable from the mechanisms underlying the effects of extinction on priming via diffuse contextual or discrete CS cues.

4. Discussion

In this report we have directly assessed the effectiveness of an extinction training protocol to reduce primed reinstatement as compared with an equivalent period of enforced abstinence. This was a prerequisite step for us in pursuing our intent to investigate the mechanisms underlying the effectiveness of extinction training to reduce reinstatement. We have found that in addition to reducing active lever responding when placed back into the drug

paired environment (Figure 2.2B), extinction training can also significantly reduce the primed drug seeking response following exposure to either CS or US cues (Figure 2.3).

In the rat reinstatement model of craving and/or relapse, the presentation of discrete, drug associated stimuli can be used to prime the reinstatement of drug seeking behavior [24]. Since an early study that employed the use of CS cues presented in a noncontingent manner [7], the majority of the subsequent work in rats has used a contingent method of assessing CS induced reinstatement [26]. This issue has been a point of contention concerning the validity of the reinstatement protocol for modeling the human condition in which self-reported craving can be triggered by passive exposure to drug associated cues. As craving in humans can be induced via noncontingent presentation of these cues, it has been argued that the CS reinstatement protocol should employ this form of priming as well [16]. In our study, we have determined conditions under which a single, noncontingent presentation of the CS or US results in a significant increase in drug seeking behavior. In order for this effect to be observed, it was important to utilize a within session design and to select an appropriate period of time for the analysis of the response. By waiting until the immediate, context stimulated responding had dissipated (Figure 2.2A), the relatively low remaining level of activity could be effectively compared with the responses on the active lever triggered by the noncontingent CS or US presented at time = 40 minutes into the test session.

The neural circuitry underlying reinstatement has been the subject of several studies, recently reviewed by See [24]. Evidence suggests that the basolateral amygdala, the dorsal medial prefrontal cortex, and the lateral orbitofrontal cortex participate in the expression of reinstatement following exposure to CS cues. In addition, the dorsal hippocampus is selectively involved in reinstatement following exposure to contextual cues [11] and both the nucleus accumbens [13] and

the ventral hippocampus [30] are involved in reinstatement following exposure to drug prime. Stress induced via foot shock can evoke reinstatement [8], and this effect may involve the bed nucleus of the stria terminalis [9] and the ventral tegmental area [36]. Finally, the participation of the dorsolateral caudate putamen region is required for either context or CS cued reinstatement following periods of either abstinence or extinction [12], whereas the participation of the basolateral amygdala or the dorsomedial prefrontal cortex is not required for reinstatement following a period of abstinence (as opposed to a period of extinction, as in the earlier studies cited above). In sum, it is evident that the reinstatement evoked via these different types of priming stimuli can differentially involve distinct regions of the brain, and therefore may also recruit different mechanisms at the synaptic level for the processing, storage, and recall of such information.

In contrast to the self-administration behavioral model, the mechanisms of extinction training have been extensively investigated in other paradigms such as fear conditioning, inhibitory avoidance, spatial navigation and conditioned taste aversion [5]. Extinction likely involves new learning [4, 20, 21], and at the molecular level, both NMDAR and nonNMDAR dependent forms of synaptic plasticity are thought to contribute to this type of learning. Although some studies have indicated that extinction learning in conditioned taste aversion [3] or conditioned fear [1] does not require the activation of NMDARs, other reports have indicated a critical role for NMDARs [2, 10, 22]. Our results with cocaine self-administration have demonstrated that antagonism of NMDARs during extinction had no acute effect on responding within a session, nor was there any effect on the progression of extinction over the five day protocol. However, there was a trend in the NMDAR coagonist treated group for enhanced extinction within each daily session (particularly evident at days 16 & 18), but again there was no significant difference in the level of responding by the end of the extinction training protocol

(Figure 2.4). Thus although the (±) CPP results demonstrate that NMDAR activation is not required for the normal progression of extinction, the D-serine results suggest that NMDAR activation may facilitate extinction, at least over the initial days of training.

As far as we are aware, the mechanism(s) underlying the effects of extinction to reduce the reinstatement of drug seeking behavior have not been described. As mentioned previously, since different types of priming stimuli have been shown to involve different neural substrates, it is reasonable to propose that the effects of extinction training on these various forms of reinstatement might also be linked to different mechanisms during the extinction learning process. Our results demonstrate that although extinction training is effective in reducing the reinstatement evoked by context, CS, and cocaine priming (Figures 2B & 3), the underlying mechanism involves both NMDAR dependent and NMDAR independent components. This was illustrated in Figure 5, where only the effect of extinction on the reinstatement primed via cocaine administration was sensitive to NMDAR blockade during extinction training (Figure 2.5C), whereas extinction was equally effective despite NMDAR antagonist treatment in the case of either context or CS primed reinstatement (Figures 2.5A & B). Based on these findings, we expected that treatment with the NMDAR coagonist D-serine would enhance the effects of extinction training such that the magnitude of cocaine primed reinstatement responses would be decreased relative to the saline treated group. This expectation was not met (Figure 2.5C), and we suggest that over training is likely to occur by the end of our five day protocol such that the effects of extinction are maximal and cannot be significantly enhanced. Future studies that assess the efficacy of a shorter extinction training period will directly test this hypothesis, and indicate whether enhancing the activity of NMDARs during extinction training might be an effective means by which to significantly decrease the duration and/or number of extinction sessions required to reduce the

magnitude of the drug primed reinstatement response. In any case, the demonstration of the participation of NMDARs in the effects of extinction to reduce cocaine primed reinstatement indicates that pharmacotherapy targeting these receptors may be useful, and it is tempting to speculate that just as different brain regions are involved in mediating the various forms of primed reinstatement of drug-seeking behavior, different synaptic mechanisms (i.e. both NMDAR dependent and NMDAR independent synaptic plasticity) may also be involved in mediating the effects of extinction on primed reinstatement.

The mechanisms involved with the resulting effects of extinction training as they may relate to the self-administration of addictive substances have been investigated at the molecular level in the ventral tegmental area and nucleus accumbens regions of the brain [25]. These studies have demonstrated that an extinction training experience can restore tyrosine hydroxylase to predrug levels in the nucleus accumbens shell, whereas an equivalent abstinent period showed a persistent deficit in tyrosine hydroxylase protein levels [23]. Similarly, a down regulation of NMDA receptor NR1 subunit during abstinence is reversed by extinction [25]. Finally, an extinction induced upregulation of GluR1 AMPA receptors occurs in the nucleus accumbens shell [32], and an upregulation of tyrosine hydroxylase also has been observed in the ventral tegmental area following extinction training [23]. All of these extinction induced neuroadaptations would be expected to act in a compensatory manner in order to counteract the drug induced depression of the synapses in the nucleus accumbens shell [33]. However, it is important to consider that these changes have been measured as a result from the animal having undergone extinction, and are not necessarily direct contributors to the learning mechanisms at work during the extinction training process itself that underlie the subsequent effects on the expression of primed reinstatement.

In conclusion, we have provided a clear measure of the effectiveness of extinction training in reducing the resumption of drug seeking behavior primed by noncontingent exposure to context, CS, or drug primes as compared to an equivalent period of enforced abstinence. In addition, the effectiveness of extinction was differentially sensitive to pharmacological intervention with an NMDAR antagonist administered during the training protocol, suggesting that multiple forms of synaptic plasticity may be utilized during this form of new learning that are specific to the different types of priming and the associated neural circuitry involved in mediating the resumption of drug seeking behavior. If this is the case, addiction treatment strategies will need to take this into account when the effectiveness of any one approach is being evaluated, and suggests that significant gains may only be achieved when several therapeutic approaches are combined.

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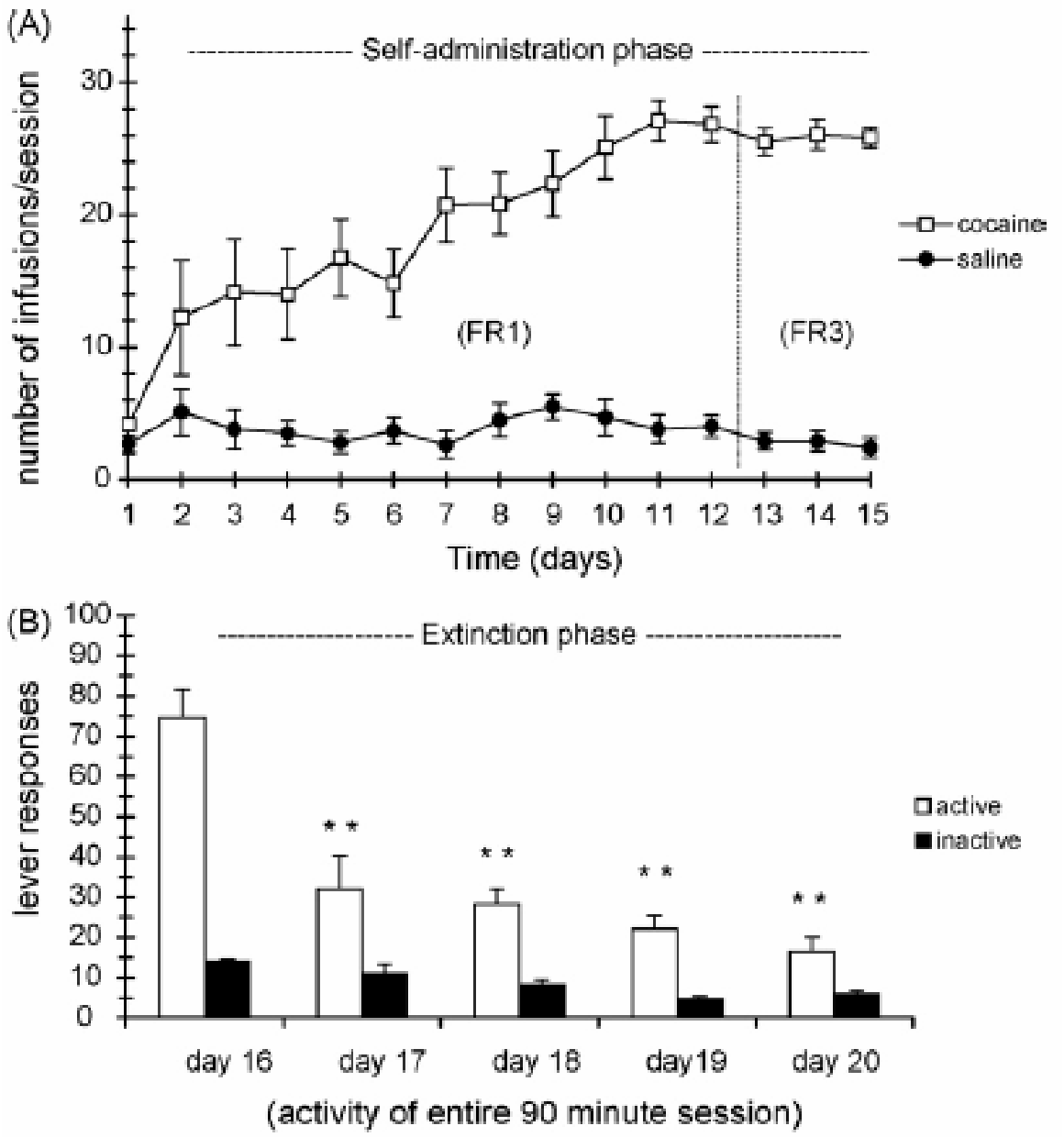

Figure 2.1. Self-administration of cocaine and extinction training.

(A) The results from one cocaine group of rats (open boxes, n=22) illustrate the average number cocaine infusions earned daily during each 90 minute session during the SA phase (days 1-15). Transition to the FR3 schedule on day 13 did not significantly alter the number of earned infusions. A saline control group of rats (filled circles, n=10) is illustrated for comparison. B)

Active and inactive lever presses of a cocaine extinguished group (n=22) are illustrated (mean ± SEM) for the 90 minute sessions on each day of extinction (days 16-20). In the absence of both CS and US the animals extinguished their drug seeking behavior over a period of 5 days. Active lever presses on extinction days 2-5 were significantly decreased (** $p<.001$, RM ANOVA, Holm-Sidak) from those measured on the first day of extinction, (i.e. protocol days 17-20 vs. 16).

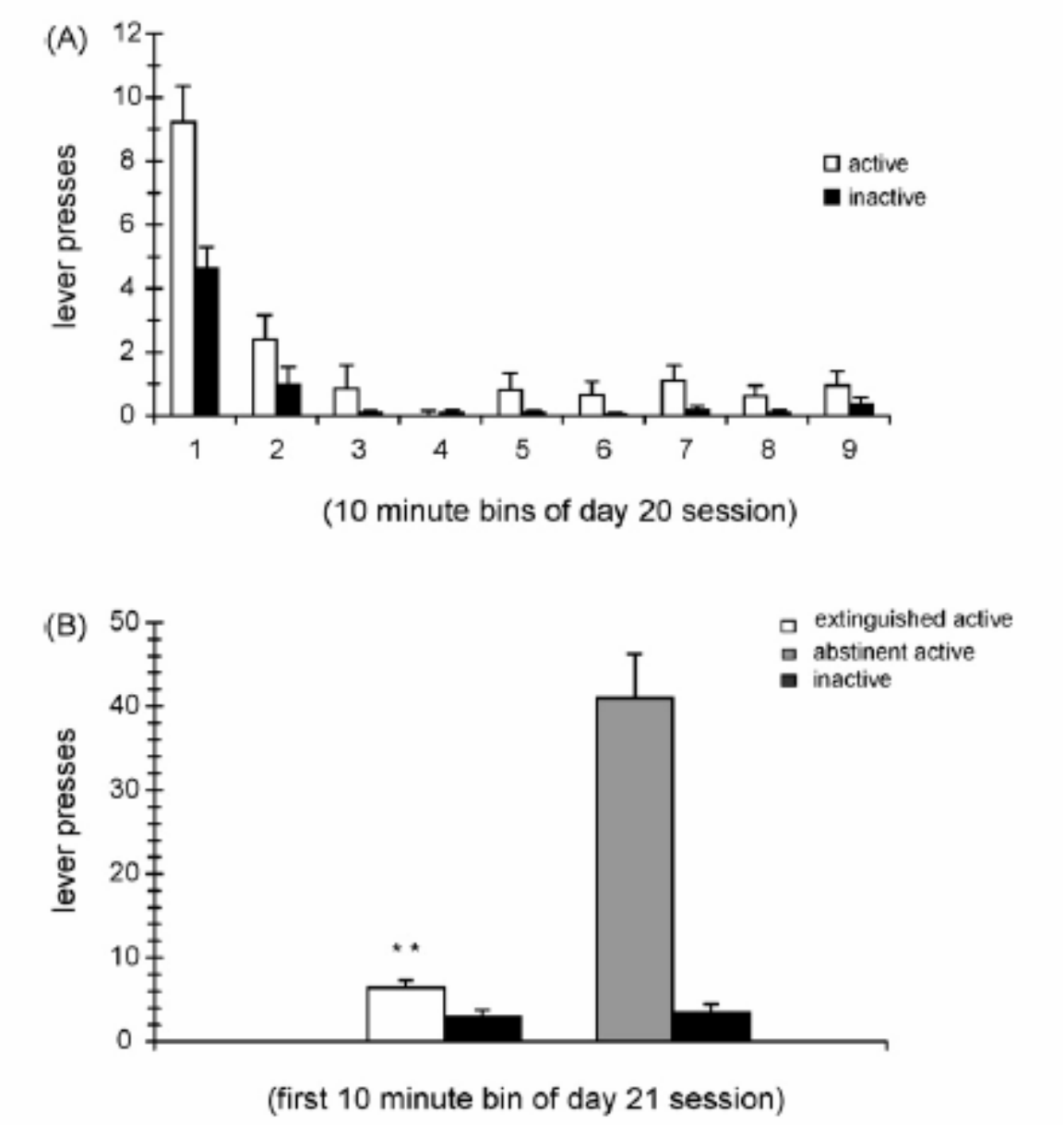

Figure 2.2. Extinction training reduces the initial response to diffuse contextual cues.

A) Extinction behavior within a session on the fifth day of extinction (protocol day 20) is illustrated using 10 minute bins. Data are the mean ± SEM of lever presses throughout the 90 minute extinction session. B) Data show the mean ± SEM of lever presses for the first 10 minute bin of the initial reinstatement test day (day 21) when the animals were placed back into the operant environment after either 5 days of extinction training (n=22) or 5 days of enforced

abstinence (n=14). Drug seeking behavior on day 21 in response to diffuse contextual cues was significantly decreased in the extinguished group as compared to the behavior of the abstinent group (** $p<.01$, unpaired t-test).

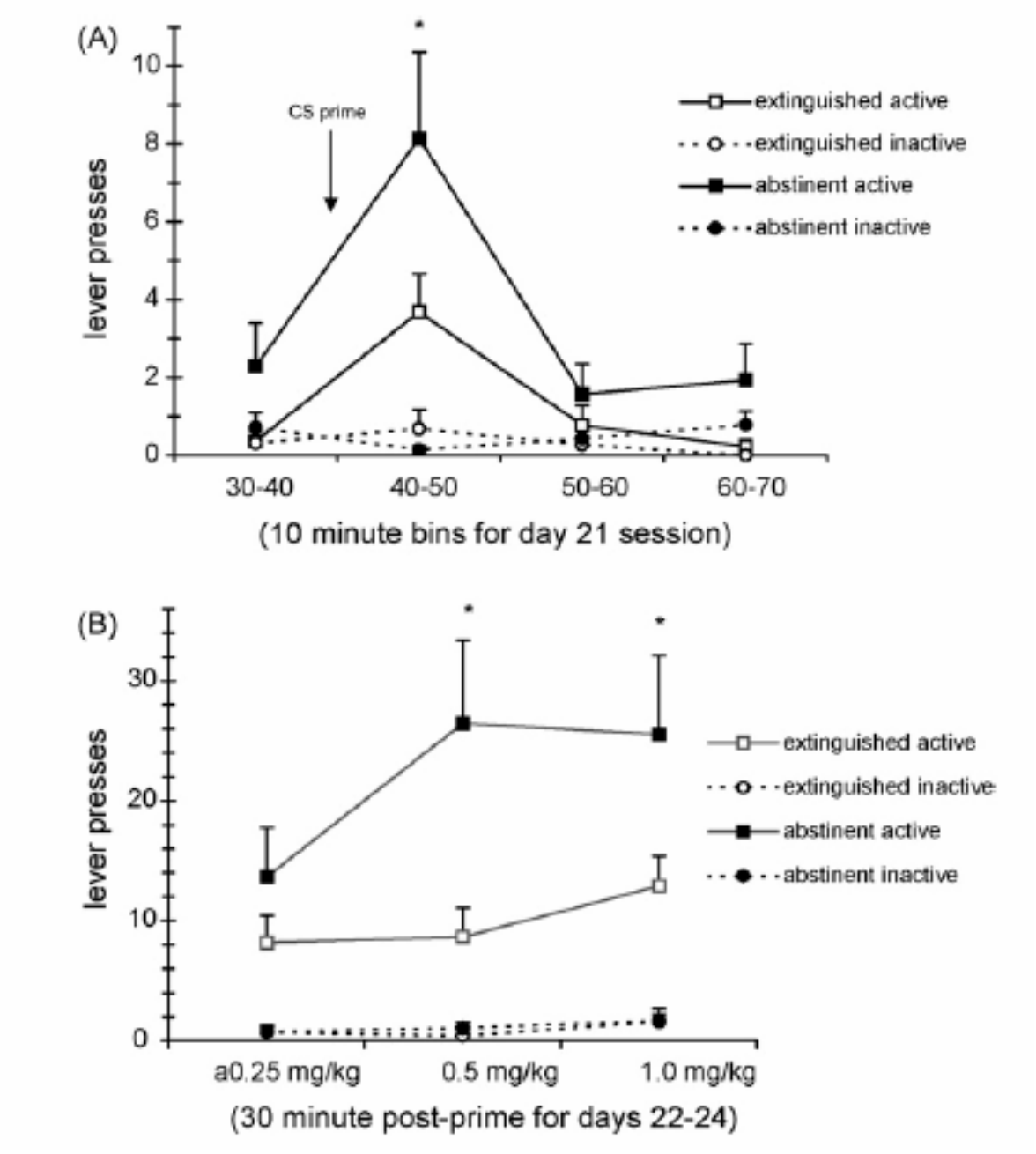

Figure 2.3. Priming with either CS or US evoked drug seeking behavior:

(A) Data show the mean ± SEM of active and inactive lever presses in 10 minute bins for 30-70 minutes of the CS prime test session on the first reinstatement test day (day 21) for the extinguished (n=22) and the abstinent groups (n=14). Lever pressing responses during the "pre priming" period (30-40 min) were minimal. The delivery of a single, noncontingent CS prime

(light and tone) at time=40 minutes reinstated the response specifically on the active lever, whereas inactive lever responses remained low throughout the session. Post prime responses on the active lever were significantly higher for both groups when compared to their respective pre prime responses (2-way ANOVA, Holm-Sidak). In addition, the abstinent group showed significantly higher responding to the single noncontingent CS prime as compared to the extinguished group (* $p<.05$, 2-way ANOVA, Holm-Sidak). **(B)** Data represent the mean ± SEM of lever presses for a 30 minute window after the single, noncontingent intravenous delivery of a drug prime at the indicated dose was administered at time=40 minutes on each of the reinstatement test days (protocol days 22-24). For both extinguished and abstinent groups, the post prime response was significantly greater than the pre prime response at all of the cocaine doses tested (data not shown) and the drug seeking responses of the abstinent animals (n=14 for 0.5 mg/kg and n=11 for 1 mg/kg) were significantly higher than those of the extinguished animals (n=21 for 0.5 mg/kg and 1 mg/kg) at priming doses of 0.5 mg/kg and 1 mg/kg (* $p<.05$, 2-way ANOVA, Holm-Sidak).

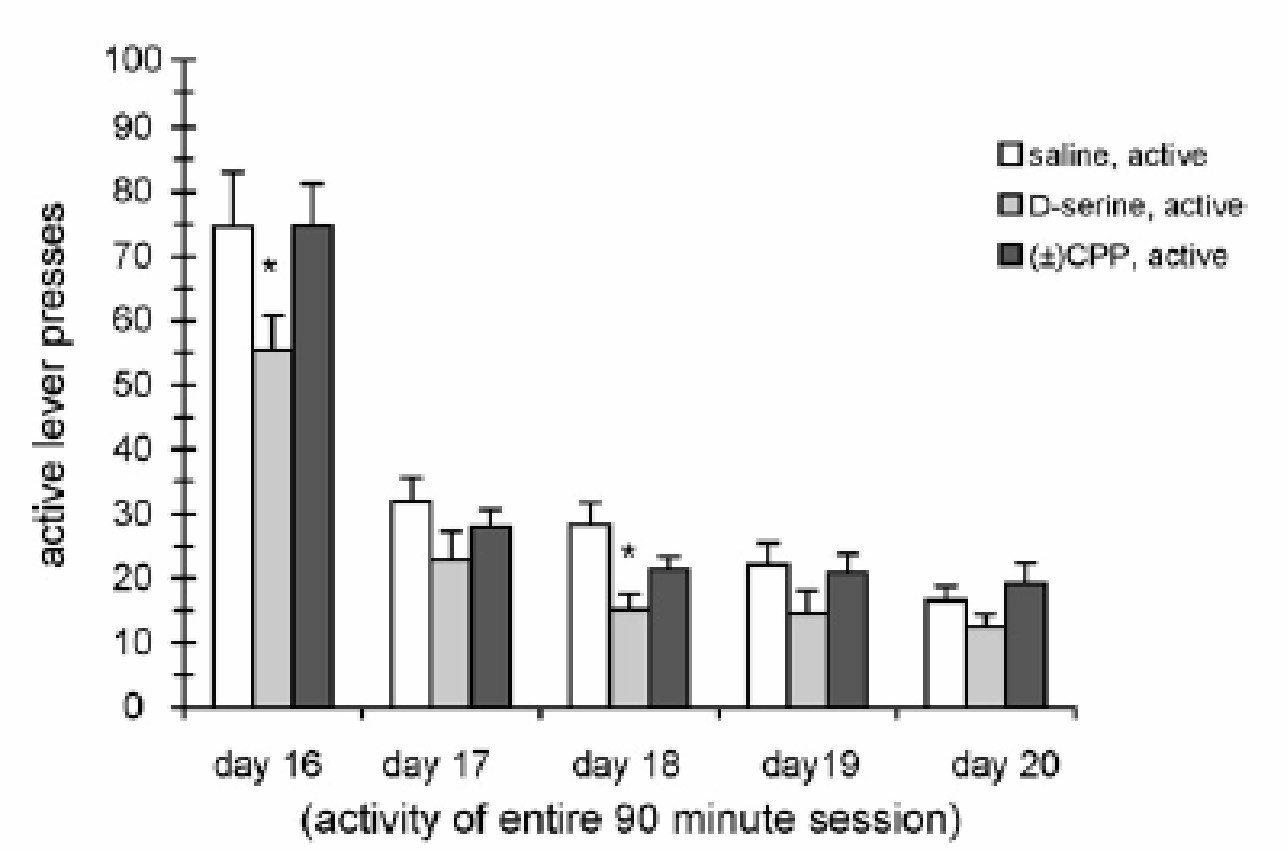

Figure 2. 4. Influence of NMDAR activity on the progression of extinction.

Data shows the mean ± SEM of active lever presses for the entire 90 minute extinction sessions on protocol days 16-20. The saline treated control group data is replotted from Fig. 1B for comparison. Active lever pressing behavior was extinguished across the 5 daily extinction sessions and responding on extinction days 2-5 was significantly decreased ($p<.001$, 2-way RM ANOVA, Holm-Sidak) from that measured on the first day of extinction (i.e. protocol days 17-20 vs. 16) within each treatment group. A trend was evident in the D-serine treated group (n=15) for an enhanced rate of extinction as compared to the animals treated with (±)CPP (n=17) or saline, and the drug seeking responses of the D-serine group were significantly lower (* $p<.05$, 2-way RM ANOVA, Holm-Sidak) on the first and third days of extinction (protocol days 16 & 18).

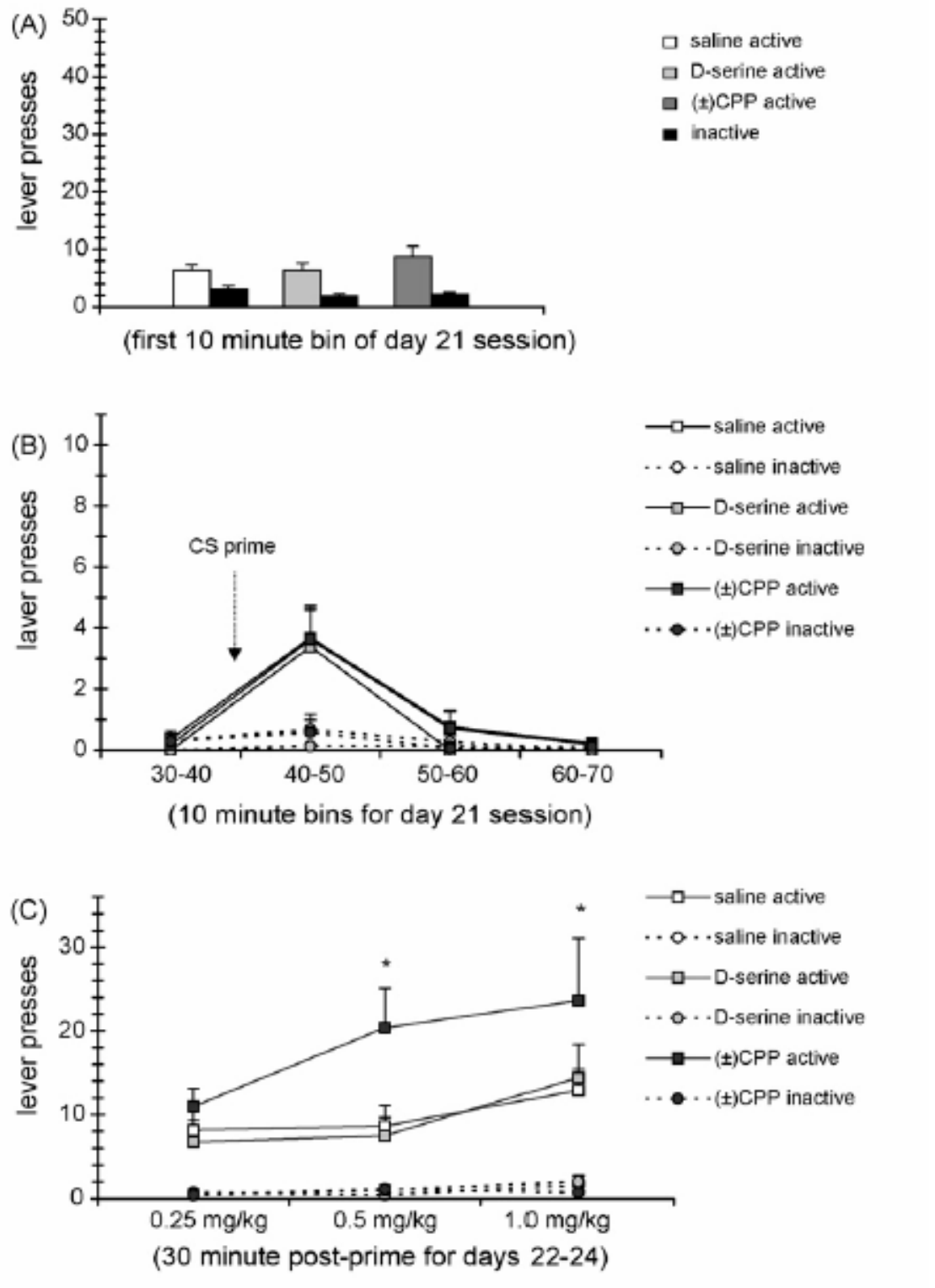
(A)
lever presses
50
40
30
20
10
0
saline active
D-serine active
(±)CPP active
inactive
(first 10 minute bin of day 21 session)
(B)
laver presses
10
8
6
4
2
0
CS prime
saline active
saline inactive
D-serine active
D-serine inactive
(±)CPP active
(±)CPP inactive
30-40
40-50
50-60
60-70
(10 minute bins for day 21 session)
(C)
lever presses
30
20
10
0
*
*
saline active
saline inactive
D-serine active
D-serine inactive
(±)CPP active
(±)CPP inactive
0.25 mg/kg
0.5 mg/kg
1.0 mg/kg
(30 minute post-prime for days 22-24)

Figure 2.5. Altering NMDAR activity during extinction had no effect on the resumption of drug-seeking following either exposure to contextual cues or the CS priming, but did affect the response to US priming:

The saline group data depicted in panels A-C. was previously designated as the "extinguished group" in Figs. 2 & 3 and is replotted here for comparison. A) Data show the mean ± SEM of lever presses for the first 10 minute bin of time on the first reinstatement test day (protocol day 21). Neither D-serine (100 mg/kg i.p., n=15) nor (±)CPP (5 mg/kg i.p., n=17) treatment during extinction training had a significant effect on responding as compared with the saline treated control group. B) Data show the mean ± SEM of active and inactive lever presses in 10 minute bins for 30-70 minutes of the CS prime test session on the first reinstatement test day (day 21) for the D-serine (n=15) and the (±)CPP (n=14) groups. Post prime responses on the active lever were significantly higher for both groups when compared to their respective pre prime responses (2-way ANOVA, Holm-Sidak). Neither treatment affected the magnitude of the post priming response as compared with the saline control groups. C) Data represent the mean ± SEM of lever presses for a 30 minute window after the single, noncontingent intravenous delivery of a drug prime at the indicated dose was administered at time=40 minutes on each of the reinstatement test days (protocol days 22-24). For both D-serine and (±)CPP groups, the post prime response was significantly greater than the pre prime response at all of the cocaine doses tested (data not shown) and the drug seeking responses of the (±)CPP animals (n=13 for 0.5 mg/kg and 1 mg/k) were significantly higher than those of the saline animals (* $p<.05$, 2-way ANOVA, Holm-Sidak).

APPENDIX-I-CHAPTER 2

THE EFFECTS OF EXTINCTION TRAINING IN REDUCING THE REINSTATEMENT OF NATURAL REWARD SEEKING BEHAVIOR: INVOLVEMENT OF NMDA RECEPTORS.

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[1]Kelamangalath, L and Wagner, J.J. To be submitted to Brain Research

A1. Introduction

It is described from as early as 1962 that the electrical stimulation of the neural system in the reward pathway of the brain mediates the natural consummatory behavior such as drinking and eating in addition to mediating the effects of the addictive rewards (Hoebel and Teitelbaum, 1962; Margules and Olds, 1962). Animals learn to self-administer natural rewards like sucrose pellets through an associative learning paradigm just like they learn to self-administer the addictive drug reward such as cocaine. During appetitive learning, dopamine neurons do not discriminate amongst various appetitive stimuli, between various sensory modalities or between primary rewards and conditioned appetitive stimuli (Schultz, 1997). Hence, the associative-learning process for acquiring the self-administration of the natural reward and the addictive drug reward utilizes a similar mechanism. But, self-administration is a situation requiring repeated learning. In such situations, the dopamine neurons are activated initially when the rewards are usually novel or unpredicted, but cease responding once the reward becomes fully predictable (Schultz et al., 1993). Inversely, as in the case of extinction, when a fully predicted reward fails to occur, dopamine neurons are depressed in their activity at exactly the time when the reward would have occurred (Schultz et al., 1993). Here it appears that dopamine neurons code the deviation or 'error' between the prediction and the actual occurrence of the reward (Schultz et al., 1995). Since the instrumental responses (lever responses) that the animals make are no longer reinforced in extinction, the animals extinguish their lever pressing behavior. During the reinstatement tests, the conditioned cue or the unconditioned reward (US-here sucrose pellet) is delivered in an unpredicted manner and this is taken as an unpredicted or novel stimulus, which has the capability to activate the dopamine neurons. The dopamine released in this way will cause the resumption of the extinguished lever pressing behavior. Hence, the

mechanism by which the learning during self-administration and extinction, and the response during reinstatement operates remains almost similar for the natural reward as well as the addictive drug reward.

In chapter-2, we have shown the involvement of NMDAR dependent synaptic plasticity mechanisms in the effects of extinction in reducing the reinstatement of drug seeking behavior (Kelamangalath et al., 2007). Since we know that the extinction learning in animals trained to self-administer sucrose pellets proceeds in the same pattern as the extinction learning in animals trained to self-administer cocaine, we hypothesize that the NMDAR dependent synaptic plasticity mechanisms involved in the effects of extinction in reducing the cocaine primed reinstatement behavior might be relevant for the sucrose pellet (natural reward) primed reinstatement behavior also. We wanted to study whether the NMDAR dependent mechanisms involved in the effects of extinction in reducing the cocaine primed reinstatement behavior is general to any kind of reward or are specific to the drugs of abuse.

A2. Materials and Methods

A2.1. Animals

Male Sprague-Dawley rats (Harlan) weighed approximately 300 g at the beginning of the experiment and were housed individually in a temperature and humidity controlled vivarium having a 12-h light:12-h dark cycle (lights off at 7:00 p.m.). They were given access to food and water *ad libitum* and were handled daily for 5 days prior to the experiment in order to diminish stress associated with handling. The housing and experimental procedures followed the *Guide for the Care and Use of Laboratory Animals* and were approved by the local ACUC at the University of Georgia.

A2.2. Self-administration environment

The operant chambers (Med associates) were equipped with two levers, one "active" and another "inactive" with lights positioned above each lever. The chambers had a rod grid floor, a house light, a speaker/tone generator (2.9 kHz, 10 dB above ambient) and were housed inside enclosures equipped with ventilation fans. A pellet dispenser was located outside the enclosure. The method for delivering a sucrose pellet was as follows. The required number of responses on the active lever delivered a sucrose pellet according to each stage of experiment. The MED-PC software program recorded the number of active lever presses, inactive lever presses and the number of sucrose pellets delivered.

A2.3. Self-administration protocol (days 1–15)

The animals were subjected to self-administration training for a period of 15 days with one session each day. Self-administration training sessions were 90 minutes in duration. Upon entry into the self-administration environment, the house light and the ventilation fan were on. In addition to triggering a pellet, active lever presses had the following programmed consequences: the house light was turned off, and the active lever light/tone (i.e. the CS) was turned on for a period of 30 seconds. Additional responses on the active lever during this 30 second period had no programmed consequences, although the program continued to count the number of active/inactive lever presses and number of pellets ingested. After this 30 second period the lever light and tone were terminated and the house light came back on. Rats were initially trained for 12 days on an FR-1 (fixed ratio schedule-1) schedule in which each active lever press outside the timeout period triggered the programmed consequences. For the last 3 days of self-administration training, an FR-3 schedule was imposed where 3 active lever presses outside the time out period were required to trigger a pellet and the CS. Each rat was placed in the same operant conditioning chamber throughout the course of the experiment.

A2.4 Extinction protocol (days 16-20):

After the 15 days of self-administration training, the animals were divided into 4 groups (balanced for sucrose pellet intake): 1) extinguished (saline), 2) extinguished ((±)CPP), 3) extinguished (D-serine), 4) abstinent (saline). All groups received i.p. injections of their respective treatments in their home cage environment. Both the extinguished group 1 and the abstinent group 4 received injections of saline (1ml/kg). Group 2 received an injection of (±)CPP (5 mg/kg) and group 3 received an injection of D-serine (100 mg/kg). Groups 1-3 underwent extinction training 4-5 hours following their respective daily pharmacological treatments, whereas group 4 rats remained in their home cages. During their extinction training sessions, they were exposed only to the environment stimuli (i.e. diffuse, contextual cues). Responses on the active lever had no programmed consequences during the extinction training phase. For protocol days 16-20, responses on both active and inactive levers, as well as the equivalent "number of pellets" were counted by the software (although pellet dispenser was not activated during this phase of training). Extinction proceeded for a period of 5 days, with one 90 minute session each day during which the animals in the extinction training groups 1-3 were taken to the operant chambers. Under these conditions, the animals extinguished their lever pressing behavior to less than 20% of their former activity during self-administration. As previously mentioned, group 4 abstinent animals remained in their home cages throughout days 16-20.

A2.5 Reinstatement tests (protocol days 21 and 22):

On days 21 and 22, all the animals (including the home cage abstinent animals), were placed back in the operant chambers for reinstatement tests. The reinstatement test session conditions were similar to an extinction session in that the animals were exposed only to the

contextual cues of the operant chamber environment and the active lever responding were not reinforced by the contingent availability of either CS or US.

Reinstatement to the contextual stimuli:

On test day 21, response to the contextual prime was assessed from active lever presses during the first 10 min in which the animals were exposed only to the contextual cues.

Reinstatement to the CS cues:

Later during the same test session on day 21, lever presses evoked in response to a CS presentation were then assessed. A single, noncontingent presentation of the CS was delivered at the 40th min of the 90 min test session. Thus the initial 40 min of the 90 min session served as an extinction period to allow lever presses initiated by exposure to contextual stimuli to subside before the CS reinstatement test. As the CS was expected to evoke an immediate response from animals, the noncontingent CS was quantified as the number of lever presses during the subsequent 10 min following the priming event (t =40–50 min).

Reinstatement to the pellet prime:

Response to the pellet prime stimulus was assessed on day 22. We tested the reinstatement of natural reward seeking behavior using a single sucrose pellet prime on day 22. A single, noncontingent sucrose pellet prime was programmed to be delivered by the pellet dispenser at the 40^{th} minute of the 90 minute session on this US reinstatement test day. Again, the initial 40 minutes of the 90 minute session served as an extinction period which allowed lever presses initiated by exposure to contextual stimuli to subside before the US reinstatement test. Natural reward seeking behavior elicited by the sucrose pellet was quantified from the number of responses on the active lever following the drug prime for 30 minutes immediately after the priming event (t=40-70 min).

A2.6. Drugs & Dosage Justification:

The NMDA receptor antagonist 3(-2 carboxipiperazin-4-yl) propyl-1-phosphonic acid ((±) CPP) and the coagonist D-serine were obtained from Sigma (St. Louis). D-serine and (±) CPP were administered in the home cage environment approximately 4 hours prior to the extinction sessions on protocol days 16-20. These compounds have long-lived effects when administered i.p. at the indicated doses (Hashimoto and Chiba, 2004; Hernandez et al, 1994), and therefore any state-dependent or acute locomotor effects were minimized by this advance treatment in the home cage environment. The D-serine dose of 100 mg/kg was chosen in order to avoid possible nephrotoxic effects at higher doses (Williams et al, 2003), while still affecting learning (Stouffer et al., 2004). The (±) CPP dose of 5mg/kg has been shown to disrupt synaptic plasticity processes in the hippocampal formation 12-24 hours following i.p. administration (Villarreal et al., 2002).

A2.7 Statistics:

The number of active lever presses, infusions and inactive lever presses were recorded for each session. These data were used to calculate the responses during each experimental session. A repeated measure ANOVA was applied to the data in figure 1B, and a 2-way repeated measures ANOVA was applied to figure 4. Planned comparisons were used for data analysis. A value of $p<.05$ was taken as significant. All the statistics were done using SigmaStat software.

A3. Results

Rats were trained to self-administer sucrose pellets in an operant chamber environment for 15 consecutive days. During the daily 90-minute sessions, rats were initially trained on an FR-1 schedule for first 12 days and switched to an FR-3 schedule for the last 3 days of self-administration training. This transition was done to increase the number of active lever pressing

responses. Animals achieved a stable self- administration of sucrose pellets by day 7 of training, and the transition to FR-3 schedule resulted in a slight dip in the learning curve, may be because the sucrose pellets are not as rewarding as cocaine (Fig. A2.1 A). This was a difference that we observed in the self-administration of cocaine vs. sucrose. On an average, each group tested in this study ingested about 50-60 pellets a day once the learning was stable. There was no significant difference in the pellet taking behavior of animals tested in this study.

After 15 days of self-administration training, the animals were subjected to either an extinction training procedure (extinction groups) in the operant chamber environment for 5 days (90 minute session on each day) in the absence of both CS and US or kept forcibly abstinent in their home cages for an equivalent period of time (abstinent group). Active and inactive lever presses were monitored during the extinction phase and the active lever presses are plotted in the second part of the figure 1 A (FigA2.1 A. days 16-20). It was found that the animals extinguish their sucrose pellet seeking behavior under these conditions. Active lever responses made on the last day of extinction were less than 20% of the responses made on the first day of extinction. This shows that the extinction training was effective in reducing the natural reward seeking behavior. This finding is consistent with our finding with the cocaine self-administered animals.

A3.2. **Reinstatement of sucrose pellet seeking behavior**

Evaluation of natural reward seeking behavior of animals within an extinction session illustrates that the majority of lever-pressing activity occurs during the initial 10 minutes in the operant chamber environment, suggesting that the environmental contextual cues are priming this response. Following this initial burst of activity, the lever pressing response diminishes rapidly as illustrated in figure A2.1B. The active lever responses are minimal by 20-30 minutes of the session and remain low for the rest of the session. This within session pattern was

observed on all extinction days (data not shown). Therefore, during the reinstatement experiments involving the non-contingent presentation of either CS or US stimuli, the priming event was delivered at time=40 min of the test session. A temporal distinction can thus be made between the natural reward seeking activity induced by introduction to the operant chamber environment (i.e. activity during the first ten minutes) versus the subsequent activity induced via non-contingent presentation of priming events delivered later within the same test session.

A3.3.The effects of extinction training versus enforced abstinence on natural reward seeking behavior.

The resumption of lever-pressing activity was induced using three forms of priming stimuli: contextual cues, conditioned cues, and sucrose pellet prime. Once the animals underwent either extinction training or enforced abstinence for a period of 5 days (days 16-20), they were tested for the resumption of natural reward seeking behavior following exposure to the contextual stimuli and the conditioned stimuli on day 21, and to the sucrose pellet prime on day 22.

The resumption of natural reward seeking behavior induced by diffuse environmental cues was assessed during the first ten minutes of the test session conducted on day 21 ((Figure A2.2A). The level of responding on the active lever in the extinguished (saline) group of rats during this period was significantly decreased ($p<0.001$, unpaired t-test) compared with responding during the same period in the abstinent (saline) group kept in the home cage environment for 5 days (10 ± 2.71 vs. 45.5 ± 8.17, respectively). These results suggest that the 5-day extinction training experience decreased the efficacy of the contextual cues present in the operant chamber environment to provoke natural reward seeking behavior. This finding with the sucrose pellet animals is consistent with that of the cocaine animals.

Until time=40 min of the day 21 reinstatement test session, the animals experienced extinction conditions. At this point, a single, non-contingent presentation of the discrete CS complex was delivered for a period of 30 seconds, and active lever responding for the next 10 min was measured as an indication of the reinstatement of natural reward seeking behavior evoked by the CS (FigureA2.2B). As previously described for extinction conditions, active lever presses had no programmed consequences at any time during these test sessions. In addition to the active lever presses, the inactive lever presses were also monitored, so as to ensure that the noncontingent CS prime was in fact inducing activity previously associated with cocaine infusion. In order to confirm that any responding due to contextual cues had subsided by the time of the CS reinstatement test, active lever pressing during a "pre-prime" ten minute period of time (t=30-40) was also assessed and compared with the cue-induced "post-prime" level of reinstatement (t=40-50). Results for both the extinguished (saline) and abstinent (saline) groups of rats tested in this manner are illustrated in figure A2.2B. These results indicate that the extinction was so effective in sucrose pellet animals that the non-contingent CS prime was not able to reinstate the reward seeking behavior in them, although a trend was evident for increased active lever presses in the abstinent animals. The post prime response among the abstinent animals was not significant as compared to the pre-prime response. This finding was against our finding with cocaine animals where the non-contingent CS was able to evoke the drug seeking behavior.

Sucrose pellet primed reinstatement was tested on the next day (protocol days 21) with a single, non-contingent sucrose pellet delivered at time=40 minute of the session. The active lever responding for the next 20 minutes was then measured as an indication of the reinstatement of drug seeking behavior evoked by the US stimuli. The results of the pellet prime are illustrated

in figure A2.2C. Among the extinguished animals, the pellet prime was not able to reinstate the natural reward seeking behavior as happened in the case of the CS prime. Because of the comparatively high pre-prime activity among abstinent animals, the post prime response did not turn out to be significant as compared to the pre prime response. But the comparison of the post prime response between the extinguished and abstinent group showed that the abstinent group responded significantly greater as compared to the extinguished ones ($p<.01$, unpaired 't' test).

A3.4 NMDAR involvement in the effects of extinction training on reinstatement behavior.

The role of NMDARs in the extinction process was also evaluated in two additional groups of extinguished rats treated with either D-serine (a coagonist of NMDAR at the glycine site), or (±) CPP (a competitive antagonist of NMDAR). These compounds were administered (D-serine: 100 mg/kg i.p., (±) CPP: 5 mg/kg i.p.) in the home cage environment prior to the extinction sessions (see Methods). In contrast to our findings with the cocaine animals, D-serine treatment prior to extinction did not facilitate extinction as compared to the saline group. Active lever presses for the entire 90 minute extinction session are shown in figureA2.3. Treatment with NMDAR drugs did not affect the extinction learning process in either way. By protocol day 20, the final extinction day, all groups exhibited similar levels of natural reward seeking behavior during the 90 minute session.

The resumption of lever-pressing activity induced by diffuse environmental cues is illustrated in Figure A2.4A during the first ten minutes of the test session conducted on protocol day 21. The level of responding on the active lever in the saline group of rats and both the D-serine and (±) CPP treated animals showed comparable levels of responding. Thus, neither positive modulation (w/D-serine) nor competitive antagonism (w/ (±) CPP) of NMDAR activity during extinction training had significant impact on the subsequently measured contextual

reinstatement as compared with the saline treated control group following a five-day extinction protocol.

In order to investigate the effects of NMDAR activity during extinction on the discrete CS-induced reinstatement, active lever pressing was again measured during a "pre-prime" ten minute period of time as well as the "post-prime" period (Figure A2.4B). Non-contingent CS prime was not able to reinstate the reward seeking behavior in animals trained to self-administer pellets irrespective of the treatments during their extinction phase.

Finally, sucrose pellet primed reinstatement was tested on the next day (protocol days 21) with a single, non-contingent delivery of the sucrose pellet at time = 40 min of the session. The active lever responding during the next 20 minutes was measured as an indication of the reinstatement of natural reward seeking behavior evoked by the US stimuli. The results are plotted in figure A2.4C. Non-contingent US (pellet) prime was not able to reinstate the reward seeking behavior in animals trained to self-administer sucrose pellets irrespective of the treatments with the NMDAR drugs during extinction. This was in contrast to the results with cocaine prime where cocaine was able to reinstate the drug seeking behavior in all the groups significantly. Further, The results with (±) CPP in cocaine animals showed that the ability of extinction to reduce drug primed reinstatement is dependent upon the activation of NMDARs during the training protocol.

In sum, altering NMDAR activity during extinction training did not affect the resumption of lever pressing activity induced by diffuse contextual cues, discrete CS cues or the US prime in sucrose pellet animals. In contrast to the findings in cocaine animals, in sucrose pellet animals, the CS cues or the US prime was not able to reinstate the natural reward seeking behavior among

the extinguished animals. Any effect of the treatment with NMDAR drugs during extinction could not be observed or explained because of this floor effect on the post prime response.

A4.Discussion

Similar to the study in chapter 1, we have directly assessed the effectiveness of an extinction training protocol to reduce primed reinstatement as compared with an equivalent period of enforced abstinence. This was a prerequisite step for us in pursuing our intent to investigate the mechanisms underlying the effectiveness of extinction training to reduce reinstatement. We found that contextual response in extinguished animals as significantly lower, compared to the abstinent ones (Fig.A2.2 A). Similarly, extinction was effective in reducing the reinstatement response to the sucrose pellet prime among the extinguished animals as compared to the abstinent animals (Fig A2.2C). With the non-contingent CS prime, although a trend was evident for a greater response in abstinent animals as compared to the extinguished ones, the results did not reach statistical significance. In sum, when compared to enforced abstinence, extinction training was effective in reducing the reinstatement response in sucrose pellet animals. This finding was consistent with our findings with cocaine animals.

The prime responses among the extinguished animals did not reach our expectations. The prime responses of the saline extinguished group itself were so low and it looked like none of the priming stimuli employed was able to trigger reinstatement. Since the responses from the control extinguished group faced this floor effect problem, the effect of pharmacological manipulation of NMDAR dependent synaptic plasticity mechanisms during extinction could not be elucidated from the responses of the treatment groups. Hence, we have to probably design a different kind of priming protocol (for example, increase the number of cue stimulus or pellets delivered for priming non- contingently) to overcome this floor effect. We have shown that the effects of

extinction in reducing the cocaine primed reinstatement response is dependent upon the NMDAR mediated synaptic plasticity mechanisms (Kelamangalath et al., 2007). With other behavioral paradigms, some investigators have shown that blocking the NMDAR activity during extinction by systemic administration of ±CPP will impair the recall of extinction, but not the actual learning process in extinction. In these cases, it is suggested that the NMDAR dependent mechanisms are important in consolidation of the memory of extinction (Santini et al., 2001; Suzuki et al., 2004). When we designed this study with natural reward, we wanted to see whether similar NMDAR dependent mechanisms operate in the effects of extinction in reducing the reinstatement response (in other words, the recall of extinction memory). Because of the floor effect on the reinstatement response among the control extinguished animals, nothing could be concluded from the reinstatement response of the animals treated with NMDAR drugs during extinction.

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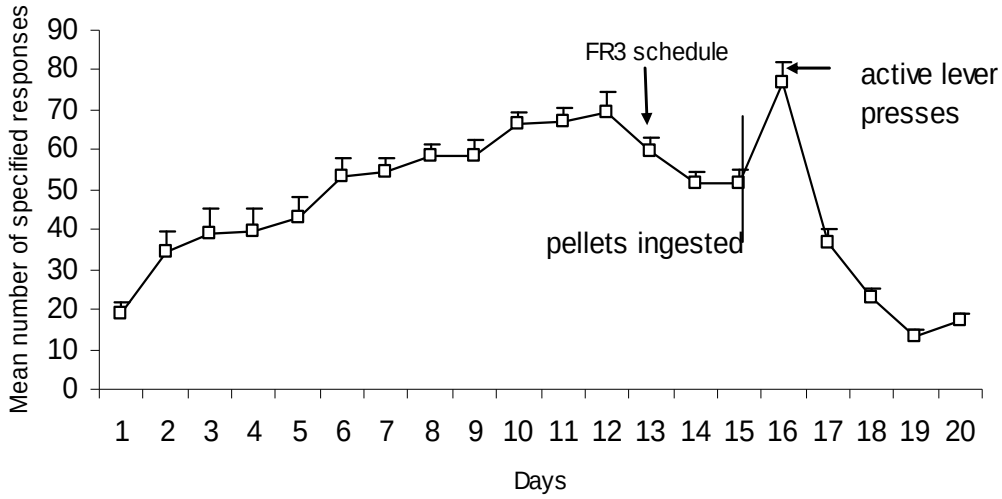

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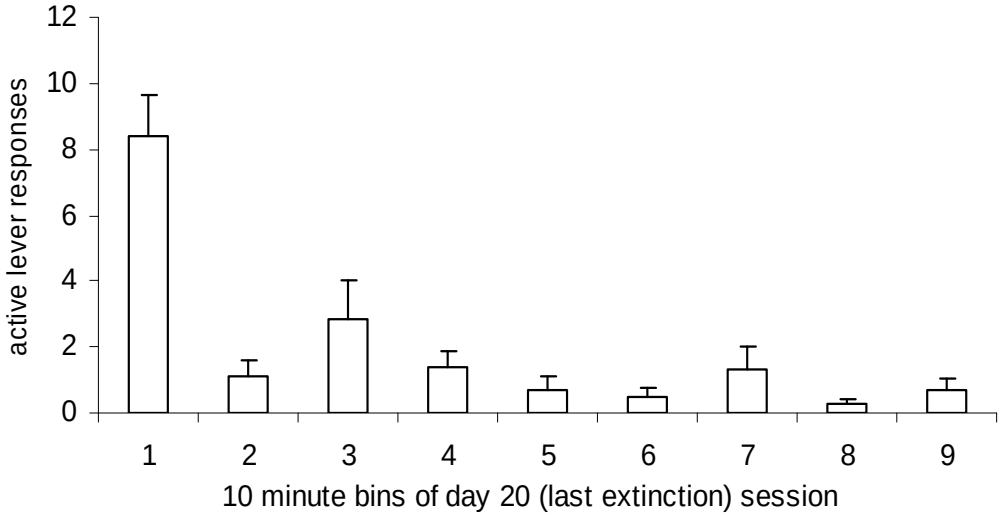


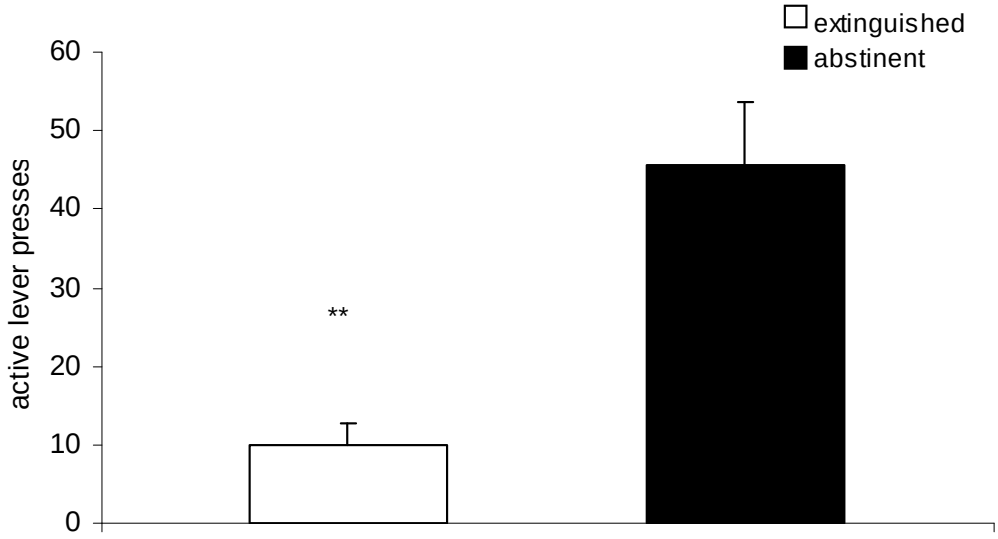
Figure A2.1. Self-administration of sucrose pellets and extinction training.

(A) The results from the sucrose pellet rats (n=24) illustrate the average number sucrose pellets taken daily during each 90 minute session during the SA phase (days 1-15). Transition to the FR3 schedule on day 13 resulted in a slight dip in the learning curve. The second part of this figure illustrates the total number of active lever presses (mean ± SEM) for the 90 minute extinction sessions on each day of extinction (days 16-20). In the absence of both CS and US the

animals extinguished their natural reward seeking behavior over a period of 5 days. Active lever presses on extinction days 2-5 were significantly decreased (** $p<.001$, RM ANOVA, Holm-Sidak) from those measured on the first day of extinction, (i.e. protocol days 17-20 vs. 16).

(B) Extinction behavior within a session on the day 5 of extinction is illustrated using 10 minute bins. Data are the mean± SEM of the active lever presses throughout the 90 minute session. Data shows that the majority of the activity occurs during the initial 10 minutes in the operant chamber environment suggesting that contextual cues are priming this activity which gets diminished later in the session.

A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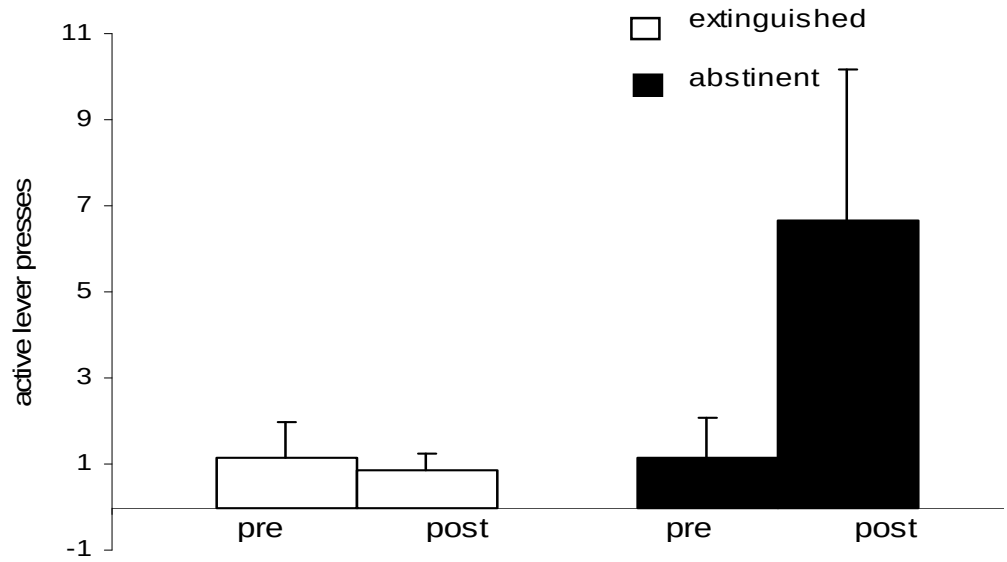
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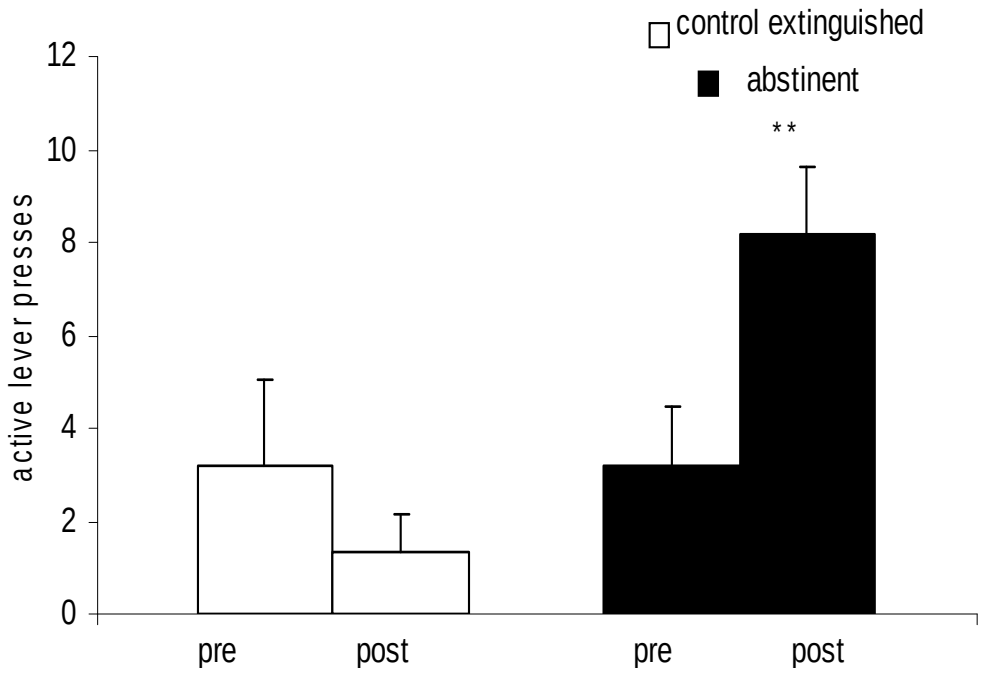


Figure A2.2. Active lever response on the reinstatement test days:

(A) Extinction training reduces the initial response to the contextual cues.

Data show the mean ± SEM of lever presses for the first 10 minute bin of the initial reinstatement test day (day 21) when the animals were placed back into the operant environment after either 5 days of extinction training (n=6) or 5 days of enforced abstinence (n=6). Natural reward seeking behavior on day 21 in response to diffuse contextual cues was significantly decreased in the extinguished group as compared to the behavior of the abstinent group (** $p<.01$, unpaired t-test).

(B) Reinstatement responses to the non-contingent CS prime.

Data show the mean ± SEM of active lever presses for10 minute period before the CS prime (labeled as pre) and 10 minute period after the CS prime on the first reinstatement test day (day 21) for the extinguished (n=6) and the abstinent groups (n=6). Lever pressing responses during the "pre priming" period (30-40 min) were minimal. The delivery of a single, non-contingent CS

prime (light and tone) at time=40 minutes was not able to reinstate the active lever response during the "post prime" (40-50 min) period significantly in both the groups.

(C) Reinstatement response to non-contingent US (sucrose pellet) prime.

Data represent the mean ± SEM of active lever presses for a 20 minute window before (pre, 20-40 min) and after (post, 40-60 min) the single, non-contingent delivery of a sucrose pellet prime at time=40 minutes on the protocol day 22. In both the extinguished and abstinent groups, sucrose pellet prime was not able to reinstate the active lever response (post prime) as compared to their pre-prime responses. A trend for increased number of active lever presses during the post prime period was evident among the abstinent group. The post prime response among the abstinent group (n=6) was significantly greater (**$p<.01$, unpaired 't' test) than the post prime response of the extinguished group (n=6).

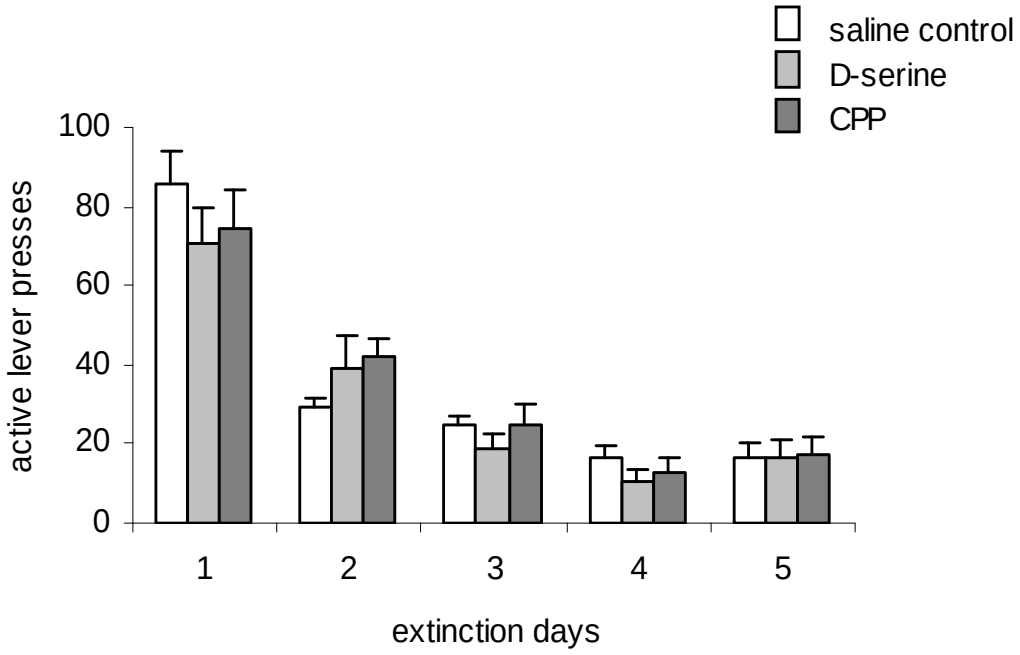
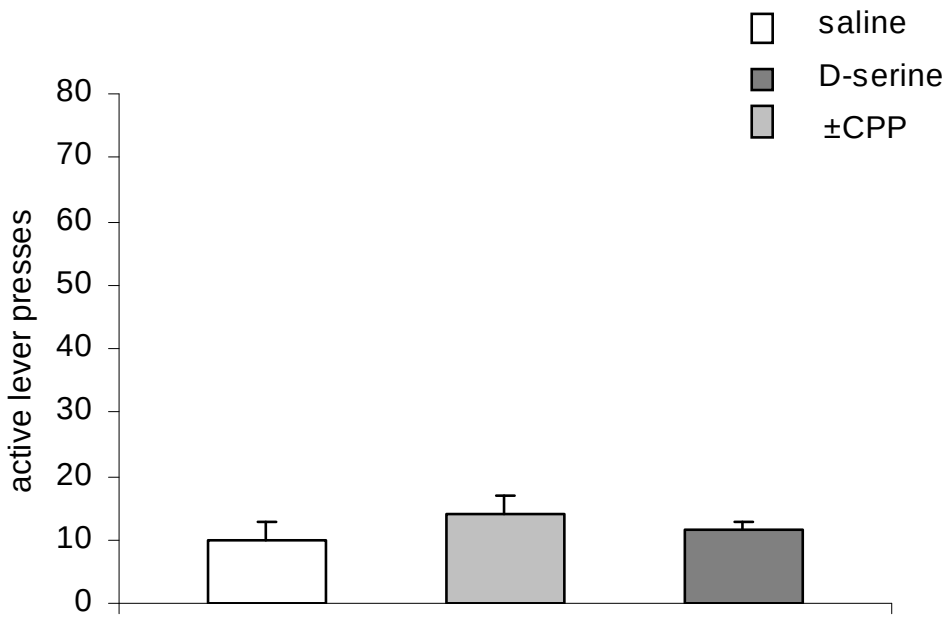


Figure A2.3. **Influence of NMDAR activity on the progression of extinction.**

Data shows the mean ± SEM of active lever presses for the entire 90 minute extinction sessions on protocol days 16-20. Active lever pressing behavior was extinguished across the 5 daily extinction sessions and responding on extinction days 2-5 was significantly decreased ($p<.001$, 2-way RM ANOVA, Holm-Sidak) from that measured on the first day of extinction (i.e. protocol days 17-20 vs. 16) within each treatment group. Treatment with D-serine (n=6) or ± CPP (n=6) during extinction did not influence the progression of extinction as compared to the saline treated control group.

A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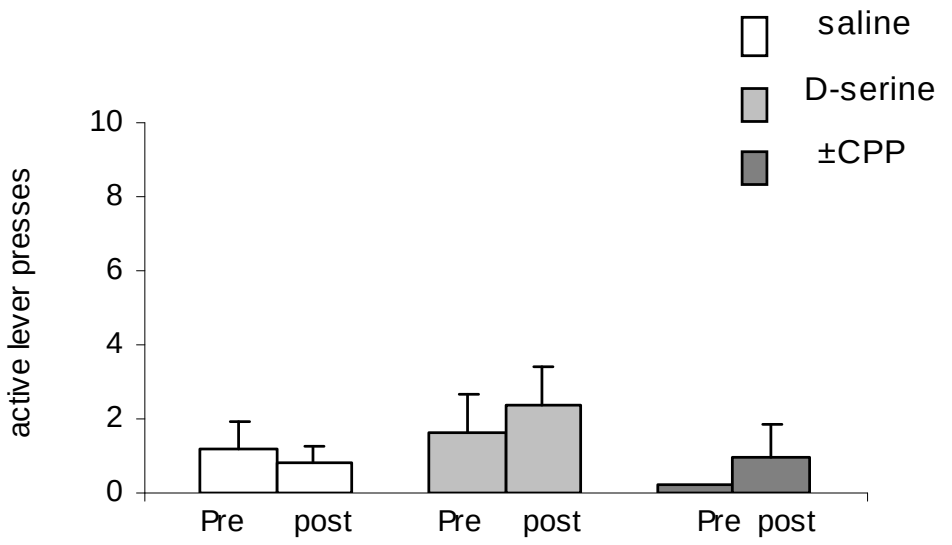
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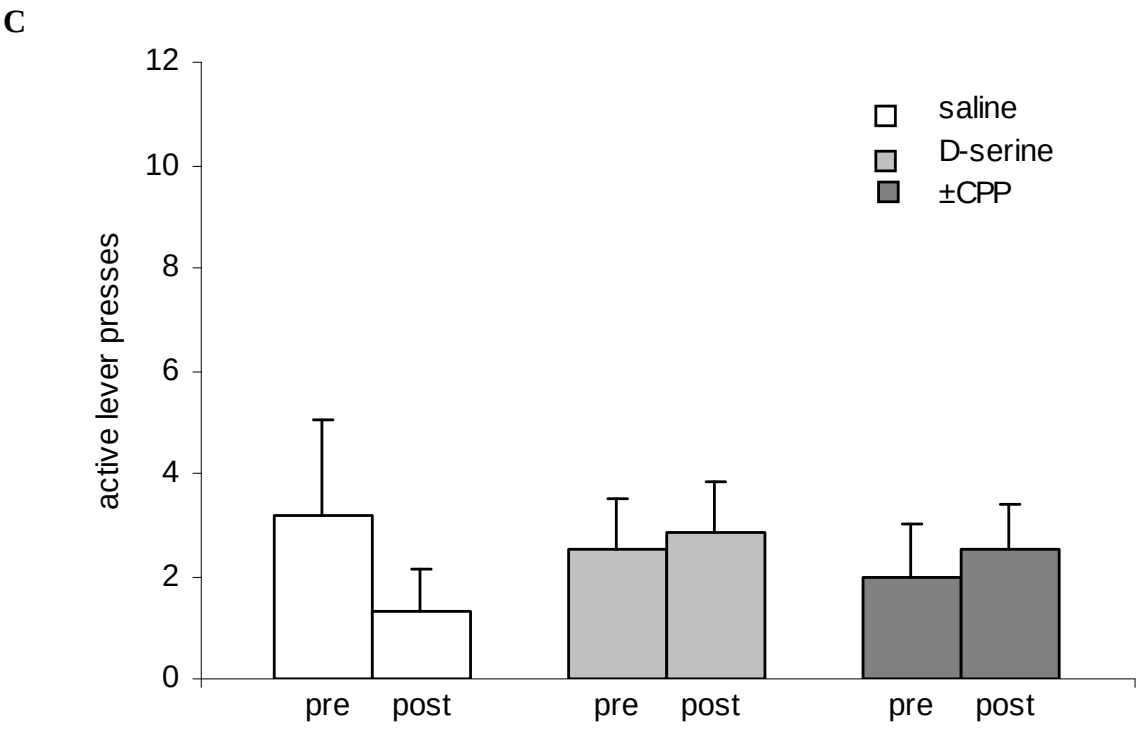

Figure A2.4. Effects of treatment with NMDAR drugs during extinction on the reinstatement response:

(A) Effects of treatment with NMDAR drugs during extinction on the contextual response.

Altering NMDAR activity during extinction had no effect on the resumption of pellet-seeking following exposure to contextual cues. Data show the mean ± SEM of lever presses for the first 10 minute bin of time on the first reinstatement test day (protocol day 21). Neither D-serine (100 mg/kg i.p., n=6) nor (±)CPP (5 mg/kg i.p., n=6) treatment during extinction training had a significant effect on responding as compared with the saline treated control group.

(B) Effects of treatment with NMDAR drugs during extinction on the CS primed response.

Altering NMDAR activity during extinction had no effect on the resumption of pellet-seeking following exposure to the non-contingent CS prime. Data show the mean ± SEM of lever presses for the 10 minute bin before the CS prime (pre) and 10 min bin after the CS prime (post) on the first reinstatement test day (protocol day 21). Non-contingent CS prime was not able to reinstate

the natural reward seeking behavior in any of the extinguished groups. Neither D-serine (100 mg/kg i.p., n=6) nor (±) CPP (5 mg/kg i.p., n=6) treatment during extinction training had a significant effect on responding as compared with the saline treated control group.

(C) Effects of treatment with NMDAR drugs during extinction on the US primed (sucrose pellet prime) response.

Altering NMDAR activity during extinction had no effect on the resumption of pellet-seeking following exposure to the non-contingent US prime. Data show the mean ± SEM of lever presses for the 20 minute bin before the US prime (pre) and 20 min bin after the US prime (post) on the second reinstatement test day (protocol day 22). Non-contingent US prime was not able to reinstate the natural reward seeking behavior in any of the extinguished groups. Neither D-serine (100 mg/kg i.p., n=6) nor (±) CPP (5 mg/kg i.p., n=6) treatment during extinction training had a significant effect on responding as compared with the saline treated control group.

CHAPTER 3

FACILITATION OF THE ACTIVITY OF NMDA RECEPTORS DURING EXTINCTION ATTENUATES COCAINE-INDUCED REINSTATEMENT IN SELF-ADMINISTRATION MODEL.

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Abstract

We have previously shown that the systemic administration of a competitive antagonist (± CPP) of the N-methyl D-aspartate receptor (NMDAR) during extinction reduce the effectiveness of extinction training on cocaine induced reinstatement response (Kelamangalath et al., 2007). In this report, we demonstrate that facilitating the NMDAR mediated mechanisms during extinction by the systemic administration of a full agonist at the strychnine insensitive glycine site of NMDAR complex, D-serine at 100mg/kg prior to and immediately after extinction enhance the effectiveness of a sub-maximal extinction experience on the cocaine induced reinstatement response.

1. Introduction:

Vulnerability to relapse is one of the common formidable challenges in the treatment of drug addiction. Drug associated environments, discrete conditioned stimuli and exposure to cocaine elicit craving in addicts (Foltin and Haney, 2000; Ehrman et al., 1992). As different neurobiological substrates are implicated in relapse induced by different stimuli (Shalev et al., 2002; Kalivas and McFarland, 2003; Sun and Rebec, 2003; Fuchs et al., 2005; Fuchs et al., 2006), different molecular mechanisms might be responsible for relapse induced by different stimuli. Extinction therapy in the form of cue exposure treatments has been utilized in treatments across most drugs of abuse (Ehrman et al., 1998, Drummond and Glautier, 1994; Raw and Russel, 1980), but the clinical success of these treatments has been less than promising leading some researchers to investigate other behavioral techniques to be used in combination with cue exposure treatment to improve the effectiveness of such treatments (Cooney et al., 1983). Though initially extinction was conceptualized as unlearning, now extinction is almost proved to involve new learning (Bouton, 2004). This new learning can be dependent upon the activation of

N-methyl-D-aspartate receptors (NMDARs), and therefore either blocking NMDARs with antagonists or enhancing NMDAR activity with coagonists would be expected to affect the ability of an extinction training experience to alter the response to primed reinstatement. For example, conditioned fear has been used to demonstrate that NMDAR antagonists administered prior to extinction sessions can significantly inhibit extinction (Baker and Azorlosa, 1996; Falls et al., 1992; Santini et al.,2001), and recent reports have indicated that treatment with D-cycloserine (DCS, an NMDAR coagonist) can facilitate extinction of conditioned fear (Ledgerwood et al.,2003; Walker et al., 2002). Together, these findings indicate an involvement of NMDARs in the learning process that occurs during an extinction training experience. Recent research with human participants suggests that D-cycloserine (DCS) can enhance exposure therapy (extinction) of acrophobia (Ressler et al., 2004) and social phobia (Hofmann et al., 2006). All these observations indicate that this drug can enhance the effectiveness of extinction therapy.

To date, as far as we are aware, in the addiction literature, the effects of D-serine in facilitating the extinction and thus reducing the chances of relapse have not been documented using the self-administration model. The closest we could find in the addiction literature are some findings based on the conditioned place preference paradigm using D-cycloserine. This study using D-cycloserine in extinction of the conditioned place preference was the first one to show that, D-cycloserine's facilitatory effect on extinction is not only limited to aversive conditioning, but can be applied to appetitive conditioning as well (Botreau et al., 2006). They found that systemic administration of D-cycloserine immediately after extinction training had a facilitatory effect on extinction, but not when administration was delayed for 4 hours after

training, confirming the role of DCS in memory consolidation. They also showed that intra-amygdalar infusions of DCS were able to block conditioned place preference completely.

D-serine is one of the endogenous coagonists at the strychnine insensitive glycine site of the NMDAR complex. D-serine is described as a full agonist of the NMDAR while D-cycloserine is described as a partial agonist at the same site. The affinity of D-serine to this glycine site on the NMDAR is about 34-fold as compared to D-cycloserine (Furukawa and Gouaux, 2003). D-serine is being tested in schizophrenia models and subjects where a hypofunction of the NMDARs are reported. The administration of D-serine is reported to improve the negative, positive and cognitive symptoms of schizophrenic subjects (Tsai et al., 1998). With these reasons in mind, and because it can be used at a relatively high dose without causing neurotoxicity, we were inclined to use D-serine instead of D-cycloserine in our study. In our previous report, we have shown that blocking the NMDARs during extinction with a competitive antagonist of NMDAR, (±) CPP impaired the effectiveness of extinction in reducing the drug primed reinstatement. We were not able to see an effect of the treatment with D-serine during extinction on the reinstatement due to the overtraining effect of extinction training. Hence, we hypothesized that D-serine treatment along with a sub-maximal level of extinction training would be able to delineate the effects of facilitation of NMDAR activity during extinction on reinstatement, in particular the drug induced reinstatement. We reduced the number of extinction training days from 5 to 1 and altered the reinstatement test to assess different forms of priming stimuli (contextual drug stimuli, non-contingent CS and the single non-contingent intravenous drug prime) at different time points of a single 120 minute session. We have found that the systemic administration of D-serine prior to extinction at a dose of 100mg/kg did not affect the extinction learning process as compared to the saline treated control. During the

reinstatement test, we observed that the treatment with D-serine prior to and post extinction did not affect the reinstatement to the contextual drug stimuli and the single non-contingent CS prime, but enhanced the effectiveness of extinction training to reduce the drug induced reinstatement. The observations from this study complemented our findings from the first study with the NMDAR antagonist and add support for the involvement of NMDAR mediated mechanisms in the effects of extinction on drug induced reinstatement in the self-administration and reinstatement model of cocaine relapse. The findings from this study support the idea of facilitating the NMDAR mediated mechanisms during extinction as a promising adjunct pharmacotherapy to psychotherapy in preventing the occurrence of relapse.

2. Materials and Methods:

2.1. Animals:

Male Sprague-Dawley rats (Harlan) weighed approximately 300 g at the beginning of the experiment and were housed individually in a temperature and humidity controlled vivarium having a 12 hour light/dark cycle (lights off at 7:00 P.M.). They were given access to food and water *ad libitum* and were handled daily for 5 days prior to the surgery in order to diminish stress associated with handling. The housing and experimental procedures followed the *Guide for the Care and Use of Laboratory Animals* and were approved by the local ACUC at the University of Georgia.

2.2. Jugular catheterization protocol:

The animals were anesthetized using a combination of ketamine (70 mg/kg), xylazine (10 mg/kg) and acepromazine (1 mg/kg) administered i.p. Depth of anaesthesia was assessed by monitoring respiration rate and palpebral and pedal withdrawal reflexes. Under anesthesia, the right jugular vein was isolated. The catheter was exteriorized by passing it subcutaneously to the

base of the skull, where it was connected to a modified 22 gauge cannula. A silastic catheter (Dow Corning) was then inserted into the vein (4-5 cm) and secured in position with silk sutures (6/0). The animal was then placed in a stereotaxic frame (Stoelting), where the right-angled cannula (Plastics One) was mounted to the top of the skull using dental cement and 4 screws. Immediately after surgery and once daily for 5 days, the animals were treated with gentamicin at a dose of 5 mg/kg, i.v. The catheters were flushed every day with saline prior to each self-administration session and with heparin (10 USP/ml) after the session to maintain the patency of the catheter. Catheter patency was verified daily by drawing blood from the catheter.

2.3. Self-administration environment:

The operant chambers (Med associates) were equipped with 2 levers, one "active" and another "inactive" with lights positioned above each lever. The chambers had a rod grid floor, a house light, a speaker/tone generator (2.9 kHz, 10 dB above ambient) and were housed inside enclosures equipped with ventilation fans. A syringe pump was located outside the enclosure. The method for delivering a cocaine infusion was as follows: The modified 22 gauge cannula mounted on the rat's skull was connected to a liquid swivel with PE-50 tubing protected by a metal spring. The swivel was connected with tygon tubing to the syringe mounted in the infusion pump. Infusion volumes were calculated according to the animal's weight. For cocaine animals, the syringes mounted in the infusion pump contained cocaine hydrochloride (NIDA) dissolved in normal saline at 4 mg cocaine/ml of solution. Each infusion delivered an infusion volume of 0.125 ml/kg body weight, hence the dose of cocaine self-administered was 0.5 mg/kg/infusion. The MED-PC software program recorded the number of active lever presses, inactive lever presses and the number of infusions.

2.4. Self-administration protocol (days 1-15):

The animals having patent indwelling catheters were subjected to self-administration training for a period of 15 days with one session each day. Self-administration training sessions were 90 minutes in duration. Upon entry into the self-administration environment, the house light and the ventilation fan were on. In addition to triggering an infusion, active lever presses had the following programmed consequences: the house light was turned off, and the active lever light/tone (i.e. the CS) was turned on for a period of 30 seconds. Additional responses on the active lever during this 30 second period had no programmed consequences, although the program continued to count the number of active/inactive lever presses and infusions. This "timeout" period protected the animals from cocaine overdose. After this 30 second period the lever light and tone were terminated and the house light came back on. Rats were initially trained for 12 days on an FR-1 (fixed ratio schedule-1) schedule in which each active lever press outside the timeout period triggered the programmed consequences. For the last 3 days of self-administration training, an FR-3 schedule was imposed where 3 active lever presses outside the time out period were required to trigger an infusion and the CS. Each rat was placed in the same operant conditioning chamber throughout the course of the experiment.

2.5. Extinction protocol (day 16):

After the 15 days of self-administration training, the animals were divided into 4 groups (balanced for cocaine intake): 1) extinguished (saline), 2) extinguished (Dserine-pre extinction), 3) extinguished (D-serine-post extinction), 4) abstinent (saline) and 5) abstinent (D-serine). All groups received i.p. injections of their respective treatments in their home cage environment. Both the extinguished group 1 and the abstinent group 4 received injections of saline (1ml/kg). Group 2 received an injection of D-serine (100 mg/kg) pre-extinction and group 3 received an

injection of D-serine (100 mg/kg) post-extinction. Group 5 received D-serine i.p. (100mg/kg) on that one day of abstinence in the home cage environment. Groups 1 and 2 underwent extinction training 2-3 hours following their respective daily pharmacological treatments, whereas group 4 and 5 rats remained in their home cages. Group 3 rats received an injection of D-serine immediately after extinction training. During their extinction training sessions, the animals in the operant chambers were attached to the drug tether but exposed only to the environment stimuli (i.e. diffuse, contextual cues). Responses on the active lever had no programmed consequences during the extinction training phase. For protocol day 16, responses on both active and inactive levers, as well as the equivalent "number of infusions" were counted by the software (although as stated above, syringe pumps were not activated during this phase of training). Extinction proceeded for only a single day, with one 90 minute session during which the animals in the extinction training groups 1-3 were taken to the operant chambers. Under these conditions, the animals extinguished their lever pressing behavior to less than 20% of their former activity during self-administration. As previously mentioned, group 4 and 5 abstinent animals remained in their home cages for the day 16.

2.6. Reinstatement tests (protocol days 17):

On day 17, all the animals (including the home cage abstinent animals), were placed back in the operant chambers for reinstatement tests. The reinstatement test session conditions were similar to an extinction session in that the animals were exposed only to the contextual cues of the operant chamber environment and the active lever responding were not reinforced by the contingent availability of either CS or US.

Reinstatement to the contextual drug stimuli: On test day 17, response to the contextual prime was assessed from active lever presses during the first 10 minutes in which the animals were exposed only to the contextual cues of the drug environment.

Reinstatement to the CS cues: Later during the same test session on day 17, lever presses evoked in response to a CS presentation were then assessed. A single, noncontingent presentation of the CS was delivered at the 40th minute of the 120 minute test session. Thus the initial 40 minutes of the 120 minute session served as an extinction period to allow lever presses initiated by exposure to contextual stimuli to subside before the CS reinstatement test. As the CS was expected to evoke an immediate response from animals, the noncontingent CS was quantified as the number of lever presses during the subsequent 10 minutes following the priming event (t=40-50 min).

Reinstatement to the drug prime: Response to the drug prime stimulus was assessed later in the same 120 minute session on day 17. We tested the reinstatement of drug seeking behavior using 0.5 mg/kg of cocaine at time=80 minutes of the 120 minute session by delivering a single non-contingent intravenous cocaine infusion. Again, the 30 minutes interval after the CS prime served as an extinction period which allowed lever presses initiated by exposure to contextual stimuli and the CS prime to subside before the US reinstatement test. Drug seeking behavior elicited by cocaine was quantified from the number of responses on the active lever following the drug prime for 30 minutes immediately after the priming event (t=80-110 min).

2.7. Drugs & Dosage Justification:

Cocaine hydrochloride was a gift from NIDA (RTI). The NMDA receptor coagonist D-serine was obtained from Sigma (St. Louis). D-serine was administered in the home cage environment approximately 2-3 hours prior to the extinction sessions on protocol days 16. For

the post-extinction group, the drug was administered immediately after extinction training. D-serine has long-lived effects when administered i.p. at the indicated doses (Hashimoto and Chiba, 2004), and therefore any state-dependent or acute locomotor effects were minimized by this advance treatment in the home cage environment for the pre-extinction treatment group. The D-serine dose of 100 mg/kg was chosen in order to avoid possible nephrotoxic effects at higher doses (Williams et al., 2003), while still affecting learning (Stouffer et al., 2004).

2.8. Statistics: The number of active lever presses, infusions and inactive lever presses were recorded for each session. These data were used to calculate the responses during each experimental session. ANOVA was applied to the data to find an effect of treatment and/or condition (extinction vs. abstinence). The two factors taken into consideration for the 2- way ANOVA were: 1) either trial (pre vs. post priming responses) or days and 2) either condition (extinction vs. abstinence) or treatment, as the case may be. A value of $p<.05$ was taken as significant, being determined from the post hoc test or planned comparisons. All the statistics were done using SigmaStat software.

3. Results

3.1. Cocaine self-administration and extinction of the drug seeking behavior:

Animals having indwelling jugular catheters were trained to self-administer cocaine in an operant chamber environment for 15 consecutive days. During the daily 90-minute sessions, rats were initially trained on an FR-1 schedule for first 12 days and switched to an FR-3 schedule for the last 3 days of self-administration training. The transition to the FR-3 schedule was done to increase the number of active lever pressing responses. Animals typically achieved stable self-administration by day 10 of training, and the FR-3 schedule did not significantly alter the number of earned infusions per session (Figure 3.1). Over the entire fifteen day self-administration

training phase the average total number of infusions earned was 340 ± 16, or the equivalent of approximately 11.3 mg/kg/day of cocaine per animal. There was no significant difference in the average number of infusions earned per animal among the five different groups of self-administration rats utilized for the reinstatement studies described in this report (data not shown).

After 15 days of SA, the animals were subjected to either an extinction training phase (extinction groups) in the same operant chamber environment for 90 minute session in the absence of both CS and US (i.e. the active lever had no programmed consequences) for only one day (Figure 3.2 A, protocol day 16), or they were kept forcibly abstinent in their home cage environment (abstinent group). Active and inactive lever presses were monitored during the extinction sessions and it was found that the animals extinguish their drug seeking behavior under these conditions.

3.2. Facilitation of NMDAR activity in the effects of extinction training on reinstatement behavior.

The role of NMDARs in the extinction process was also evaluated in two groups of extinguished rats, one group treated with D-serine (a coagonist of NMDAR at the glycine site) at a dose of 100 mg/kg i.p in the home cage environment prior to the extinction session (see Methods) and the control group treated with saline i.p before extinction training in the similar manner. The animals were subjected to only one day of extinction training (one 90 minute extinction session) so as to give a sub-maximal level of extinction for the animals in this study. The lever responses during the extinction session are illustrated in figure 3.2A. D-serine treatment prior to extinction (facilitating the NMDAR activity during the extinction) training did not enhance the extinction learning process as compared to the saline treated control group. There was one more extinction group in this study which received the D-serine treatment at the

same dose (100mg/kg i.p.) immediately after the extinction training. The extinction response from this group is also plotted in figure 3.2A so as to show that, there was no significant difference in the drug seeking behavior (during extinction) of the animals tested in this study as compared to the saline controls.

3.3. Reinstatement of drug seeking behavior:

Evaluation of drug seeking behavior within this single extinction session illustrates that the majority of lever pressing activity occurs during the initial ten minutes in the operant chamber environment, suggesting that environmental contextual cues are priming this response. Following this initial burst of activity, active lever pressing diminishes rapidly. As illustrated in the figure 3.2B, active lever responses are minimal (< 4) by 20-30 minutes and remain low for the remainder of the 90 minute session. Therefore, during the reinstatement experiments involving the non-contingent presentation of either CS or US stimuli, the first priming event was delivered after waiting for 40 minutes of the test session. A temporal distinction can thus be made between the drug-seeking activities induced by introduction to the operant chamber environment (i.e. activity during the first ten minutes) versus the subsequent activity induced via non-contingent presentation of priming events delivered later within the same test session.

3.4. Effects of D-serine administration before and after extinction training on the reinstatement behavior.

The resumption of lever-pressing activity was induced using three forms of priming stimuli: contextual cues, conditioned cues, and drug prime. Once the animals underwent either extinction training or enforced abstinence for a day (day 16), they were tested for the resumption of drug seeking behavior following exposure to the contextual stimuli and the conditioned stimuli and to cocaine on day 17 at different time points of the same test session.

3.5. Effects of facilitating NMDAR activity during extinction training and post extinction training on the contextual reinstatement

The resumption of drug seeking induced by diffuse environmental cues was assessed during the first ten minutes of the test session conducted on day 21 ((Figure 3.3A). The level of responding on the active lever in the extinguished (saline and D-serine (pre and post extinction)) group of rats during this period was significantly decreased ($p<0.001$, one way ANOVA) compared with responding during the same period in the abstinent (saline and D-serine) group kept in the home cage environment for one day. These results suggest that the single day of extinction training experience decreased the efficacy of the contextual cues present in the operant chamber environment to provoke drug seeking behavior. Among the extinguished groups, the contextual response observed in the saline treated control group and the group treated with D-serine (prior to extinction and post extinction) was not significantly different from each other.

3.6. Effects of facilitating NMDAR activity during extinction training and post extinction training on the CS induced reinstatement

Until time=40 min of the day 21 reinstatement test session, the animals experienced extinction conditions. At this point, a single, non-contingent presentation of the discrete CS complex was delivered for a period of 30 seconds, and active lever responding for the next 10 min was measured as an indication of the reinstatement of drug seeking behavior evoked by the CS (Figure 3.3 B). As previously described for extinction conditions, active lever presses had no programmed consequences at any time during these test sessions. In addition to the active lever presses, the inactive lever presses were also monitored, so as to ensure that the non-contingent CS prime was in fact inducing activity previously associated with cocaine infusion. Inactive lever presses averaged to less than 1 in the post prime responses of all the groups (data not

shown). In order to confirm that any responding due to contextual cues had subsided by the time of the CS reinstatement test, active lever pressing during a "pre-prime" ten minute period of time (t=30-40) was also assessed and compared with the cue-induced "post-prime" level of reinstatement (t=40-50). Results for both the extinguished (saline and both the D-serine treated groups) and abstinent (saline and D-serine) groups of rats tested in this manner are illustrated for 10 minutes before and following the CS priming event. Paired 't' tests within each group indicates that the non-contingent CS prime was able to reinstate the drug seeking behavior (active lever response) in all the groups except the group treated with D-serine during abstinence.

Table 3.1. Results of the paired't' tests (post-prime vs. pre-prime) for the non-contingent cue prime response within each group.

Saline-treated control extinction	**Pre-extinction D-serine**	**Post-extinction D-serine**	**D-serine abstinent**	**Saline-treated control abstinent**
6.58 ± 1.53 vs. 1.16 ± 0.51	5.5 ± 1.47 vs. 0.86 ± 0.71	3.47 ± 0.71 vs. 0.53 ± 0.53	4.43 ± 1.28 vs. 1.93 ± 0.69	6.93 ± 2.43 vs. 1.57 ± 0.68
$P < 0.01$	$P < 0.01$	$P < 0.001$	$P = .097$	$P < 0.05$

Comparisons of the post-prime responses between different groups did not reveal a significant effect of treatment or condition (extinction or abstinence). This indicates that one day of extinction was not effective in reducing the reinstatement response as compared to the equivalent period of enforced abstinence in any of the extinguished groups. The lack of a significant effect among both the D-serine treated extinguished groups as compared to the saline treated control extinguished group indicates that facilitating the NMDAR activity during extinction learning or post extinction learning did not alter the drug seeking behavior evoked by the non-contingent CS prime significantly.

3.7. Effects of facilitating NMDAR activity during extinction training and post extinction training on the US induced (cocaine prime) reinstatement

Finally, cocaine-induced reinstatement was tested at time=80 minutes of the reinstatement test session on day 21. A single, non-contingent intravenous infusion of drug was delivered at a dose of 0.5mg/kg at time=80 min of the session. The active lever responding during the next 30 minutes was measured as an indication of the reinstatement of drug seeking behavior evoked by the US stimuli. The postprime response (80-110 min) on the active lever was compared to the 30 minute preprime response (50-80 min) to assess the reinstatement within each group as illustrated in figure 3.3 C. Inactive lever presses averaged to less than 1 in the post prime responses of all the groups (data not shown).

The analysis of the data by two way- ANOVA at the priming dose of 0.5mg/kg showed an effect of trial, F_{trial} (1, 130) = 89.821 and treatment, ($F_{treatment}$(4, 30)=5.61. Two-way ANOVA was done by ignoring the factor "condition" (extinction vs. abstinence) which was of low interest for this particular experiment. Paired 't' tests within each group indicates that the non-contingent US prime was able to reinstate the drug seeking behavior (active lever response) in all the groups except the group treated with D-serine during the post extinction period.

Table 3.2.Results of the paired 't' tests (post-prime vs. pre-prime) for the non-contingent cocaine prime response within each group.

Saline treated Control extinction group	**Pre-extinction D-serine group**	**Post-extinction D-serine group**	**D-serine abstinent group**	**Saline treated Control abstinent group**
16.313±2.42 vs. 1.88±0.57	9.15±2.14 vs. 3.33±0.85	7.6±2.65 vs. 2.06±0.81	16.25±2.00 vs. 2.67±0.80	21.29±2.86 vs. 4.36±1.24
P<.001	*P*<.05	*P*=.084	*P*<.001	*P*<.001

Table 3.3. The effect of D-serine treatment (pre and post extinction) on drug prime response-Planned comparisons (unpaired‘t’ tests) of the post-prime responses.

Control abstinent (Saline) 21.2857±2.856	**D-serine abstinent** 16.25±2.003	**Control (saline) extinguished** 16.3125±2.42	**D-serine pre-extinction treatment** 9.1538±2.144	**D-serine post-extinction treatment** 7.6±2.6525
D-serine abstinent 16.25±2.003		*P*=.981	*P*<.05	*P*<.01
Control (saline)extinguished 16.3125±2.42	*P*=.981		*P*<.01	*P*<.01
D-serine pre-extinction treatment 9.1538±2.144	*P*<.01	*P*<.01		*P*=.56
D-serine post-extinction treatment 7.6±2.6525	*P*<.01	*P*<.01	*P*=.56	
Control abstinent (Saline) 21.2857±2.856	*P*=.071	*P*=.055	*P*<.001	*P*<.001

Planned comparisons of the post-prime responses of the saline treated control groups, that is the comparison between the saline extinction group and the saline abstinent group was not significant indicating that one day of extinction training was not effective in reducing the reinstatement response to the drug prime as compared to the saline treated abstinent group. Planned comparison of the post prime responses between the saline treated control groups (both extinguished and abstinent) and the D-serine treated extinguished groups (both the groups treated with D-serine prior to extinction and postextinction) showed significance. This indicates that facilitating the NMDAR activity during extinction and post extinction enhances the effects of

extinction in reducing the drug primed reinstatement. Comparison of the postprime responses of both the D-serine treated extinguished groups with that of the D-serine treated abstinent group also showed statistical significance indicating the advantage of combining D-serine treatment along with extinction as compared to the D-serine administration alone during abstinence. The post prime response of the D-serine treated abstinent group was not significantly different as compared to the saline treated control abstinent group supporting the idea that the D-serine treatment independent of extinction training does not have an effect in reducing the drug primed reinstatement. There was no significant difference observed between the postprime responses of the group treated with D-serine prior to extinction and the group treated with D-serine post extinction indicating that facilitating the NMDAR function in general along with extinction training can enhance the effectiveness of extinction training in reducing the drug primed response.

In sum, facilitation of NMDAR activity along with the extinction training could not enhance the effectiveness of extinction training on the reinstatement induced by the contextual drug stimuli or the non-contingent CS prime, but could enhance the effectiveness of extinction in reducing the drug primed response.

4. Discussion

In this study we have found that facilitating the NMDAR mediated synaptic plasticity mechanisms during extinction training and postextinction training can enhance the effectiveness of extinction training in reducing the drug-induced reinstatement. However, this effect was not observed on the reinstatement response induced by the contextual drug stimuli and the non-contingent CS prime. D-serine treatment independent of extinction training could not reduce the drug-induced reinstatement response (as in D-serine treated abstinent animals) and this

demonstrates that D-serine is effective in reducing the drug primed reinstatement only when combined with extinction training. Extinction likely involves new learning (Bouton et al., 2004; Rescorla and Heth, 1975), and at the molecular level, both NMDAR and non-NMDAR dependent forms of synaptic plasticity are thought to contribute to this type of learning.

In our previous study we have already shown findings supporting the above concept. We have shown that systemic administration of an NMDAR antagonist, (±) CPP during extinction does not impair the acquisition of extinction, but affects the recall of extinction when tested on a drug free day (Kelamangalath et al., 2007). Similar findings have been reported by other investigators with systemic administration of the NMDAR antagonist, (±) CPP during extinction, supporting the role of NMDAR mediated mechanisms in consolidation of memory of extinction (Santini et al., 2001; Suzuki et al., 2004). These investigators utilized a massed extinction trial to avoid extinction training over several days so that they will be able to distinguish between the impairments in acquisition vs. memory consolidation. In such cases, where administration of a drug prior to extinction does not affect the extinction learning as such, but affects the recall of the memory of the extinction learning later when tested, the drugs are supposed to affect the consolidation of the memory of extinction learning (Quirk and Muller, 2007). When extinction trial was carried out over several days in the presence of a non-competitive antagonist of NMDAR, MK-801, it was reported to impair the acquisition of extinction learning. In this case, it was not possible to distinguish between the impairments in acquisition vs. consolidation of extinction memory (Baker and Azorlosa, 1996; Cox and Westbrook, 1994). Similarly, in our previous study, extinction trial was carried out for 5 consecutive days and here the D-serine group showed a significant enhancement in the rate of extinction learning and we were not sure whether D-serine was affecting the acquisition or consolidation or both. The results of the

reinstatement response were also not significantly different from the extinguished control group, possibly because of the overtraining effect due to 5 days of extinction (Kelamangalath et al., 2007). Hence, it was important in this study to adopt a sub-maximal extinction protocol to investigate the effects of D-serine on extinction and reinstatement. For this purpose, we restricted extinction into a one day session in this study. We selected two time points for administration of D-serine, one before the extinction session and another immediately after extinction session using this sub-maximal extinction protocol. After the one day of extinction training, the animals were tested for reinstatement response to different kinds of priming stimuli the next day at different time points of a single 120 minute reinstatement session. The administration of D-serine prior to extinction training, and thus facilitating the NMDAR mediated mechanisms during extinction learning did not enhance the extinction learning process as such , but enhanced the effectiveness of extinction training in reducing the drug primed reinstatement to a similar degree as the group treated with D-serine immediately after extinction training. We found that D-serine administered immediately after the extinction training as equally effective as the D-serine administered 2-3 hours prior to extinction training. D-serine treatment postextinction might be bringing this effect on drug primed response by helping in the consolidation of the extinction memory. D-serine is reported to have long lived effects in the brain (Hashimoto and Chiba, 2004), we believe that bioavailability of D-serine from the pre-extinction treatment remains significant even after the extinction training process and helps in pharmacologically enhancing the consolidation of the memory of extinction.

To study whether the D-serine treatment itself can help in reducing the reinstatement response independent of extinction, we studied the effect of D-serine in a group of abstinent animals and the result was compared to that of a saline abstinent group. D-serine treatment

independent of extinction was not found to reduce the reinstatement response. Since no new learning is happening in the period of enforced abstinence, the NMDAR mediated mechanisms are not recruited for the drug to act and produce an effect. This suggests that D-serine is advantageous in reducing the chances of relapse only when combined with extinction therapy.

In contrast to the self-administration behavioral model, the mechanisms involved in the learning and memory of extinction and the mechanisms involved in the effects of extinction on reinstatement have been extensively investigated in other paradigms such as fear conditioning, inhibitory avoidance, spatial navigation and conditioned taste aversion (Cammarota et al., 2005). A large body of literature in the field of extinction comes from the fear conditioning model. Recently, it is shown that extinction of conditioned fear responses can be facilitated by injections of the partial NMDAR agonist, D-cycloserine (D-4-amino-3-isoxazolidone), that acts at the strychnine –insensitive glycine-recognition site of the NMDA receptor complex, and which does not produce any obvious neurotoxicity in rats (Watson et al., 1990). Walker et al (2002), concluded that the administration of D-cycloserine either systemically or directly into the amygdala prior to extinction training and then tested on a drug free day showed a dose dependent enhancement in extinction performance compared to the control rats that did not receive any extinction training. This indicated that the facilitatory effect was specific to extinction and did not result from general dampening of fear expression itself in the presence of D-cycloserine. This complemented their earlier work demonstrating that NMDAR antagonist blocked extinction. Another study reported that administration of D-cycloserine prior to and immediately after extinction significantly enhanced extinction as indicated by the lower response to reinstatement when tested (Ledgerwood et al., 2003). These investigators observed that the systemic injection of D-cycloserine before and after extinction training significantly enhanced extinction, and the

dose-response curve was found to be linear. They selected different time points for the injection of D-cycloserine post extinction and they found that the administration at 30 minutes as the most effective one in reducing the reinstatement as compared to the injection at 240 minutes, again supporting the role of NMDAR mediated mechanisms in memory consolidation as previously reported by Santini et al. 2001). These authors concluded that D-cycloserine acts at acquisition and/ or consolidation of extinction memory.

Reinstatement is one reason for relapse, renewal is another. Renewal is the recovery of extinguished responding that occurs when the context is changed after extinction (Bouton, 2004). This emphasizes the context specificity of extinction which can include any physical or temporal stimuli as well as internal drug states or emotions (Bouton, 2004). The study on the effect of D-cycloserine on the renewal effect (Woods and Bouton, 2006) showed that D-cycloserine at a dose of 30mg/kg significantly facilitated extinction learning, but did not make extinction any less dependent on context since the drug was not able to prevent renewal.

It is well supported in aversive conditioning that facilitation of NMDAR activity in extinction learning enhances the effectiveness of incomplete extinction therapy. Hence, we directed our efforts to study whether similar mechanisms operate in appetitive conditioning using the reinstatement model for drug relapse. To this point, investigators in the field of drug addiction have primarily been studying the mechanisms and neural substrates involved in the actual reinstatement process itself. It is very clear that extinction reduces reinstatement as compared to enforced abstinence, but as far as we are aware, the mechanisms underlying the effects of extinction on reinstatement in the self-administration model have not been reported. Our first study was an attempt to address this issue and we found that blocking NMDAR activity during extinction impairs the effects of extinction on reducing the cocaine-induced reinstatement.

This current study was designed to complement the prior one, using D-serine either prior to extinction or immediately after extinction to enhance the effects of extinction on cocaine induced reinstatement.

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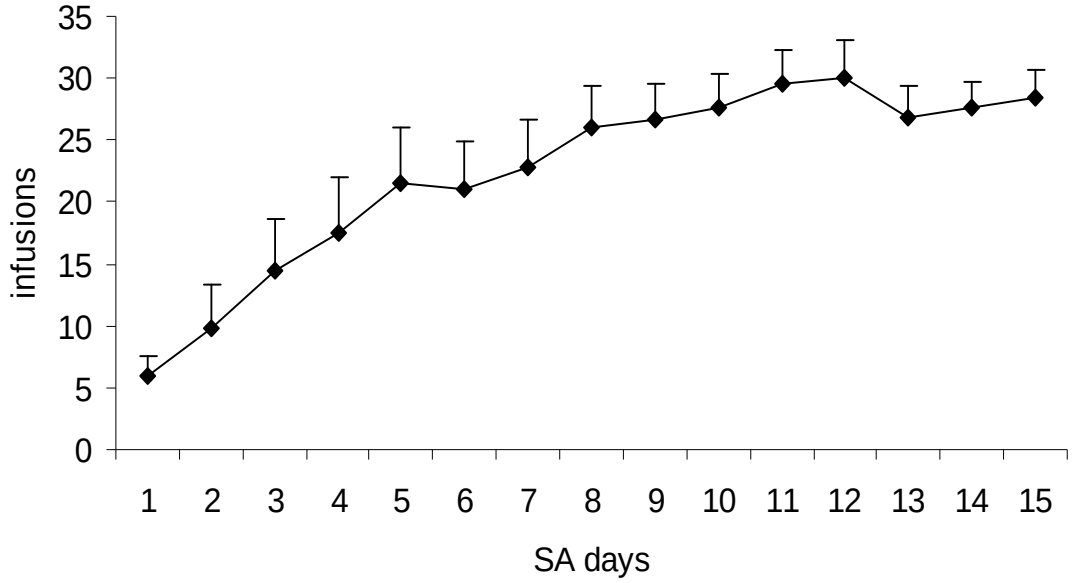
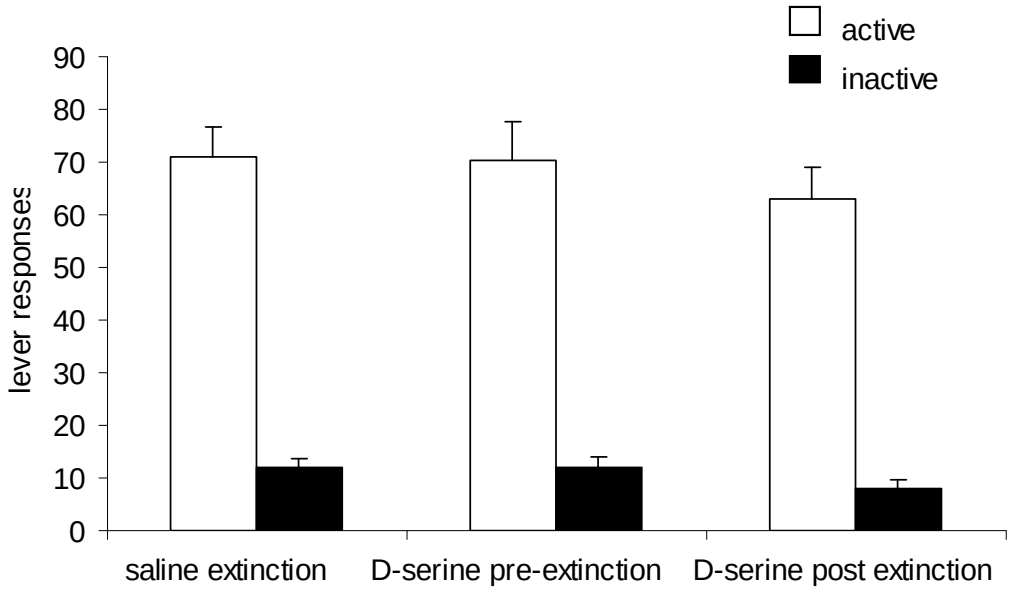


Figure 3.1. Self-administration of cocaine.

The results from the cocaine self-administered rats (n=75) illustrate the average number cocaine infusions earned daily during each 90 minute session during the SA phase (days 1-15). Transition to the FR3 schedule on day 13 did not significantly alter the number of earned infusions.

A


active
inactive
lever responses
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10
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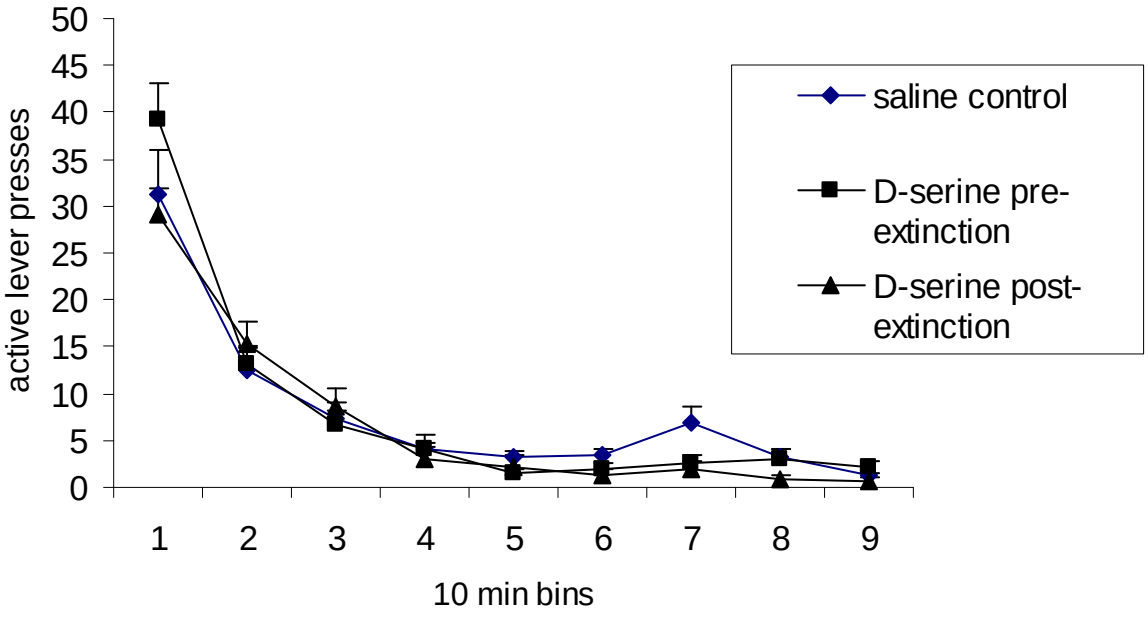
saline extinction
D-serine pre-extinction
D-serine post extinction

B


active lever presses
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40
35
30
25
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15
10
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1
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3
4
5
6
7
8
9
10 min bins
saline control
D-serine pre-extinction
D-serine post-extinction

Figure 3.2. Lever responses on the extinction training days:

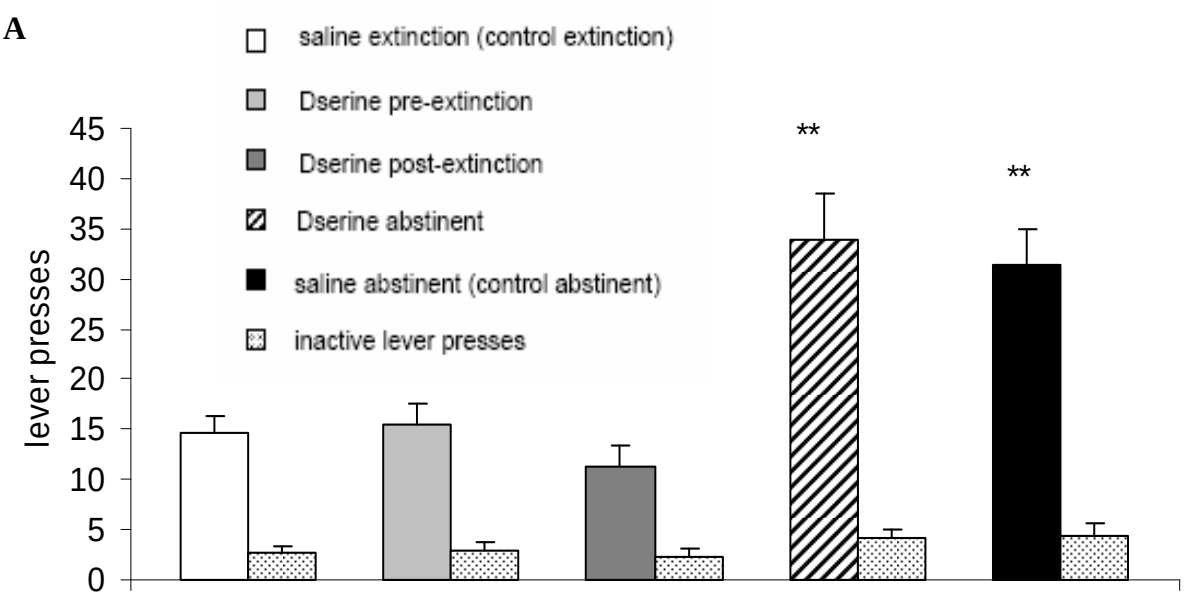
(A) Active and inactive lever response for the 90 minute extinction session.

Active and inactive lever presses of the saline (control) extinction group (n=18), group treated with D-serine prior to extinction (D-serine pre-extinction, n=15) and group treated with D-serine immediately after extinction (D-serine post-extinction, n=15) are illustrated (mean ± SEM) for the 90 minute sessions on the single day of extinction (day16). D-serine treatment prior to extinction did not affect the extinction performance compared to the saline extinction group. The three extinguished groups did not differ significantly in their drug seeking behavior on the day of extinction.

(B) Progression of extinction in 10 minute bins for the 90 minute extinction session.

Active lever presses (mean ± SEM) in 10 minute bins for the 90 minute extinction session for the saline extinction (n=18), D-serine pre-extinction (n=15) and D-serine post-extinction (n=15) groups. In the absence of CS and US, the animals extinguish their lever pressing behavior during extinction. All the groups were extinguished at a similar rate and by time=40 minutes, the average response was below 4 or 5 lever presses in all the groups and remained low from thereafter. The extinction pattern shows that majority of the lever pressing activity occurs in the initial 10 minutes and gets diminished soon after and remains low for the rest of the session.

A


saline extinction (control extinction)
Dserine pre-extinction
Dserine post-extinction
Dserine abstinent
saline abstinent (control abstinent)
inactive lever presses
lever presses
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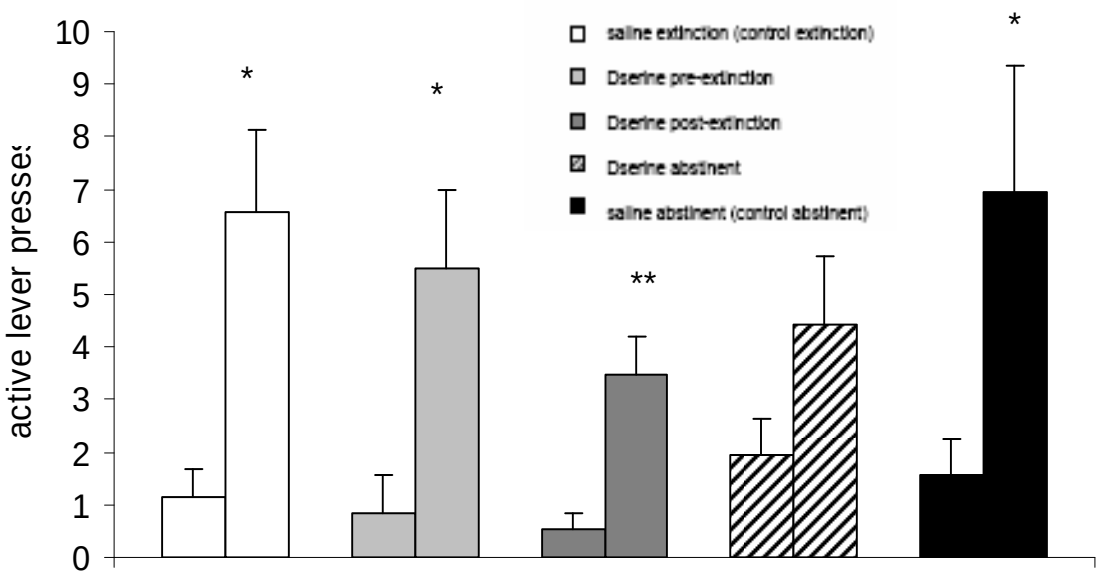
B


saline extinction (control extinction)
Dserine pre-extinction
Dserine post-extinction
Dserine abstinent
saline abstinent (control abstinent)
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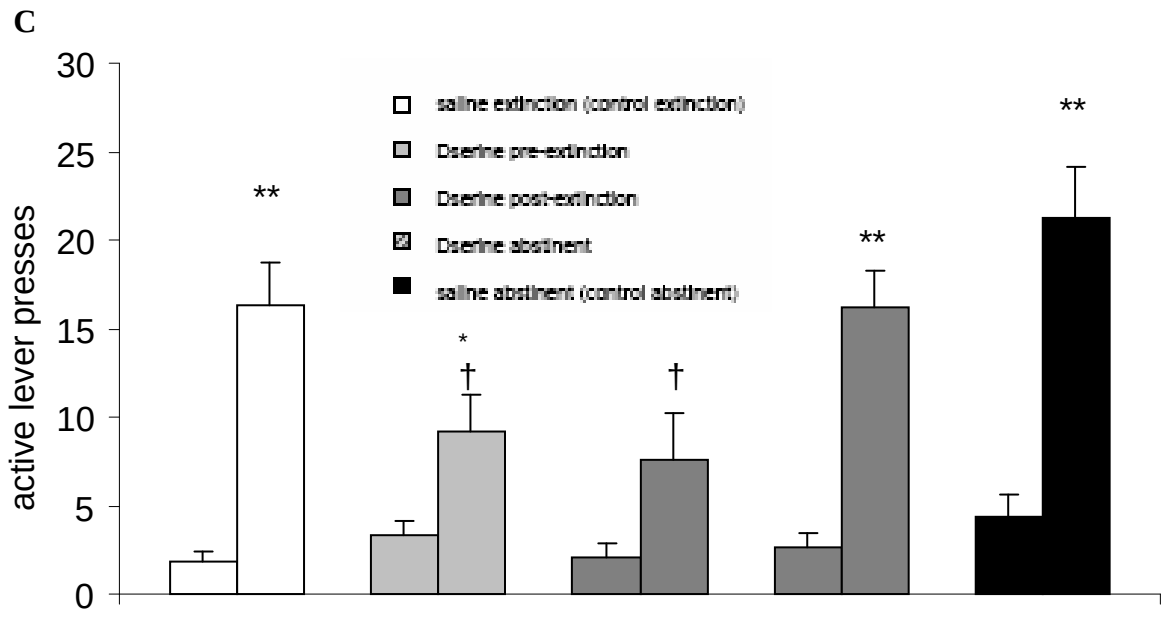

Figure 3.3. Facilitating the NMDAR activity during extinction had no effect on the resumption of drug-seeking following either exposure to contextual cues or the CS priming, but did affect the response to US priming:

(A) Effects of pre-extinction and post-extinction D-serine treatment on the reinstatement response induced by the contextual drug stimuli.

Data shows the mean ± SEM of lever presses for the first 10 minute time period on the reinstatement test day (day 17) when the animals were placed back into the operant environment after the single day of extinction training or enforced abstinence. Data shows that the extinction training experience reduces the reinstatement response induced by the contextual drug stimuli in the saline extinction (n=18), D-serine pre-extinction (n=15) and D-serine post-extinction (n=15) as compared to the saline abstinent (n=15) and the D-serine abstinent (n=14) group ($p<.001$, unpaired‘t’ test). Facilitation of NMDAR activity during extinction (pre and post extinction) did not enhance the effects of extinction on contextual response when compared to the saline

extinction group. Facilitating the activity of NMDARs during enforced abstinence did not affect the contextual response as compared to the saline abstinent group.

(B) Effects of pre-extinction and post-extinction D-serine treatment on the reinstatement response induced by the non-contingent CS prime.

Data show the mean ± SEM of the active ever presses for the10 minute bin of time before (30-40 min) the non-contingent CS prime and for the 10 minute bin of time after the CS prime (40-50 min) for each group on the reinstatement test day (protocol day 17). Neither D-serine treatment before extinction (100 mg/kg i.p., n=14) nor the D-serine treatment immediately after extinction (100mg/kg i.p., n=15) had a significant effect on responding as compared with the saline extinction (n=18) group. Post prime responses on the active lever were significantly higher for all the groups except the D-serine abstinent group when compared to their respective pre prime responses (paired't' test, *p<.05 or p<.01 and **p<.001). Facilitating the activity of NMDARs during extinction (both pre-extinction and post-extinction D-serine treated groups) affected the magnitude of the post priming response as compared with the saline extinction group or the abstinent groups (both D-serine abstinent, n=14 and saline abstinent, n=14).

(C) Effects of pre-extinction and post-extinction D-serine treatment on the reinstatement response induced by the non-contingent US (cocaine prime at 0.5mg/kg dose) prime.

Data show the mean ± SEM of the active ever presses for the30 minute bin of time before (50-80min) the non-contingent US prime and for the 30 minute bin of time after the US prime (80-110 min) for each group on the reinstatement test day (protocol day 17). D-serine treatment before extinction (100 mg/kg i.p., n=12) and the D-serine treatment immediately after extinction (100mg/kg i.p., n=15) had a significant effect on responding (*p<.05, unpaired't' test) as compared with the saline extinction (n=16) group. Post prime responses on the active lever were

significantly higher for all the groups except the D-serine post extinction treatment group when compared to their respective pre prime responses (paired 't' test, *p<.05, **p<.001). Facilitation of the NMDAR activity during extinction by the pre extinction and the post extinction treatment enhanced the effects of extinction training on drug induced reinstatement as compared to the saline treated control extinction group. Administration of D-serine during abstinence (n=12) did not affect the drug primed response as compared to the saline abstinent group (n=14).

CHAPTER 4

EFFECTS OF D-SERINE ADMINISTRATION DURING EXTINCTION ON THE REINSTATEMENT OF DRUG SEEKING BEHAVIOR IN RATS SELF-ADMINISTERED COCAINE IN A LONG ACCESS PROTOCOL

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[1]Kelamangalath, L and Wagner, J.J. To be submitted to Journal of Neuroscience

Abstract

We have found that facilitating the N-methyl D-aspartate receptor (NMDAR) mediated synaptic plasticity mechanisms during extinction enhance the effectiveness of extinction training on cocaine induced reinstatement response in animals trained to self-administer cocaine in a short access protocol. In this report, utilizing the long access model of self-administration, we demonstrate that facilitating the NMDAR mediated mechanisms during extinction by the systemic administration of D-serine at 100mg/kg enhance the effectiveness of an extinction training experience on the cocaine-induced reinstatement. Pharmacological facilitation of the NMDAR activity had no effect on the reinstatement response induced by the contextual drug stimuli and the non-contingent CS prime. Since the D-serine treatment during extinction was effective in reducing the drug primed reinstatement with the long access model of self-administration, this pharmacological intervention during extinction suggests to be a promising strategy for enhancing the effectiveness of extinction therapy in drug addiction.

1. Introduction

Drug addiction is a disease characterized by transition from recreational use to compulsive drug taking (Cami and Farre, 2003; O'Brien, 2005). Drug addiction is often accompanied by increased drug seeking and intake and difficulty in discontinuing the drug use. There are a number of animal models developed for studying different aspects of drug addiction. The most common model that the investigators use for self-administration is the short access model where cocaine is only available for a limited time period. Some researchers in the field of addiction have the opinion that this model is not sufficient to show the characteristic features of addiction in human beings like binge taking of drugs (Roberts et al., 2007) because the short access model always result in a stable pattern of drug taking. Studies in this area led to the

development of a new protocol for self-administration termed the “long access” model in which cocaine is available for up to 6-7 hours per day (Ahmed and Koob, 1998). This model was successful in showing the progressive increase in drug intake when the animals were switched from the short to long access protocol. The long access model of self-administration was able to show the escalation of drug intake especially in their first hour of self-administration compared to the short access model. This is similar to the binge pattern of drug intake in addiction.

Therefore, any study in the drug addiction field will be incomplete with out making use of the long access model. Our previous work in short access model has shown complementary findings in regard to the NMDAR mediated mechanisms in the effects of extinction on drug seeking behavior, that the activation of the NMDAR mediated mechanisms during extinction is necessary for the extinction to be effective on drug induced reinstatement (Kelamangalath et al., 2007) and facilitation of NMDAR activity during extinction enhances the effectiveness of extinction on drug-induced reinstatement (Ch 3). In this study we were interested to investigate the effects of D-serine administration during extinction on drug induced reinstatement behavior in rats trained to self-administer cocaine following exposure to a long access protocol. As discussed earlier, the molecular mechanisms underlying the effects of extinction on reinstatement are relatively understudied in the self-administration and reinstatement model. However, this has been studied in detail in behavioral paradigms based on aversive conditioning. Several of these reports have focused on NMDAR mediated mechanisms in making an incomplete extinction effective in reducing the reinstatement in aversive conditioning paradigms such as fear conditioning (reviewed by Myers and Davis, 2002). D-cycloserine was the most widely used drug to facilitate the extinction learning in the fear conditioning studies, based on the concept that extinction involves new learning (Bouton , 2005) and that this new learning is dependent on

the recruitment of the NMDAR mediated mechanisms. D-cycloserine (D-4-amino-3-isoxazolidone), binds at the strychnine–insensitive glycine-recognition site of the NMDA receptor complex, and does not produce any obvious neurotoxicity in rats at maximally effective doses (Hood et al., 1989; Watson et al., 1990). It has been shown that extinction of conditioned fear responses can be facilitated by injections of this partial NMDAR agonist. Walker et al (2002), concluded that the administration of D-cycloserine either systemically or directly into the amygdala prior to extinction training and then tested on a drug free day showed a dose dependent enhancement in extinction performance compared to the control rats that did not receive any extinction training. Other investigators have shown that the mechanism of consolidation of extinction memory shifts from NMDAR independent to NMDAR dependent form once the extinction learning happens (Santini et al., 2001; Ledgerwood et al., 2003).

Based on the concept that extinction involves new learning, and that the new learning is dependent on the recruitment of NMDAR mediated mechanisms, we decided to pursue our studies on extinction by pharmacologically manipulating the NMDAR activity during extinction by administering D-serine prior to extinction training in the long access cocaine animals. D-serine is supposed to be more effective pharmacologically than D-cycloserine at the glycine site of NMDAR because of the additional hydrogen bond formed by D-serine at the NR-1 ligand binding core. This makes the affinity of D-serine to be 34 times that of D-cycloserine (Furukawa and Gouaux, 2003). D-serine has long-lived effects in the brain (Hashimoto and Chiba, 2004) and can be administered at relatively high doses without renal toxicity (Williams et al., 2003).

2. Materials and Methods:

2.1. Animals:

Male Sprague-Dawley rats (Harlan) weighed approximately 300 g at the beginning of the experiment and were housed individually in a temperature and humidity controlled vivarium having a 12 hour light/dark cycle (lights off at 7:00 P.M.). They were given access to food and water *ad libitum* and were handled daily for 5 days prior to the surgery in order to diminish stress associated with handling. The housing and experimental procedures followed the *Guide for the Care and Use of Laboratory Animals* and were approved by the local ACUC at the University of Georgia.

2.2. Jugular catheterization protocol:

The animals were anesthetized using a combination of ketamine (70 mg/kg), xylazine (10 mg/kg) and acepromazine (1 mg/kg) administered i.p. Depth of anaesthesia was assessed by monitoring respiration rate and palpebral and pedal withdrawal reflexes. Under anesthesia, the right jugular vein was isolated. The catheter was exteriorized by passing it subcutaneously to the base of the skull, where it was connected to a modified 22 gauge cannula. A silastic catheter (Dow Corning) was then inserted into the vein (4-5 cm) and secured in position with silk sutures (6/0). The animal was then placed in a stereotaxic frame (Stoelting), where the right-angled cannula (Plastics One) was mounted to the top of the skull using dental cement and 4 screws. Immediately after surgery and once daily for 5 days, the animals were treated with gentamicin at a dose of 5 mg/kg, i.v. The catheters were flushed every day with saline prior to each self-administration session and with heparin (10 USP/ml) after the session to maintain the patency of the catheter. Catheter patency was verified daily by drawing blood from the catheter.

2.3. Self-administration environment:

The operant chambers (Med associates) were equipped with 2 levers, one "active" and another "inactive" with lights positioned above each lever. The chambers had a rod grid floor, a house light, a speaker/tone generator (2.9 kHz, 10 dB above ambient) and were housed inside enclosures equipped with ventilation fans. A syringe pump was located outside the enclosure. The method for delivering a cocaine infusion was as follows: The modified 22 gauge cannula mounted on the rat's skull was connected to a liquid swivel with PE-50 tubing protected by a metal spring. The swivel was connected with tygon tubing to the syringe mounted in the infusion pump. Infusion volumes were calculated according to the animal's weight. For cocaine animals, the syringes mounted in the infusion pump contained cocaine hydrochloride (NIDA) dissolved in normal saline at 4 mg cocaine/ml of solution. Each infusion delivered an infusion volume of 0.125 ml/kg body weight, hence the dose of cocaine self-administered was 0.5 mg/kg/infusion. The MED-PC software program recorded the number of active lever presses, inactive lever presses and the number of infusions.

2.4. Self-administration protocol (days 1-22):

The animals having patent indwelling catheters were subjected to self-administration training for a period of 22 days with one session each day. The first 15 days the Self-administration training sessions were 90 minutes in duration and is called the short access protocol. The next 7 days the self-administration was extended up to 6 hours and is called the long access protocol. Upon entry into the self-administration environment, the house light and the ventilation fan were on. In addition to triggering an infusion, active lever presses had the following programmed consequences: the house light was turned off, and the active lever light/tone (i.e. the CS) was turned on for a period of 30 seconds. Additional responses on the

active lever during this 30 second period had no programmed consequences, although the program continued to count the number of active/inactive lever presses and infusions. This "timeout" period protected the animals from cocaine overdose. After this 30 second period the lever light and tone were terminated and the house light came back on. Rats were initially trained for 10 days on an FR-1 (fixed ratio schedule-1) schedule in which each active lever press outside the timeout period triggered the programmed consequences. For the next 12 days of self-administration training, an FR-3 schedule was imposed where 3 active lever presses outside the time out period were required to trigger an infusion and the CS. Each rat was placed in the same operant conditioning chamber throughout the course of the experiment.

2.5. Extinction protocol (days 23-27):

After the 15 days of self-administration training, the animals were divided into 3 groups (balanced for cocaine intake): 1) extinguished (saline), 2) extinguished (D-serine), 3) abstinent (saline). All groups received i.p. injections of their respective treatments in their home cage environment. Both the extinguished group 1 and the abstinent group 3 received injections of saline (1ml/kg). Group 2 received an injection of D-serine (100 mg/kg). Groups 1 and 2 underwent extinction training 2 hours following their respective daily pharmacological treatments, whereas group 3 rats remained in their home cages. During their extinction training sessions, the animals in the operant chambers were attached to the drug tether but exposed only to the environment stimuli (i.e. diffuse, contextual cues). Responses on the active lever had no programmed consequences during the extinction training phase. For protocol days 23-27, responses on both active and inactive levers, as well as the equivalent "number of infusions" were counted by the software (although as stated above, syringe pumps were not activated during this phase of training). Extinction proceeded for a period of 5 days; with one 90 minute session

each day during which the animals in the extinction training groups 1and 2 were taken to the operant chambers. Under these conditions, the animals extinguished their lever pressing behavior to less than 20% of their former activity during self-administration. As previously mentioned, group 3 abstinent animals remained in their home cages throughout days 23-27.

2.6. Reinstatement tests (protocol days 28-30):

On days 28-30, all the animals (including the home cage abstinent animals), were placed back in the operant chambers for reinstatement tests. The reinstatement test session conditions were similar to an extinction session in that the animals were exposed only to the contextual cues of the operant chamber environment and the active lever responding were not reinforced by the contingent availability of either CS or US.

Reinstatement to the contextual drug stimuli:

On test day 28, response to the contextual prime was assessed from active lever presses during the first 10 minutes in which the animals were exposed only to the contextual cues of the drug environment.

Reinstatement to the CS cues:

Later during the same test session on day 28, lever presses evoked in response to a CS presentation were then assessed. A single, noncontingent presentation of the CS was delivered at the 40^{th} minute of the 120 minute test session. Thus the initial 40 minutes of the 120 minute session served as an extinction period to allow lever presses initiated by exposure to contextual stimuli to subside before the CS reinstatement test. As the CS was expected to evoke an immediate response from animals, the noncontingent CS was quantified as the number of lever presses during the subsequent 10 minutes following the priming event (t=40-50 min).

Reinstatement to the drug prime:

Response to the drug prime stimulus was assessed on days 28-30. We tested the reinstatement of drug seeking behavior using 3 different doses (0.25 mg/kg, 0.5 mg/kg and 1 mg/kg) of cocaine on three consecutive days (day 28, 29 and 30), respectively. The 0.25 mg/kg was tested in the latter half of the 120 minute session on day 28. A single, noncontingent drug prime was programmed to be infused intravenously by the syringe pump at the 80th minute of the 120 minute session on the first reinstatement test day. Thereafter on day 29 and 30 ,the drug prime sessions were of 90 minutes duration and a single non-contingent infusion was delivered at time=40 minutes of the session at 0.5 and 1 mg/kg dose respectively. Again, the initial 40 minutes of the 90 minute session served as an extinction period which allowed lever presses initiated by exposure to contextual stimuli to subside before the US reinstatement test. Drug seeking behavior elicited by the different doses of cocaine was quantified from the number of responses on the active lever following the drug prime for 30 minutes immediately after the priming event (t= 80-110 min on day 28 and t=40-70 min on day 29 and 30).

2.7. Drugs & Dosage Justification:

Cocaine hydrochloride was a gift from NIDA (RTI). The NMDA coagonist D-serine was obtained from Sigma (St. Louis). D-serine and was administered in the home cage environment approximately 2 hours prior to the extinction sessions on protocol days 16-20. D-serine has long-lived effects when administered i.p. at the indicated doses (Hashimoto and Chiba, 2004). The D-serine dose of 100 mg/kg was chosen in order to avoid possible nephrotoxic effects at higher doses (Williams et al., 2003), while still affecting learning (Stouffer et al., 2004).

2.8. Statistics:

The number of active lever presses, infusions and inactive lever presses were recorded for each session. These data were used to calculate the responses during each experimental session. A one way repeated measures ANOVA was applied for figure 4. 2A. 2-way ANOVA was used for the other data analysis. The two factors taken into consideration for the 2- way ANOVA were: 1) either trial (pre vs. post priming responses) or days and 2) either condition (extinction vs. abstinence) or treatment, as the case may be. A value of $p<.05$ was taken as significant, being determined from the Holm-Sidak post hoc test method. All the statistics were done using SigmaStat software.

3. Results

3.1. Cocaine self-administration and extinction of the drug seeking behavior:

Animals having indwelling jugular catheters were trained to self-administer cocaine in an operant chamber environment for 22 consecutive days. Rats were initially trained on an FR-1 schedule for first 10 days and switched to an FR-3 schedule for the next 12 days of self-administration training. In this, the last 7 days (from day 16-22), the animals were on a long access protocol and the session duration was extended to 6 hours from 90 minutes. The transition to the FR-3 schedule was done to increase the number of active lever pressing responses. Animals typically achieved stable self-administration by day 10 of training (data not shown), and the FR-3 schedule did not significantly alter the number of earned infusions per session. When the animals were switched from the short access FR-3 schedule to the long access FR3 schedule, the animals showed a significant escalation in the average number of infusions earned during the first hour of self-administration (figure 4.1A). Analysis by one way repeated measures ANOVA showed a significant effect of treatment (short vs. long access), $F(11,256)=4.651$, $P<.001$.

Further planned comparisons of the average number of infusions earned during the first hour of the long access on each day with the total average of the first hour infusions on short access days revealed a significant effect of escalation on most of the long access days from day 2 of the long access session (unpaired 't' test, $p<.05$ for LgA day 2 and 3, $p<.01$ for LgA days 5,6 and 7). During the 90 minute sessions, the animals took 25-30 infusions (empty squares) on an average and during the long access (6 hour session), the animals self-administered around 110-120 infusions (filled squares) per session (figure 4.1B, data shown only for the FR-3 days). There was no significant difference in the average number of infusions earned per animal among the three different groups of self-administration rats utilized for the reinstatement studies described in this report (data not shown).

3.2. NMDAR involvement in the effects of extinction training on reinstatement behavior.

After 22 days of SA, the animals were subjected to either an extinction training phase (extinction groups) in the same operant chamber environment for 90 minute sessions in the absence of both CS and US (i.e. the active lever had no programmed consequences) for a period of 5 days (Figure 4.2A, protocol days 23-27), or they were kept forcibly abstinent in their home cage environment (abstinent group).

The effect of facilitation of NMDARs in the extinction process was evaluated in the rats treated with D-serine (a coagonist of NMDAR at the glycine site). D-serine was administered at a dose of 100 mg/kg i.p in the home cage environment prior to the extinction sessions (see Methods).

Active and inactive lever presses were monitored during the extinction sessions and it was found that the animals extinguish their drug seeking behavior under these conditions. Evaluation of drug seeking behavior between extinction sessions demonstrates that lever

pressing activity observed on second-fifth days of extinction is significantly decreased as compared with the first day of extinction ($p<.001$, two way repeated measures ANOVA, Holm-Sidak) with in each group. Conversely, the second-fifth days' activities were not significantly different from each other. There was no significant difference observed between the extinction performance of the control extinguished and the D-serine treated group on any of the extinction days. By protocol day 27, the final extinction day, both the groups exhibited similar levels of drug-seeking behavior during the 90 minute session.

3.3. Reinstatement of drug seeking behavior:

Evaluation of drug seeking behavior within an extinction session illustrates that the majority of lever pressing activity occurs during the initial ten minutes in the operant chamber environment, suggesting that environmental contextual cues are priming this response. Following this initial burst of activity, active lever pressing diminishes rapidly. As illustrated during the fifth extinction session on protocol day 27 (Figure 4.2B), active lever responses are minimal (< 1) by 20-30 minutes and remain low for the remainder of the 90 minute session. This within session response pattern was observed during all extinction days (data not shown). Therefore, during the reinstatement experiments involving the noncontingent presentation of either CS or US stimuli, the priming event was delivered at time=40 min of the test session. A temporal distinction can thus be made between the drug-seeking activities induced by introduction to the operant chamber environment (i.e. activity during the first ten minutes) versus the subsequent activity induced via noncontingent presentation of priming events delivered later within the same test session. For the first reinstatement test session of 120 minutes, after the delivery of the CS prime at time=40 minutes, we waited for about 30 minutes for the CS primed response to subside before the delivery of the drug prime at 0.25mg/kg dose.

3.4. The effects of D-serine administration during extinction training on the reinstatement of drug seeking behavior.

The resumption of lever-pressing activity was induced using three forms of priming stimuli: contextual cues, conditioned cues, and drug prime. Once the animals underwent either extinction training or enforced abstinence for a period of 5 days (days 23-27), they were tested for the resumption of drug seeking behavior following exposure to the contextual stimuli, conditioned stimuli and the drug prime at 0.25mg/kg dose of cocaine on day 28. The reinstatement response to cocaine doses of 0.5mg/kg and 1mg/kg was tested on days 29 and 30 respectively.

The resumption of lever-pressing activity induced by diffuse environmental cues is illustrated in Figure 4.3A, and was assessed during the first ten minutes of the test session conducted on protocol day 28. The contextual response shown by the 5 day control (saline) abstinent group was significantly higher compared to the response in the saline treated and the D-serine treated extinguished groups (unpaired 't' test, $p<.001$) confirming the effectiveness of extinction in reducing the contextual reinstatement as compared to enforced abstinence. The level of responding on the active lever in both the extinguished groups of rats (saline treated and D-serine treated rats) during this period was similar. Thus, the facilitation of NMDAR activity during extinction training did not have a significant impact on the subsequently measured contextual reinstatement as compared with the saline treated control group following a five-day extinction protocol.

In order to investigate the effects of facilitation of NMDAR activity during extinction on the discrete CS-induced reinstatement, active lever pressing was measured during a "pre-prime" ten minute period of time as well as the "post-prime" period (Figure 4.3B). Analysis by paired 't'

test showed that the post-priming activity following the CS prime was significantly greater than the pre-priming activity for the control extinguished ($p<.01$), the D-serine extinguished ($p<.05$) and the control abstinent ($p<.05$) groups. Although active lever pressing activity was significantly increased in the ten minute period immediately following the CS prime in all the groups tested, there was no significant difference between the post-prime responses of these groups. Thus extinction was not effective in reducing the CS primed response significantly compared to abstinence in long access cocaine animals and NMDAR facilitation during extinction did not have a significant effect on the cue prime response compared to the saline treated control extinguished groups.

Finally, cocaine-induced reinstatement was tested on 3 consecutive days (protocol days 28-30) at 3 different priming doses (0.25mg/kg, 0.5mg/kg, 1mg/kg) with a single, noncontingent intravenous infusion. The prime at 0.25 mg/kg was tested on day 28 itself at time=80 min of the 120 min session. Further doses of cocaine were tested with a 90 min reinstatement test session on the 2 consecutive days and the prime was delivered at time=40 min of the session. The active lever responding during the next 30 minutes was measured as an indication of the reinstatement of drug seeking behavior evoked by the US stimuli. The dose-response results are plotted to demonstrate the shift in the sensitivity to cocaine-induced drug seeking activity (Figure 4.3C). Treatment with D-serine during extinction had a significant effect on reinstatement at two of the priming doses tested (0.5 and 1 mg/kg) as compared with the saline treated group. At the 0.5mg/kg priming dose of cocaine, the 2-way ANOVA confirmed an effect of trial, $F_{trial}(1,42)=20.25$, an effect of treatment, $F_{treatment}(2,42)=7.985$ and an effect of interaction of trial and treatment, $F_{interaction}(2,42)=3.545$. The post hoc analysis of this data showed that the 30 minute post priming response was significantly greater than the pre priming response for the

saline control (t=2.584, p=.01) and the control abstinent group (t=4.358, p=.000) groups. The post prime response of the control abstinent group was significantly greater than the post prime responses of the control extinguished (t=2.254, p<.05) and the D-serine treated extinguished groups (t=4.698, p=.017). Again the post prime response of the control extinguished group was significantly greater than the post-prime response of the D-serine treated extinguished groups (t=2.215, p<.05). The analysis of the data at the priming dose of 1mg/kg showed an effect of trial, (F_{trial} (1,40=24.69), treatment, $F_{treatment}$ (2,40)=5.787)and an effect of interaction between these two factors $F_{interaction}$(2,40)=3.725). The post-priming responses were significantly greater than the pre priming responses for both the control extinguished (t=3.202, p=.003) and the control abstinent group (t=4.581, p=0.000). Pairwise comparison between the post-prime responses of the different treatments showed that the control extinguished (t=2.650, p<.01) and the control abstinent group (t=4.279, p=0.000) responded significantly greater than the D-serine treated extinguished group. The post-prime responses of the control (saline) extinguished group and the control abstinent group were not significantly different from each other. These results indicate that the ability of extinction to reduce drug primed reinstatement is enhanced by the facilitation of the NMDAR activity during extinction.

4. Discussion

The results from the previous study on the effects of D-serine treatment during extinction on drug induced reinstatement in the short access model (Ch 3) formed the basis for this study. Since the long access model of self-administration is proposed to be a better model for addiction, we wanted to investigate the effects of D-serine treatment during extinction on reinstatement utilizing the long access model.

As stated in the introduction, the short access model results in stable intake of drugs whereas the long access model is supposed to show an escalation in the drug intake modeling the binge taking of drugs as often happens in addiction (Ahmed and Koob, 1998; Liu et al., 2005; Kitamura et al., 2006). In this study, when the animals were switched to a long access protocol, we observed the escalation effect. The transition to long access protocol in this study also helped in increasing the time spent in the cocaine environment and increased the amount of cocaine self-administered. Similar findings have been reported by other investigators. Some investigators hypothesized that the laboratory animals can regulate the self-administration of psychostimulants within a session, regardless of the unit dose and maintain a constant level of drug intake (Lynch et al., 1998; Panlilio et al., 2006). This implies that escalation might not be observed at all the doses of cocaine tested. Most of the studies utilizing the long access protocol used a FR-1 schedule of cocaine self-administration and were able to show the escalation during the first hour of intake when the animals were switched from a short to long access session (Wee et al., 2006; Knackstedt et al., 2007). In our study, we started training the animals on a FR-1 schedule, but from day 11 of self-administration, they were transitioned to a FR-3 schedule so as to increase their lever pressing behavior. This was required in our study because of the non-contingent form of priming protocol we were employing for the reinstatement session which is reported to model human condition of relapse better (Katz and Higgins, 2003). In addition to the escalation effect that was observed, transition to long access protocol also helped in increasing the drug intake by at least 4 times than in the short access protocol.

After the 22 days of self-administration, the animals were subjected to extinction training for 5 days or kept as forcibly abstinent in their home cages for an equivalent time period. The extinction behavior of the saline treated control animals and the D-serine treated animals were

not different significantly on any of the extinction days. The 5 days extinction protocol was adopted for the long access cocaine animals based on a report indicating that the long access animals are more resistant to extinction (Ahmed et al., 2000). Hence our concept was that even 5 days of extinction would be less effective in reducing the reinstatement response in these animals and this would allow the effects of D-serine treatment during extinction to become more evident during reinstatement tests. The previous study with one day of extinction training already showed that the treatment with D-serine prior to and post extinction were equally effective in reducing the reinstatement responses. Hence, in the present study, we decided to treat the animals prior to extinction only so that we could cover the effects of D-serine treatment on the actual extinction learning if there is any, and the effect of D-serine on post-extinction memory consolidation because D-serine is reported to have long lived effects in the brain (Hashimoto and Chiba, 2004). As before, the facilitation of the NMDAR activity by D-serine treatment prior to extinction did not have an effect on the extinction learning process as such. But, we could observe similar enhancement of the effects of extinction on drug induced reinstatement when the animals were tested on a drug free (D-serine free) day. The facilitation of NMDAR activity during extinction did not have an effect on the reinstatement response induced by the contextual drug stimuli or the non-contingent CS prime. Hence, this study was successful in replicating the effects of D-serine during extinction on drug induced reinstatement from our previous study utilizing the short access protocol.

In contrast to the self-administration behavioral model, the mechanisms of extinction training have been extensively investigated in other paradigms such as fear conditioning, inhibitory avoidance, spatial navigation and conditioned taste aversion (Cammarota et al., 2005). Hence, studies in this area are fairly novel and of potential interest because extinction therapies

are tried in drug addicts to reduce the chances of relapse. For example, the cue exposure therapy practiced in addiction treatment is aimed at diminishing the associative impact of drug cues and these procedures are derived from the basic animal research on extinction (Conklin and Tiffany, 2002). But, the clinical success of these treatments has been less promising, leading some researchers to investigate whether cue-exposure proves more effective when used in combination with cognitive-behavioral techniques such as social skills training (Cooney et al., 1983). For many years researchers believed that extinction involved unlearning the initially conditioned CS-US association (Rescorla and Wagner, 1972). The current concepts on extinction are more inline with those of Pavolv (1927), who postulated that repeated unreinforced exposure to the CS serves to mask it, not erase it (Robbins, 1990). Therefore, the conventional notion that extinction is unlearning has been replaced with the position that extinction is new learning. Thus, the learning and memory for extinction of conditioned responses might be expected to involve some neurochemical processes similar to those involved in the original conditioning of the association. At the molecular level n-methyl D-aspartate receptor mediated mechanisms are increasingly implicated in extinction learning (Baker and Azorlosa, 1996; Falls et al., 1992; Santini et al., 2001) although some nonNMDAR dependent forms of synaptic plasticity also are shown to contribute to this type of learning. For these reasons, attempts to enhance or facilitate the extinction processes and to improve the retention of this new learning has taken a prominent position in the field of behavioral extinction therapy by pharmacologically modulating the activity of NMDAR during extinction learning.

Recently, it was shown that extinction of conditioned fear responses can be facilitated by injections of the partial NMDAR agonist, D-cycloserine (D-4-amino-3-isoxazolidone), that acts at the strychnine –insensitive glycine-recognition site of the NMDA receptor complex, and

which does not produce any obvious neurotoxicity in rats (Hood et al., 1989; Watson et al., 1990). Walker et al (2002), concluded that the administration of D-cycloserine either systemically or directly into the amygdala prior to extinction training and then tested on a drug free day showed a dose- dependent enhancement in extinction performance compared to the control rats that did not receive any extinction training. Another study reported that administration of D-cycloserine prior to and immediately after extinction significantly enhanced extinction as indicated by the lower response to reinstatement when tested (Ledgerwood et al., 2003). These investigators observed that the systemic injection of D-cycloserine before and after extinction training significantly enhanced extinction, and the dose-response curve was found to be linear. The only report that we could find in the addiction literature about the effects of pharmacological facilitation of extinction on reinstatement was based on the conditioned place preference paradigm (Botreau et al., 2006). They used D-cycloserine to enhance the NMDAR activity during extinction and found that the peripheral injection of this compound facilitates the extinction of the conditioned place preference. In the same study, this finding was replicated by intra-amygdalar injections of the D-cycloserine. In the self-administration and reinstatement model, we showed that blockade of the NMDAR activity during extinction impairs the effects of extinction on drug induced reinstatement (Kelamangalath et al., 2007) and the findings from this study complement this previous finding because we could show that the facilitation of NMDAR activity during extinction enhances the effects of extinction on drug induced reinstatement.

In summary, we have found that administration of D-serine during extinction, in a group of animals trained to self-administer cocaine in a long access protocol was effective in enhancing the effects of extinction on drug induced reinstatement. D-serine might be bringing its effect by facilitating the consolidation of the memory of extinction after the extinction learning process. It

is shown that exposure to the drug associated cues and /or to the exposure to the drug itself is responsible for craving and relapse in human addicts. Since extinction therapy is practiced in the addiction field to reduce the chances of relapse, the investigation on the mechanisms underlying the effects of extinction on reinstatement is of potential interest. As a result, the findings from this study may contribute to the basis for the development of new pharmacotherapy as an adjunct to psychotherapy of extinction.

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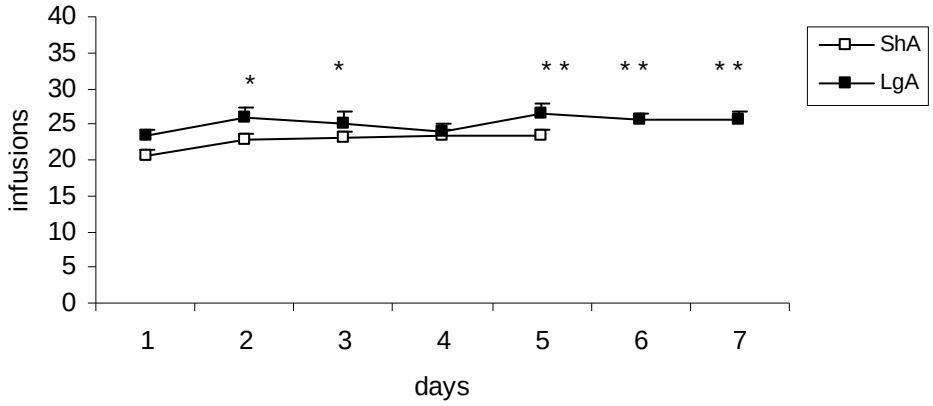

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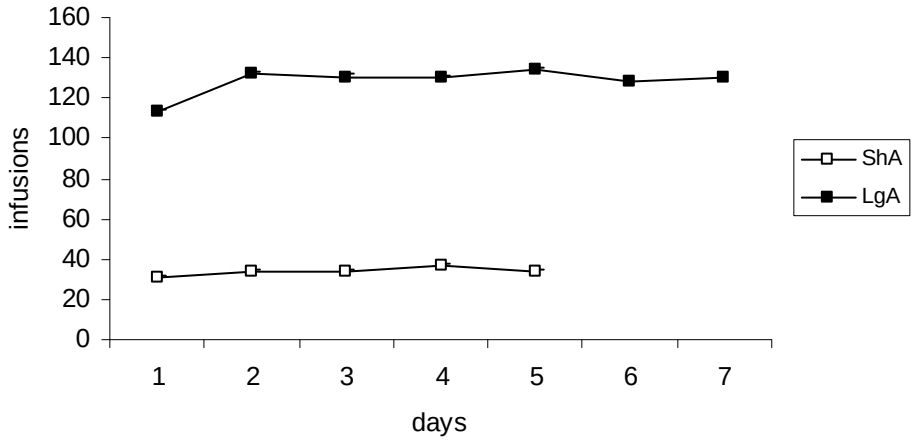

Figure 4.1. Cocaine intake during the first hour and the entire session of self-administration on the FR-3 schedule days:

(A) This figure illustrates the average number of cocaine infusions earned during the first hour of self-administration on the last 5 days of short access (empty squares) and on the 7 days of long access protocol (filled squares). The animals (n=25) showed a significant escalation in the

number of self-administered cocaine infusions on long access days 2,3,5,6 and 7 during the first hour of self-administration when switched from short to long access sessions. Planned comparisons were performed between the total average of the number of infusions for the first hour on the last 5 days of short access with the mean number of infusions for the first hour on each of the long access days.

(B) This figure illustrates the average number of cocaine infusions self-administered during the entire self-administration session on the last 5 days of short access (empty squares) and on the 7 days of long access protocol (filled squares). The animals (n=25) showed 4-5 fold increase in the total number of self administered infusions when switched from short (empty squares) to long access sessions (filled squares).

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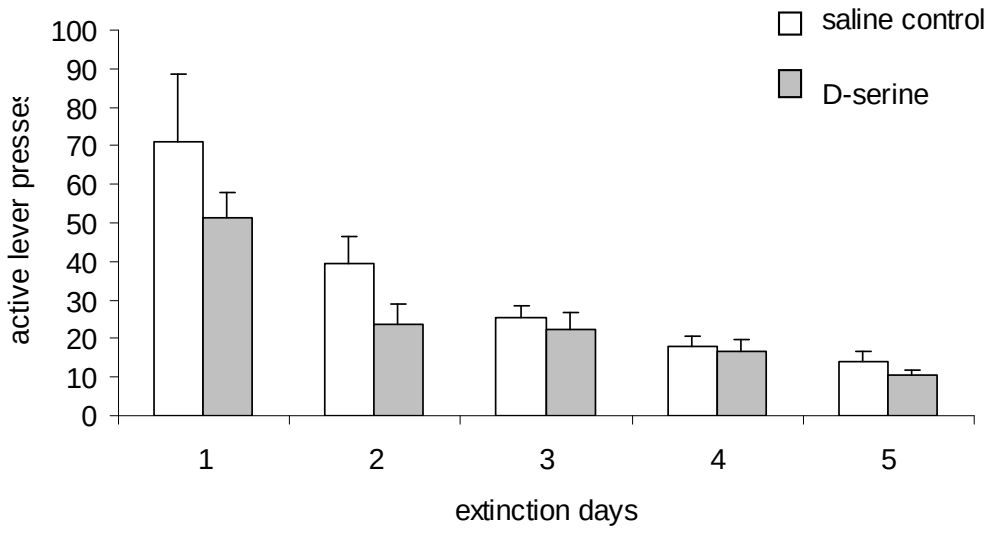

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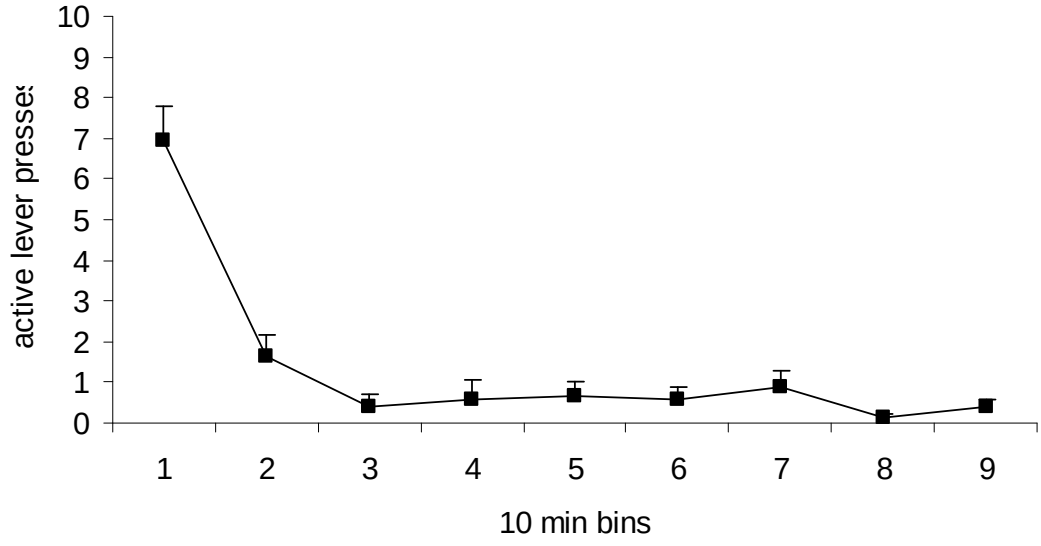


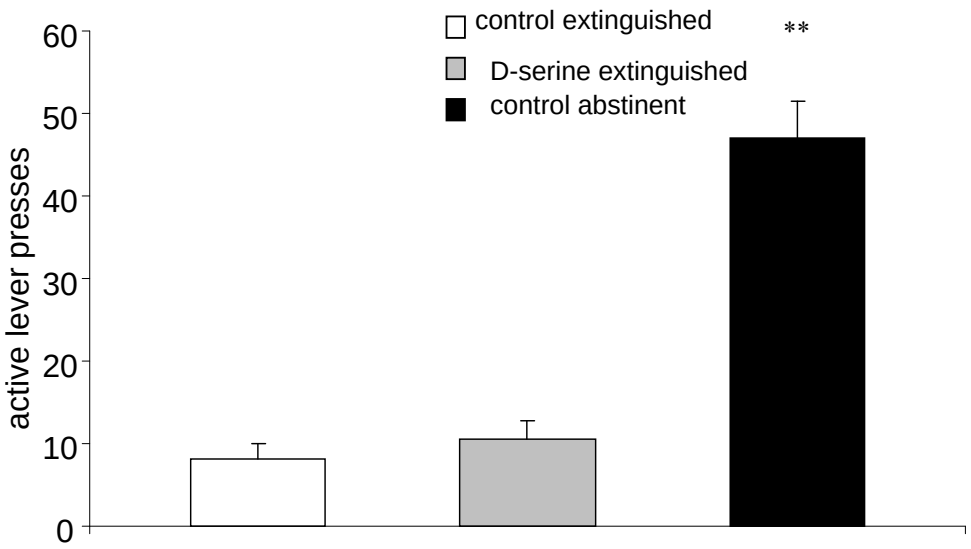
Figure 4.2 Effects of facilitation of NMDAR activity during extinction training on the progression of extinction:

(A) Data shows the mean ± SEM of active lever presses for the entire 90 minute extinction sessions on protocol days 23-27. Active lever pressing behavior was extinguished across the 5

daily extinction sessions and responding on extinction days 2-5 was significantly decreased ($p<.001$, 2-way RM ANOVA, Holm-Sidak) from that measured on the first day of extinction (i.e. protocol days 24-27 vs. 23) within each treatment group. D-serine treatment (n=9) during extinction training did not influence the progression of extinction as compared to their saline treated controls (n=8) on any day of extinction.

(B) Extinction behavior within a session on the fifth day of extinction (protocol day 27) is illustrated using 10 minute bins. Data are the mean ± SEM of lever presses throughout the 90 minute extinction session. Data shows that the majority of the lever pressing activity occurs during the initial 10 minutes of entry in to the operant chamber and then the lever pressing response diminishes rapidly and remains low for the entire session.

A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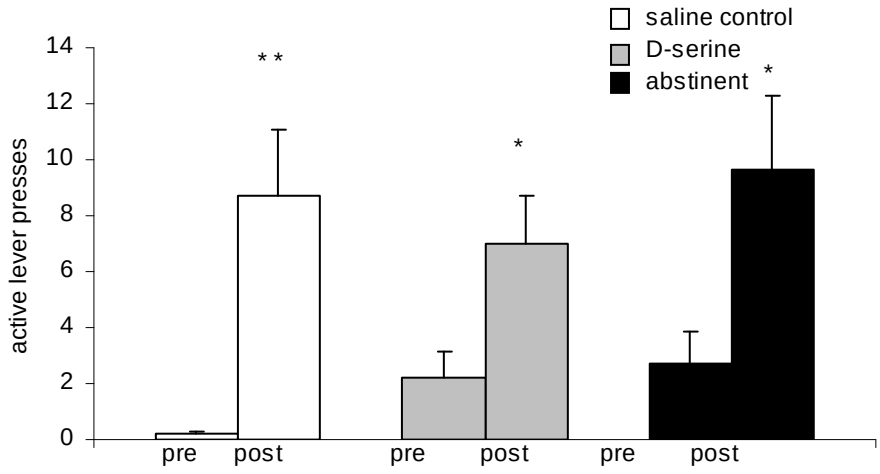
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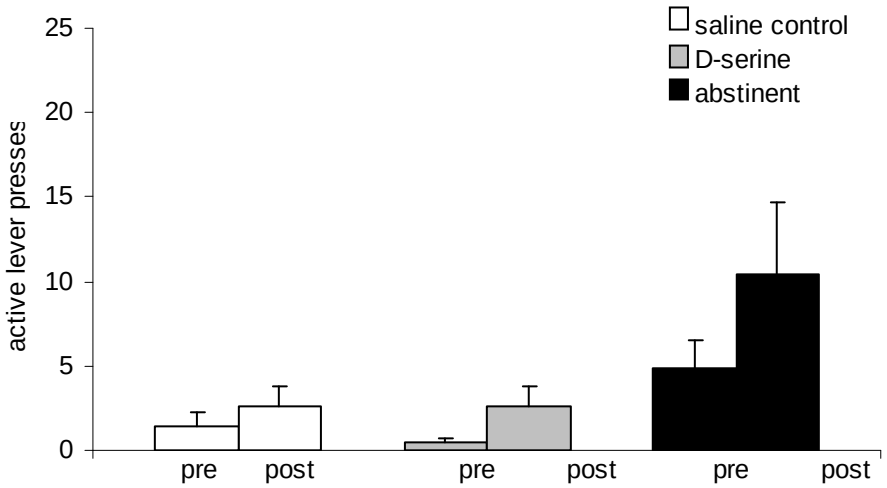
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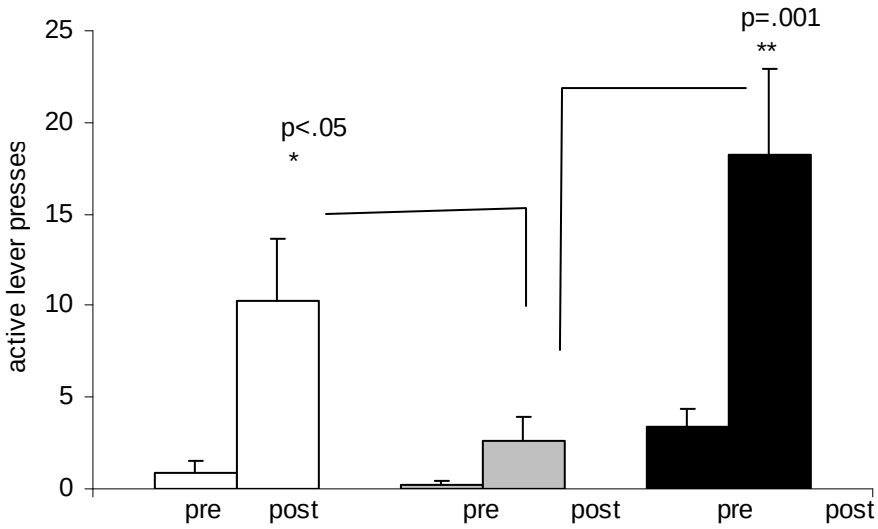
p=.001
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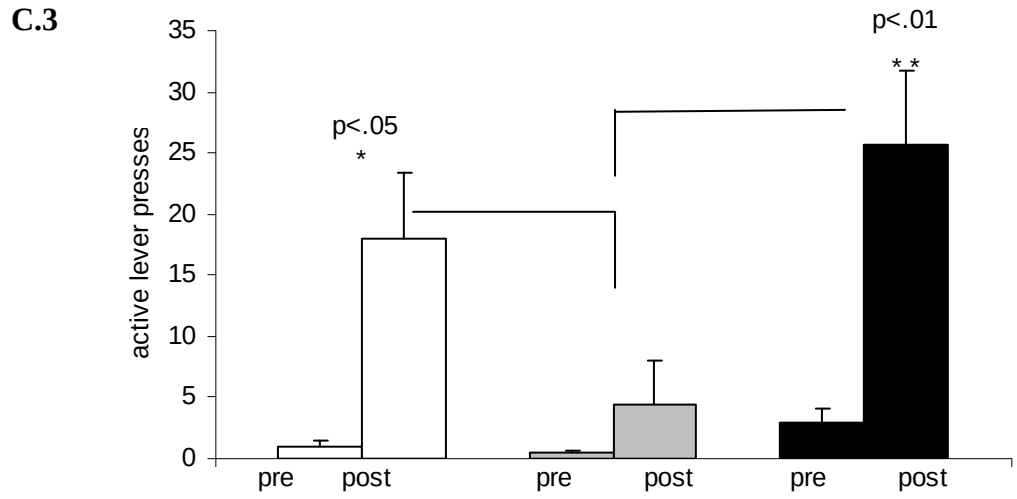

Figure 4.3. Facilitation of NMDAR activity during extinction had no effect on the resumption of drug-seeking following either exposure to contextual cues or the CS priming, but had an effect on the response to US priming.

(A) Data show the mean ± SEM of lever presses for the first 10 minute bin of time on the first reinstatement test day (protocol day 28). D-serine treatment (100 mg/kg i.p., n=9) during extinction training did not have a significant effect on responding as compared with the saline treated control group (n=8). The contextual response of the control abstinent group (n=8) was significantly higher as compared to both the extinguished groups (p<.001, unpaired't' test).

(B) Data show the mean ± SEM of active lever presses for 10 minutes before the cue prime (pre, 30-40 min) and for 10 minutes after the cue prime event (post, 40-50 min) on the first reinstatement test day (day 28) for the D-serine (n=9), control extinction (n=8) and the control abstinent (n=8) groups. Post-prime responses on the active lever were significantly higher for all the groups when compared to their respective pre-prime responses (paired't'- test, *p<.05, **p<.01). D-serine treatment during extinction did not affect the magnitude of the post priming response as compared with the saline control groups.

(C) Data represent the mean ± SEM of lever presses for a 30 minute window before (pre) and after (post) the single, noncontingent intravenous delivery of a drug prime at the dose of 0.25mg/kg, 0.5mg/kg and 1mg/kg respectively administered at time=80 minutes on the first reinstatement day (0.25mg/kg) and at time=40 minutes on the next 2 consecutive days (0.5 and 1 mg/kg test days). The post prime response was significantly greater than the pre prime response for the control extinction and control abstinent groups at cocaine doses 0.5 and 1 mg/kg, but not for the D-serine treated extinguished group. Pairwise comparisons of the post prime responses between the groups show that at the dose of 0.5mg/kg, the response of the control abstinent group (n=8 for 0.5mg/kg and n=7 for 1mg/kg) was significantly greater than that of control extinguished and D-serine treated extinguished group. At the dose of 1mg/kg, the response of the control extinguished group was not significantly different from that of the abstinent group. The reinstatement responses at the doses of 0.5 mg/kg and 1mg/kg were significantly greater for the control extinction group (n=7 for 0.5mg/kg, n=6 for 1mg/kg) compared to the D-serine treated group (n=8 at 0.5 mg/kg and 1mg/kg). (**$p<.001$, .01* $p<.05$, 2-way ANOVA, Holm-Sidak).

CHAPTER 5

EFFECTS OF BLOCKADE OF N-METHYL D-ASPARTATE RECEPTOR ACTIVITY IN THE VENTRAL HIPPOCAMPUS DURING EXTINCTION ON REINSATEMENT BEHAVIOR.

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Abstract

We have shown previously that activation of n-methyl D-aspartate (NMDAR) mediated mechanisms during extinction is required for extinction to be effective in reducing the drug primed reinstatement using systemic administration of (±) CPP, a competitive antagonist of NMDAR during extinction. In this study, we investigate whether activation of NMDAR mediated mechanisms in the ventral hippocampus is required for extinction to be effective in reducing the reinstatement responses by the intra hippocampal injections of the same drug into the ventral hippocampus prior to extinction. The intra-hippocampal infusion of (±) CPP at 100 and 200 ng dose did not affect the extinction learning process compared to the control group. The results from the reinstatement tests were not robust enough to derive any solid conclusion about the requirement of the NMDAR mediated synaptic plasticity mechanisms in the ventral hippocampus for the extinction to be effective in reducing the reinstatement response.

1. Introduction

A major challenge in the treatment of drug addiction is the relapse (O'Brien and McLellan, 1996). Development of any successful treatment strategies should involve the treatment to prevent the chances of relapse. Relapse appears to be triggered by several factors, including stress (Kosten et al., 1986; Kreek and Koob, 1998; Sinha et al., 2000), conditioned stimuli (Childress et al., 1988), and re-exposure to drug (Jaffe et al., 1989). It is important to identify the neural mechanisms and neural substrates underlying the relapse caused by these different factors in order to develop effective treatments. Substantial progress has been made in identifying the neural circuitry involved in the cue induced relapse by the neural imaging techniques such as Positron emission tomography (PET) and functional magnetic resonance imaging technique (fMRI) (Childress et al., 1999; Kilts et al., 2001). Similarly, cocaine-induced relapse is shown to

involve many other brain regions such as the nucleus accumbens, right para-hippocampus and lateral pre-frontal cortex by the use of the imaging techniques (Breiter et al., 1997). These conclusions are consistent with the data from the reinstatement studies on drug seeking behavior in animals.

Interestingly, different neural mechanisms appear to underlie the reinstatement induced by these priming stimuli, and several specific brain regions are involved in some of these events. For example, one early report identified that the basolateral region of the amygdala as being critical for the reinstatement response to conditioned stimuli (CS) priming (Meil and See, 1997). Subsequently, Grimm & See (2000) demonstrated that inactivation of the nucleus accumbens was effective in preventing drug (cocaine) induced drug seeking, but had no effect on CS induced priming of reinstatement. The opposite relationship was found to exist for inactivation of the basolateral amygdala, as tetrodotoxin infusion did not affect drug primed reinstatement. This study established a basis for multiple, discrete neuronal mechanisms in mediating the primed reinstatement of drug-seeking behavior. Additional work has found that the dorsomedial prefrontal cortex is also involved in CS induced reinstatement (McLaughlin and See, 2003), and that inactivation of the dorsomedial prefrontal cortex, the basolateral amygdala or the dorsal hippocampus can inhibit context induced reinstatement (Fuchs et al., 2005).

Another likely component of this circuitry is the ventral subiculum, an extension of the ventral hippocampus known to play a role in goal directed behavior. Lesions of the ventral subiculum is reported to block the potentiating effects of amphetamine infused locally to the nucleus accumbens on operant responding reinforced by CS previously paired with sucrose reinforcement (Everitt et al., 2001). Stimulation of ventral subiculum by electrical or chemical means is reported to reinstate cocaine or amphetamine seeking behavior in rats (Vorel et al.,

2001; Taepavarapruk and Philips, 2003). We suggest that, as different neural substrates are involved in the drug seeking behavior induced by different stimuli, different mechanisms might be involved in different forms of reinstatement.

An extinction protocol is always included in the animal reinstatement model of relapse. It is well accepted that extinction is effective in reducing the levels of reinstatement as compared to enforced abstinence. But, the molecular mechanisms that might be responsible for bringing this effect of extinction on reinstatement for drugs of abuse are unknown. Using other behavioral models, evidence indicates that new learning is occurring during the extinction training experience (Pavolov, 1927; Bouton et al., 2006; Rescorla and Heth, 1975). This new learning can be dependent upon the activation of n-methyl-d-aspartate receptors (NMDARs), and either blocking NMDARs with antagonists or enhancing NMDAR activity with coagonists would be expected to affect the ability of an extinction training experience to alter the response to primed reinstatement. Our previous work in this particular field has shown that activation of the N-methyl D-aspartate mechanisms during extinction is essential for the extinction training to be effective in reducing the drug induced reinstatement (Kelamangalath et al., 2007).

The hippocampus is known to be involved in the formation of new memories (Scoville and Milner, 1957) and 90% of the neurons in the hippocampus are glutamatergic in nature (Freund and Buzsaki, 1996).). It is shown that ventral subiculum (VSUB) stimulation enhances VTA dopamine neuron firing by an indirect way (Legault et al, 2000; Todd and Grace, 1999). NAc dopamine increase after VSUB stimulation depends on VTA glutamate because it has been reported that the NAc dopamine release is blocked by glutamate receptor antagonist kynurenic acid applied into the VTA (Legault et al, 2000). Since our previous finding suggests a glutamatergic mechanism and since hippocampus is shown to involve in the formation of new

memories, we hypothesized that activation of the NMDAR mediated mechanisms in the ventral hippocampus during extinction might be necessary for the extinction training to be effective in reducing the reinstatement response.

2. Materials and Methods

2.1. Animals:

Male Sprague-Dawley rats (Harlan) weighed approximately 300 g at the beginning of the experiment and were housed individually in a temperature and humidity controlled vivarium having a 12 hour light/dark cycle (lights off at 7:00 P.M.). They were given access to food and water *ad libitum* and were handled daily for 5 days prior to the surgery in order to diminish stress associated with handling. The housing and experimental procedures followed the *Guide for the Care and Use of Laboratory Animals* and were approved by the local ACUC at the University of Georgia.

2.2. Jugular catheterization protocol:

The animals were anesthetized using a combination of ketamine (70 mg/kg), xylazine (10 mg/kg) and acepromazine (1 mg/kg) administered i.p. Depth of anaesthesia was assessed by monitoring respiration rate and palpebral and pedal withdrawal reflexes. Under anesthesia, the right jugular vein was isolated. The catheter was exteriorized by passing it subcutaneously to the rump region of the animal where it was connected to a modified 22 gauge cannula. A silastic catheter (Dow Corning) was then inserted into the vein (4-5 cm) and secured in position with silk sutures (6/0). The cannula was mounted on the back of the animal and secured with nylon sutures safely. The wiremesh around the cannula helped the cannula to get adhesioned closely to the subcutaneous region as the healing occurs.

Immediately after surgery and once daily for 5 days, the animals were treated with gentamicin at a dose of 5 mg/kg, i.v. The catheters were flushed every day with saline prior to each self-administration session and with heparin (10 USP/ml) after the session to maintain the patency of the catheter. Catheter patency was verified daily by drawing blood from the catheter.

2.3. Cannula implantation protocol (ventral hippocampus)

The animal was then placed in a stereotaxic frame (Stoelting) for the cannulation of the ventral hippocampus. The surgical site was prepared by swabbing with iodide and alcohol, a 15mm mid-line incision was made and the scalp and temporal muscles were reflected back. Guide cannulas were implanted via the holes drilled with the help of a hand drill on either half of the skull following the co-ordinates for ventral hippocampus. The guide cannula was secured with dental acrylic and 4 stainless steel screws, two on either side. Once the dental acrylic was hardened, the incision was closed around the guide cannula with interrupted Ethicon sutures. A dummy cannula cut to the same size as the guide cannula was inserted through the guide cannula and screwed in place to maintain the patency of the guide cannula. Animals were moved from the stereotaxic frame and placed on a heated isothermal pad (35^0C) and they were monitored until completely recovered. They were allowed at least one week for recovery before any testing is performed.

2.4. Co-ordinates for the guide cannula placements:

For intrahippocampal infusions in to the ventral hippocampus , two guide cannulas were placed according to these co-ordinates, -5.5mm AP and ±5.4mm ML. The guide cannulas were allowed to protrude in to a depth of -5.0 mm DV, leaving the cannula 1 mm above the subiculum. Injections were delivered with the use of an internal cannula, protruded 1mm beyond the depth of the guide cannula, so that the drug infused will be deposited in the subiculum.

2.5. Self-administration environment:

The operant chambers (Med associates) were equipped with 2 levers, one "active" and another "inactive" with lights positioned above each lever. The chambers had a rod grid floor, a house light, a speaker/tone generator (2.9 kHz, 10 dB above ambient) and were housed inside enclosures equipped with ventilation fans. A syringe pump was located outside the enclosure. The method for delivering a cocaine infusion was as follows: The modified 22 gauge cannula mounted on the rat's skull was connected to a liquid swivel with PE-50 tubing protected by a metal spring. The swivel was connected with tygon tubing to the syringe mounted in the infusion pump. Infusion volumes were calculated according to the animal's weight. For cocaine animals, the syringes mounted in the infusion pump contained cocaine hydrochloride (NIDA) dissolved in normal saline at 4 mg cocaine/ml of solution. Each infusion delivered an infusion volume of 0.125 ml/kg body weight, hence the dose of cocaine self-administered was 0.5 mg/kg/infusion. The MED-PC software program recorded the number of active lever presses, inactive lever presses and the number of infusions.

2.6. Self-administration protocol (days 1-15):

The animals having patent indwelling catheters were subjected to self-administration training for a period of 15 days with one session each day. Self-administration training sessions were 90 minutes in duration. Upon entry into the self-administration environment, the house light and the ventilation fan were on. In addition to triggering an infusion, active lever presses had the following programmed consequences: the house light was turned off, and the active lever light/tone (i.e. the CS) was turned on for a period of 30 seconds. Additional responses on the active lever during this 30 second period had no programmed consequences, although the program continued to count the number of active/inactive lever presses and infusions. This

"timeout" period protected the animals from cocaine overdose. After this 30 second period the lever light and tone were terminated and the house light came back on. Rats were initially trained for 12 days on an FR-1 (fixed ratio schedule-1) schedule in which each active lever press outside the timeout period triggered the programmed consequences. For the last 3 days of self-administration training, an FR-3 schedule was imposed where 3 active lever presses outside the time out period were required to trigger an infusion and the CS. Each rat was placed in the same operant conditioning chamber throughout the course of the experiment.

2.7. Extinction protocol (days 16-18):

After the 15 days of self-administration training, the animals were divided into 3 groups (balanced for cocaine intake): 1) extinguished (saline), 2) extinguished ((±) CPP-100ng), 3, extinguished ((±) CPP-200ng). All groups received respective treatments by intra-hippocampal injections 2 hours prior to extinction training.

Procedure for intra-hippocampal injections:

The respective treatments were delivered by intra-cranial injections to a conscious, restrained animal by using a 28 gauge internal cannula. Prior to injection, the patency of the guide cannula was confirmed using a spare internal cannula. The maximum volume injected to one side was restricted to 0.5µl and was administered with the help of a 10µl Hamilton syringe connected to the internal cannula through a PE-50 tubing. The injection was given at a rate of 0.1µl/min using a micro infusion pump. A total of 5 minutes was taken for the injection and after the injection; the internal cannula was left in position for about 1 min to allow the drug to diffuse into the intended brain region and then withdrawn slowly. The dummy cannula was replaced and the animal was returned to the home cage. All the groups underwent extinction training (90 min extinction sessions for 3 days) following their respective daily pharmacological treatments.

During their extinction training sessions, the animals in the operant chambers were attached to the drug tether but exposed only to the environment stimuli (i.e. diffuse, contextual cues). Responses on the active lever had no programmed consequences during the extinction training phase. For protocol days 16-18, responses on both active and inactive levers, as well as the equivalent "number of infusions" were counted by the software (although as stated above, syringe pumps were not activated during this phase of training). Extinction proceeded for a period of 3 days, with one 90 minute session each day during which the animals were taken to the operant chambers. Under these conditions, the animals extinguished their lever pressing behavior to less than 20% of their former activity during self-administration.

2.8. Reinstatement tests (protocol days 19):

On day 19, all the animals were placed back in the operant chambers for reinstatement tests. The reinstatement test session conditions were similar to an extinction session in that the animals were exposed only to the contextual cues of the operant chamber environment and the active lever responding were not reinforced by the contingent availability of either CS or US.

2.9. Reinstatement to the contextual drug stimuli:

On test day 19, response to the contextual prime was assessed from active lever presses during the first 10 minutes in which the animals were exposed only to the contextual cues of the drug environment.

2.10. Reinstatement to the CS cues:

Later during the same test session on day 19, lever presses evoked in response to a CS presentation were then assessed. A single, non-contingent presentation of the CS was delivered at the 40^{th} minute of the 120 minute test session. Thus, the initial 40 minutes of the 120 minute session served as an extinction period to allow lever presses initiated by exposure to contextual

stimuli to subside before the CS reinstatement test. As the CS was expected to evoke an immediate response from animals, the noncontingent CS was quantified as the number of lever presses during the subsequent 10 minutes following the priming event (t=40-50 min).

Reinstatement to the drug prime: Response to the drug prime stimulus was assessed later in the same 120 minute session on day 19. We tested the reinstatement of drug seeking behavior using 0.5 mg/kg of cocaine at time=80 minutes of the 120 minute session by delivering a single non-contingent intravenous cocaine infusion. Again, the 30 minutes interval after the CS prime served as an extinction period which allowed lever presses initiated by exposure to contextual stimuli and the CS prime to subside before the US reinstatement test. Drug seeking behavior elicited by cocaine was quantified from the number of responses on the active lever following the drug prime for 30 minutes immediately after the priming event (t=80-110 min).

2.11. Drugs & Dosage Justification:

Cocaine hydrochloride was a gift from NIDA (RTI). The NMDA receptor antagonist 3(-2 carboxipiperazin-4-yl) propyl-1-phosphonic acid ((±) CPP) was obtained from Sigma (St. Louis). (±) CPP at a dose of 100 ng and 200ng were administered approximately 2 hours prior to the extinction sessions on protocol days 16-18. The doses were selected based on the previous reports (Escobar et al., 1998). In intra-cranial injections, usually state dependent learning is not discussed as a potential problem as opposed to systemic treatments. (±) CPP at the indicated doses did not produce any acute locomotor effects. This is well supported by the extinction performance of the drug treated groups as compared to the performance of the saline treated groups.

2.12. Statistics:

The number of active lever presses, infusions and inactive lever presses were recorded for each session. These data were used to calculate the responses during each experimental session. A value of $p<.05$ was taken as significant, being determined from planned comparisons. All the statistics were done using SigmaStat software

3. Results

This was only a preliminary study designed to evaluate the involvement of NMDAR mediated mechanisms in the ventral hippocampus responsible for the effects of extinction on the reinstatement. For this reason, we decided to avoid having an abstinent group as a positive control for the effects of extinction on reinstatement. We were interested to see whether the effects of extinction on reinstatement are altered by the intra-hippocampal injections of (±) CPP during extinction. From our previous studies, we have observed that the animals' lever pressing response diminishes to a floor level by about day 3 of extinction training. Keeping this observation in mind, we decided to reduce the number of extinction days to 3 from 5 for this study, so that we can limit the days of intra-hippocampal injections to 3 and this will help us in avoiding any possible damage to the brain tissues due to frequent intracranial injections. Though we tried 2 different doses of (±) CPP (high-200ng and low-100ng), we did not observe any difference in the extinction or reinstatement behavior of the groups treated with high and low dose. Hence the results from both the groups are combined together.

3.1. Cocaine self-administration and extinction of the drug seeking behavior:

Animals having indwelling jugular catheters were trained to self-administer cocaine in an operant chamber environment for 15 consecutive days. During the daily 90-minute sessions, rats were initially trained on an FR-1 schedule for first 12 days and switched to an FR-3 schedule for

the last 3 days of self-administration training. The transition to the FR-3 schedule was done to increase the number of active lever pressing responses. Animals typically achieved stable self-administration by day 10 of training, and the FR-3 schedule did not significantly alter the number of earned infusions per session (Figure5.1A). Over the entire fifteen day self-administration training phase the average total number of infusions earned was 316 ± 44, or the equivalent of approximately 10.53 mg/kg/day of cocaine per animal. Average number of infusions earned per animal per day is shown for the last 3 days of self-administration (Figure 5.1B). There was no significant difference in the average number of infusions earned per animal among the two different groups of self-administration rats utilized for the reinstatement studies described in this report. (data not shown).

3.2. Blockade of NMDAR activity in the ventral hippocampus in the effects of extinction training on reinstatement behavior.

The requirement of NMDAR activity in the ventral hippocampus, in the extinction process was evaluated in the group of extinguished rats given intra-hippocampal injections of (±) CPP before extinction training. After 15 days of SA, the animals were subjected to an extinction training phase in the same operant chamber environment for 90 minute session in the absence of both CS and US (i.e. the active lever had no programmed consequences) for only three days (Figure 5.2, protocol day 16,17 and 18). Active and inactive lever presses were monitored during the extinction sessions and it was found that both the groups extinguish their drug seeking behavior under these conditions similarly. The extinction learning process was not affected by blocking the NMDAR activity in the ventral hippocampus during extinction. This finding was similar to that we observed with the peripheral injection of (±) CPP at 5mg/kg dose.

3.3. Reinstatement of drug seeking behavior:

Evaluation of drug seeking behavior within this single extinction session illustrates that the majority of lever pressing activity occurs during the initial ten minutes in the operant chamber environment, suggesting that environmental contextual cues are priming this response. Following this initial burst of activity, active lever pressing diminishes rapidly. Active lever responses are minimal (< 4) by 20-30 minutes and remain low for the remainder of the 90 minute session (data not shown). Therefore, during the reinstatement experiments involving the non-contingent presentation of either CS or US stimuli, the first priming event was delivered after waiting for 40 minutes of the test session. A temporal distinction can thus be made between the drug-seeking activities induced by introduction to the operant chamber environment (i.e. activity during the first ten minutes) versus the subsequent activity induced via non-contingent presentation of priming events delivered later within the same test session.

3.4. Effects of blockade of NMDAR activity in the ventral hippocampus during extinction training on the reinstatement behavior.

The resumption of lever-pressing activity was induced using three forms of priming stimuli: contextual cues, conditioned cues, and drug prime. Once the animals underwent extinction training for a period of 3 days (day 16,17 and 18), they were tested for the resumption of drug seeking behavior following exposure to the contextual stimuli and the conditioned stimuli and to cocaine on day 19 at different time points of the same test session. On the reinstatement test day, the animals were drug free.

3.5. Effects of blockade of NMDAR activity in the ventral hippocampus during extinction on the contextual reinstatement

The resumption of drug seeking induced by diffuse environmental cues was assessed during the first ten minutes of the test session conducted on day 19((Figure 5.3A). The level of responding on the active lever for the (±)CPP treated group was found to be significantly greater than that of the saline treated control group ($p<.05$, unpaired 't' test). These results suggest that the NMDAR blockade during extinction training experience impaired the efficacy of the extinction training to decrease the response provoked by the contextual cues present in the operant chamber.

3.6. Effects of blocking NMDAR activity in ventral hippocampus during extinction training on the CS induced reinstatement

Until time=40 min of the day 19 reinstatement test session, the animals experienced extinction conditions. At this point, a single, non-contingent presentation of the discrete CS complex was delivered for a period of 30 seconds, and active lever responding for the next 10 min was measured as an indication of the reinstatement of drug seeking behavior evoked by the CS (Figure 5.3B). As previously described for extinction conditions, active lever presses had no programmed consequences at any time during these test sessions. In addition to the active lever presses, the inactive lever presses were also monitored, so as to ensure that the non-contingent CS prime was in fact inducing activity previously associated with cocaine infusion. Inactive lever presses averaged to less than 1 in the post prime responses of all the groups (data not shown). In order to confirm that any responding due to contextual cues had subsided by the time of the CS reinstatement test, active lever pressing during a "pre-prime" ten minute period of time (t=30-40) was also assessed and compared with the cue-induced "post-prime" level of

reinstatement (t=40-50). Results for both the extinguished (saline and (±) CPP treated groups) groups of rats tested in this manner are illustrated for 10 minutes before and following the CS priming event. Paired 't' tests within each group indicates that the non-contingent CS prime was not able to reinstate the drug seeking behavior in the (±) CPP treated group and in the saline treated control group. We were not able to conclude anything from the pairwise comparison of the post priming data between the 2 groups, though the response of the (±) CPP group turned significantly greater ($p<.05$, unpaired 't' test) compared to the control group since we had the floor effect problem in response to the CS prime in the control group.

3.7. Effects of blocking NMDAR activity in the ventral hippocampus during extinction training on the US induced (cocaine prime) reinstatement

Finally, cocaine-induced reinstatement was tested at time = 80 minutes of the reinstatement test session on day 19. A single, non-contingent intravenous infusion of drug was delivered at a dose of 0.5mg/kg at time=80 min of the session. The active lever responding during the next 30 minutes was measured as an indication of the reinstatement of drug seeking behavior evoked by the US stimuli. The post-prime response (80-110 min) on the active lever was compared to the 30 minute pre prime response (50-80 min) to assess the reinstatement within each group as illustrated in figure 5.3C. Inactive lever presses averaged to less than 1 in the post-prime responses of all the groups (data not shown).

The post prime responses in both the groups were not significant compared to the pre prime responses in this preliminary study. However, the reinstatement response of the cocaine prime was in the expected direction for the (±) CPP group compared to the saline treated control group. This study has to be repeated with more animals to reach at any solid conclusion about the

requirement of NMDAR activity in the ventral hippocampus during extinction for extinction to be effective in reducing the levels of reinstatement response.

4. Discussion

This was a preliminary study designed to study the requirement of NMDAR activity in the ventral hippocampus during extinction for the extinction to be effective in reducing the reinstatement response. Though we had 2 groups treated with (±) CPP during extinction by intra hippocampal injections, (one low dose 100ng and another high dose 200ng) we ended up combining the data from both the groups because they were not different from each other. We did not observe any acute locomotor effects after the injections of the drug and their extinction performance was similar to that observed in the saline treated control group. The animals in the treatment and control groups were extinguished at a similar rate before they were tested for the reinstatement response on a drug ((±) CPP) free day.

The results from the contextual reinstatement response was unexpected because of the significantly greater activity for the group extinguished under (±) CPP treatment as compared to the control group. Ventral hippocampus is not reported to involve in contextual reinstatement, although the dorsal hippocampus is (Fuchs et al., 2005). Hence, our assumption here is that at least some quantity of the drug infused intracranially might have diffused dorsally due to the osmotic effect and reached the dorsal hippocampus. This might have blocked the activity of NMDAR in the dorsal hippocampus during extinction. Since this is only an assumption at this point, we can not conclude anything from this effect of treatment on contextual response.

The data from the non-contingent CS prime-induced reinstatement test was also not conclusive since the control group had the floor effect problem in primed response. Since an effect of priming was not evident for the control group, it is difficult to conclude any effect of

treatment on the CS prime response. For the non-contingent US (cocaine) induced reinstatement test, there was some effect of priming in both the groups though they were not significant. The US-induced response in the (±) CPP treated group was shifted in the expected direction compared to the response of the control group. Ventral hippocampus is reported to be an important neurobiological substrate mediating the CS-and US-induced reinstatement by inactivation studies of the region using bilateral infusion of lidocaine (Sun and Rebec, 2003) and baclofen-muscimol (Rogers and See, 2007).

From this study, we were not able to derive any solid conclusion about the requirement of NMDAR activation in the ventral hippocampus during extinction for the extinction to be effective in reducing the reinstatement response. This study has to be repeated with more animals to reach at any solid conclusion regarding the necessity of this particular NMDAR dependent mechanism in the ventral hippocampus for the efficacy of extinction on reinstatement.

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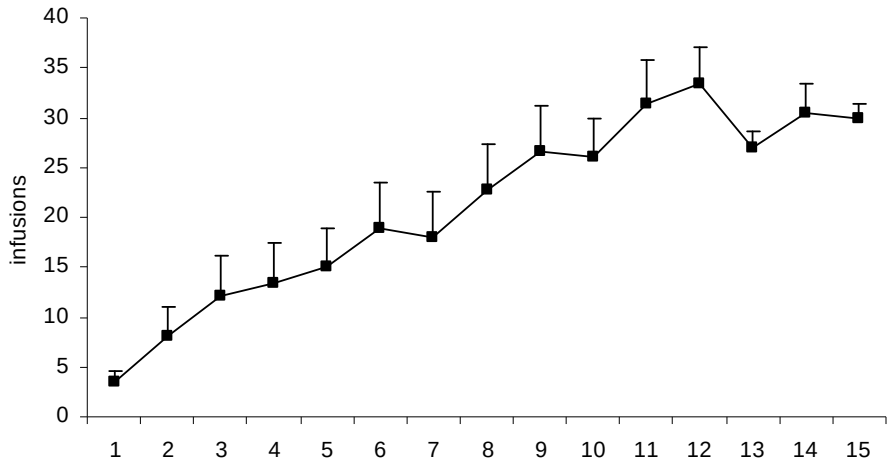

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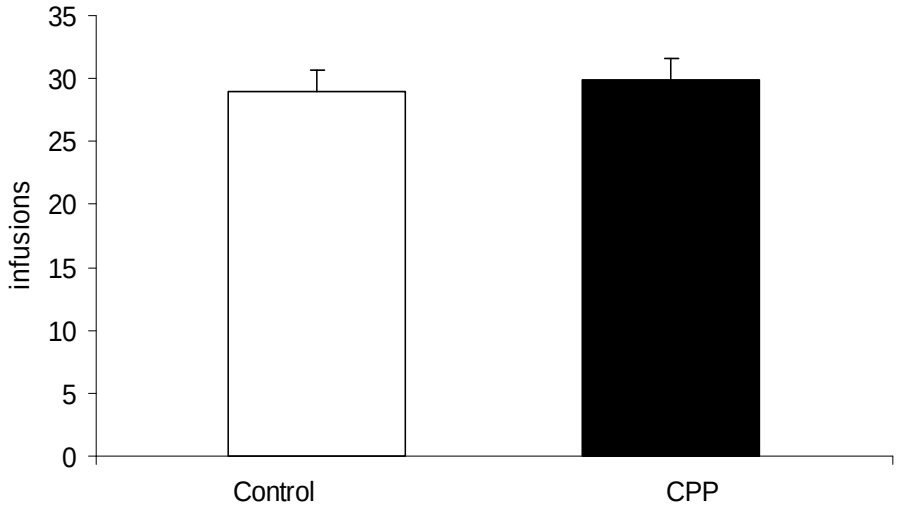

Figure 5.1 Self-administration of cocaine:

(A) The results from the cocaine self-administered rats (n=11) illustrate the average number cocaine infusions earned daily during each 90 minute session during the SA phase (days 1-15).

(B) Data illustrate the average number of cocaine infusions self-administered on the last 3 days of self-administration phase for the saline treated control group (n=4) and the (±) CPP treated

group. The 2 groups self administered relatively similar number of infusions on the last 3 days of SA phase.

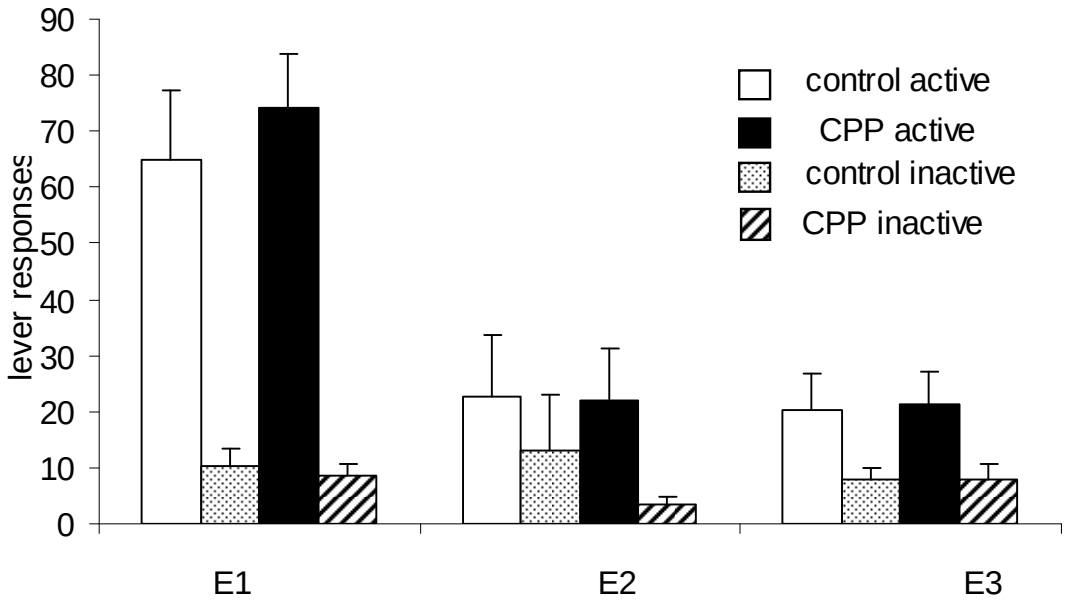

Figure 5.2 Progression of extinction in the control and the (±) CPP treated groups:

Active and inactive lever presses of the cocaine extinguished groups (n=4 for control and n=7 for (±) CPP groups) are illustrated (mean ± SEM) for the 90 minute sessions on each day of extinction (days 16-18). In the absence of both CS and US the animals extinguished their drug seeking behavior over a period of 3 days. Both the control and the treatment groups extinguished at a similar rate. Blocking the NMDAR activity during extinction in the ventral hippocampus did not affect the extinction learning process compared to the performance of the control group.

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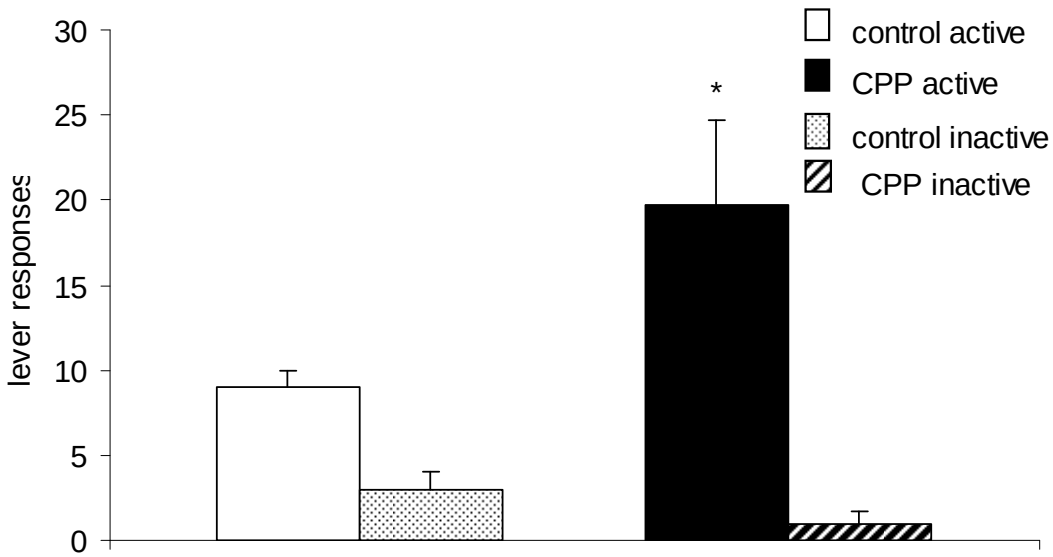
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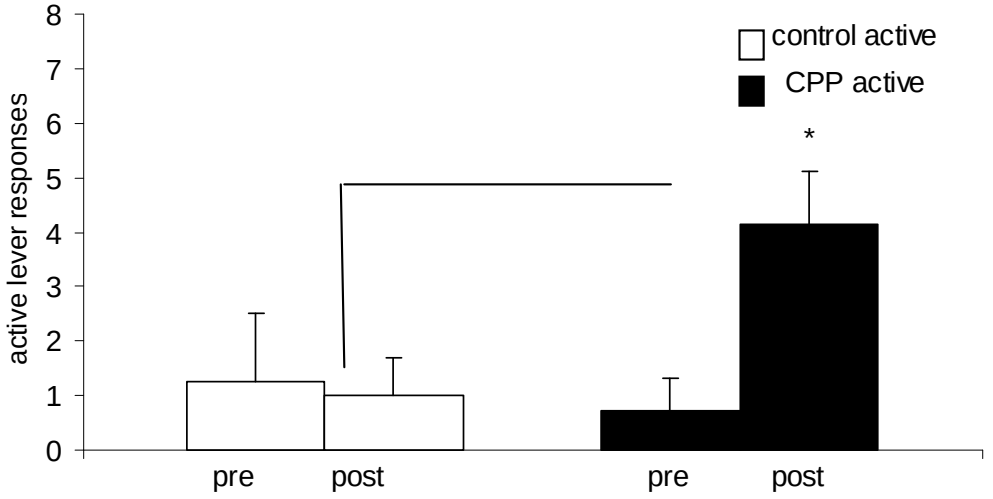
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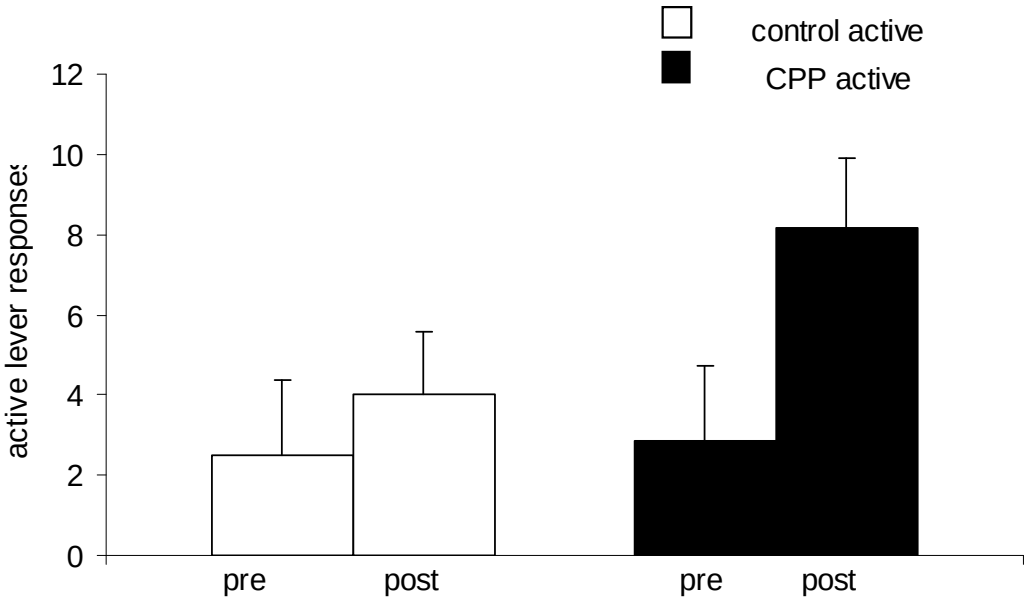

Figure 5.3 Effects of blocking the NMDAR activity in the ventral hippocampus during extinction on the resumption of drug-seeking following exposure to contextual cues, the CS priming and on the response to US priming:

(A) Data show the mean ± SEM of lever presses for the first 10 minute bin of time on the first reinstatement test day (protocol day 19). The intra-hippocampal injection of (±)CPP- during extinction training had a significant effect on responding to the contextual drug stimuli as compared with the saline treated control group ($p<.05$, unpaired 't' test).

(B) Data show the mean ± SEM of active lever presses for 10 minutes before the cue prime (30-40 min, pre) and for 10 minutes after the cue prime (40-50 min, post) on the protocol day 19 for the control (n=4) and the (±) CPP groups (n=7). The non-contingent CS prime was not able to reinstate the drug seeking behavior in the control and the (±) CPP group. Comparison of the post-prime responses between the groups showed that the response of the(±) CPP group as significantly greater compared to that of the control group ($p<.05$, unpaired 't' test).

(C) Data represent the mean ± SEM of lever presses for a 30 minute window before (pre, 50-80 min) and 30 min window after (80-110 min) the single, noncontingent intravenous delivery of a drug prime at 0.5mg/kg dose was administered at time=80 minutes on the reinstatement test day (protocol day 19). For both control (n=4) and (±) CPP group (n=7), the post-prime response was not significantly greater than the pre-prime response at the cocaine dose tested. However, the drug seeking response of the (±) CPP animals was in the expected direction when the post-prime responses of the two groups were considered.

CHAPTER 6

EFFECTS OF ABSTINENCE OR EXTINCTION ON REINSTATEMENT OF COCAINE-SEEKING AS A FUNCTION OF WITHDRAWL DURATION AND TRAINING LATENCY.

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[1]Kelamangalath, L and Wagner, J.J. Submitted to Neurobiology of Learning and Memory, 10/15/2008

Abstract

In this report we have tried to study the persistence of the effectiveness of extinction training and whether the timing of the initiation of extinction training matters for extinction to remain effective in reducing the reinstatement, by utilizing a between subject design. In addition to comparing the reinstatement responses between the groups extinguished and tested at different points of time, we have compared the reinstatement responses of the extinguished groups with that of their time matched abstinent controls also. We found that the abstinent animals incubate their drug seeking behavior in response to the contextual cues of the drug environment, but not to the conditioned cues and to the non-contingent drug prime. When the animals undergone extinction training were tested after 3 weeks of their last exposure to the extinction context, they showed the effect of spontaneous recovery in response to the contextual drug stimuli and to the non-contingent cocaine prime but not to the non-contingent CS prime. Another group of animals extinguished after 3 weeks of enforced abstinence since last day of self-administration when tested after the extinction, showed that the extinction still remained effective in reducing the reinstatement response compared to enforced abstinence. The study suggests that there is not a critical time period for extinction training to be performed for that to be effective in reducing reinstatement and this implies that extinction therapy can be performed in human drug addicts anytime during abstinence as extinction always is advantageous to prevent the chances of relapse compared to enforced abstinence.

1. Introduction

The reinstatement of drug-seeking behavior is a commonly utilized rodent model for studying the aspects of human addiction such as craving and relapse (Shaham et al., 2003); however several issues of interest concerning the characterization of the model remain to be investigated. For example, although the general effectiveness of extinction training is well accepted, the protocols employed have varied widely in terms of number of training sessions, the interval of abstinence following self-administration, the timing of reinstatement test sessions, etc. One item of interest as it may relate to the treatment of human addicts concerns the persistence of extinction effects to reduce drug-seeking behavior. It would be necessary to know what the expected time course of effectiveness would be in order to be able to determine an appropriate treatment schedule. A second area of interest concerns the timing of the initiation of the extinction training protocol with respect to the last drug exposure. Again, practical issues relating to the treatment of human addicts would include determining whether a critical window of time exists following the cessation of drug use, for such a time would influence the effectiveness of any intervention.

As compared with extinction training, enforced abstinence is not an effective means by which to reduce reinstatement of drug-seeking, as increasing periods of withdrawal have been shown to enhance both self-reported cravings in humans and drug-seeking behavior in rodent reinstatement tests (Grimm et al., 2001 & Tran Nguyen et al., 1998). This "incubation effect" has been suggested to be an important component of the persisting susceptibility for relapse (Grimm et al., 2001). In order to compare the effectiveness of extinction over time, it is necessary to have time-matched abstinence controls to account for any influence of incubation that could confound interpretation of the extinction training results. As a consequence, using this

comparative design we have also obtained results from these abstinent control groups for three different forms (context, CS, drug) of primed resumption of drug-seeking behavior, and found that incubation is not evident in all of these cases.

With respect to the rat model of reinstatement of drug-seeking, we are aware of only one published study that systematically assessed the persistence of the effectiveness of extinction training (Ciano & Everitt 2002), and none that characterize the temporal component for the initiation of extinction training. This report provides evidence demonstrating that the effectiveness of extinction training to reduce reinstatement can persist at least three weeks, and that a three week delay in the initiation of extinction training does not prevent reductions in reinstatement behavior. The significance of both of these observations depends on the form of reinstatement being assessed. These preclinical findings using the rodent self-administration/reinstatement model demonstrate that behavioral therapies based on extinction principles can exhibit both the persistence and temporal characteristics consistent with practical benefits for the treatment of addiction in humans.

2. Materials and Methods

Male Sprague-Dawley rats (Harlan) weighed approximately 300 g at the beginning of the experiment and were housed individually in a temperature and humidity controlled vivarium having a 12 hour light/dark cycle (lights off at 7:00 P.M.). They were given access to food and water *ad libitum* and were handled daily for 5 days prior to the surgery in order to diminish stress associated with handling. The housing and experimental procedures followed the *Guide for the Care and Use of Laboratory Animals* and were approved by the local ACUC at the University of Georgia.

Jugular vein catheterization: The animals were anesthetized using a combination of ketamine (75 mg/kg), xylazine (10 mg/kg) and acepromazine (1 mg/kg) administered i.p. Depth of anaesthesia was assessed by monitoring respiration rate and palpebral and pedal withdrawal reflexes. Under anesthesia, the right jugular vein was isolated and cleaned. The catheter was exteriorized by passing it subcutaneously to the base of the skull, where it was connected to a modified 22 gauge cannula. A silastic catheter (Dow Corning) was then inserted into the vein (4-5 cm) and secured in position with silk sutures (6/0). The animal was then placed in a stereotaxic frame (Stoelting), where the right-angled cannula (Plastics One) was mounted to the top of the skull using dental cement and 4 screws. Immediately after surgery, and once daily for 5 days, the animals were treated with gentamicin at a dose of 5 mg/kg (i.v.). The catheters were flushed every day with saline prior to each self-administration session and with heparin (10 USP/ml) after the session to maintain the patency of the catheter. Catheter patency was verified daily by drawing blood from the catheter.

The experiment was performed in an operant chamber environment. The operant chambers (Med associates) were equipped with 2 levers, one “active” and another “inactive” with lights positioned above each lever. The chambers had a rod grid floor, a house light, a speaker/tone generator (2.9 kHz, 10 dB above ambient) and were housed inside enclosures equipped with ventilation fans. A syringe pump was located outside the enclosure. The method for delivering a cocaine infusion was as follows: The modified 22 gauge cannula mounted on the rat’s skull was connected to a liquid swivel with PE-50 tubing protected by a metal spring. The swivel was then connected with tygon tubing to the syringe mounted in the infusion pump. Infusion volumes were calculated according to the animal’s weight. For cocaine animals, the syringes mounted in the infusion pump contained cocaine hydrochloride (NIDA) dissolved in

normal saline at 4 mg cocaine/ml of solution. Each infusion delivered an infusion volume of 0.125 ml/kg body weight; hence the dose of cocaine self-administered was 0.5 mg/kg/infusion. The MED-PC software program recorded the number of active lever presses, inactive lever presses and the number of infusions.

The animals having patent indwelling catheters were subjected to self-administration training for a period of 15 days with one session each day. Self-administration training sessions were 90 minutes in duration. Upon entry into the self-administration environment, the house light and the ventilation fan were on. In addition to triggering an infusion, active lever presses had the following programmed consequences: the house light was turned off, and the active lever light/tone (i.e. the CS) was turned on for a period of 30 seconds. Additional responses on the active lever during this 30 second period had no programmed consequences, although the program continued to count the number of active/inactive lever presses and infusions. This "timeout" period protected the animals from cocaine overdose. After this 30 second period the lever light and tone were terminated and the house light came back on. Rats were initially trained for 12 days on an FR-1 (fixed ratio schedule-1) schedule in which each active lever press outside the timeout period triggered the programmed consequences. For the last 3 days of self-administration training, an FR-3 schedule was imposed where 3 active lever presses outside the time out period were required to trigger an infusion and the CS. Each rat was placed in the same operant conditioning chamber throughout the course of the experiment.

After the 15 days of self-administration (SA) training, the animals were divided into 5 groups (balanced for cocaine intake): 1) extinguished starting the day after SA, continued for five days, and then tested one day after this "early" extinction (EET1). 2) extinguished starting the day after SA, continued for five days, and then tested twenty four days after early extinction

(EET24). 3) extinguished starting twenty four days after SA, continued for five days, and then tested one day after this "late" extinction (LET1). 4) abstinent for six days after SA and tested on this day with out any prior extinction training experience (A6). 5) abstinent for thirty days and tested on this day with out any prior extinction training experience (A30). During their extinction training sessions, the animals in the operant chambers were attached to the drug tether but exposed only to the environment stimuli (i.e. diffuse, contextual cues) of the operant chamber. Responses on the active lever had no programmed consequences during extinction training. During the extinction sessions, responses on both active and inactive levers, as well as the equivalent "number of infusions" were counted by the software (although as stated above, syringe pumps were not activated during this phase of training). Extinction training proceeded over a period of 5 days, with one 90 minute session each day during which the animals in the extinction training groups (EET1, EET24 and LET1) were taken to the operant chambers. Under these conditions, the animals extinguished their lever pressing behavior to less than 20% of their former activity during self-administration. Thus, extinction training was started either "early"-one day after the self-administration phase (EET1 and EET24) or "late"-after waiting 24 days after the last SA day (LET1). As previously mentioned, the abstinent group animals remained in their home cages until their test day except for the A30 group which were moved to an alternate environment for 90 minutes on last 5 days of abstinence (days 25-29) before they were tested on day 30. On each group's reinstatement test day, the animals were placed back in the operant chambers for reinstatement tests. The reinstatement test session conditions were similar to an extinction session in that the animals were exposed only to the contextual cues of the operant chamber environment and the active lever responding were not reinforced by the contingent availability of either CS or US.

On each group's first reinstatement test day, response to the diffuse contextual cues was assessed from active lever presses during the first 10 minutes during which the animals were exposed only to the contextual cues of the drug environment. Later during the same test session, lever presses evoked in response to a CS presentation were then assessed. As the CS is expected to evoke an immediate response from animals, we delivered a single, non-contingent presentation of the CS at the 40th minute of the 90 minute test session. Thus, the initial 40-minutes of the 90 minute session served as an extinction period to allow lever presses initiated by exposure to contextual stimuli to subside before the CS reinstatement test. The response to the non-contingent CS was quantified as the number of responses on the active lever over ten minutes following the priming event (t = 40-50 min), and compared with those from the 10 minutes prior to the priming event (t = 30-40 min).

Response to the drug prime stimulus was assessed on the second test day. We tested the reinstatement of drug seeking behavior using the same dose as the self-administered dose (0.5 mg/kg) of cocaine. A single, noncontingent drug prime was programmed to be infused intravenously by the syringe pump at the 40th minute of the 90 minute session on this US reinstatement test day. Again, the initial 40 minutes of the 90 minute session served as an extinction period which allowed lever presses initiated by exposure to contextual stimuli to subside before the US reinstatement test. Drug seeking behavior elicited by the cocaine prime was quantified as the number of responses on the active lever over 30 minutes following the drug prime (t = 40-70 min), and compared with those from the 30 minutes prior to the priming event (t = 10-40 min).

In experiment 1, abstinent groups of rats were tested for their responses to the contextual stimuli, non-contingent cue prime and the non-contingent drug prime as a function of the

withdrawal period (A6 vs. A30). Also note that, as described above, these abstinent animals experienced extinction conditions during the first 40 min of the reinstatement test sessions. In experiment 2, both the persistence of the effects of extinction (EET1 vs. EET24) and the effectiveness of delayed extinction training (EET1 vs. LET1) to reduce the reinstatement responses were assessed. The animals in experiment 2 were subjected to the same series of reinstatement tests as described for the animals in the experiment 1.

Drugs: Cocaine hydrochloride was a gift from NIDA (RTI).

3. Results

3.1. Self-administration training

The mean ± SEM of the infusions during the last 3 days of cocaine self-administration training for the five different groups tested in this study was 27.7±1.7. There was no significant difference in the mean number of infusions self administered by the five different groups of animals ($p>.05$, one way ANOVA). A saline self-administration group from a previous study averaged less than 3 infusions/day (Kelamangalath et al., 2007).

3.2. Experiment 1: Drug-Seeking Response in Abstinent Rats

Three different groups of animals kept abstinent for different withdrawal periods were tested for their reinstatement response to the diffuse contextual cues present in the drug taking environment. Two of these groups of abstinent animals were also tested for their reinstatement response to a single, non-contingent presentation of either the compound CS or an intravenous drug infusion as described previously (Kelamangalath et al., 2007). None of the abstinent animals tested in this experiment were exposed to extinction conditions (i.e. they remained in their home cages) until their respective reinstatement test sessions were initiated.

3.3. Response to the diffuse contextual cues:

Drug seeking behavior in response to contextual cues present in the self-administration chamber was assessed on the initial reinstatement test by quantifying the active lever responses during the first 10 minutes of the session. Figure 6.2A illustrates the non-reinforced responses on the active and inactive levers following different periods of withdrawal in the groups tested. The A1 group was tested after one day of abstinence, group A6 was tested after 6 days of abstinence and group A30 was tested after 30 days of abstinence. Analyses by one way ANOVA showed a significant effect of withdrawal period on active lever responding in the A30 group as compared to the A6($p<.05$, $t=3.044$) and A1($p<.05$, $t=4.781$) groups. Responses on the inactive lever were not different among the abstinence groups tested in this experiment. These results indicate that the drug seeking behavior in response to the diffuse contextual drug cues can incubate over time in abstinent animals, in agreement with previous reports (Tran-Ngyuen, et al. 1998; Grimm et al, 2001).

3.4. Response to the non-contingent CS:

The A6 and A30 groups were also tested for their response to the non-contingent CS (light/tone) during the first reinstatement test session. Figure 6.2B illustrates the active and inactive lever presses for both the 10 minutes before ("pre") and 10 minutes after ("post") delivery of the compound CS for the A6 and A30 groups. In the A6 group, the post prime response on the active lever was significantly greater than that of the pre prime response ($p<.05$, one way ANOVA, Holm-Sidak). Similarly, in the A30 group, the post prime response was significantly greater as compared to the pre prime response ($p<.01$, one way ANOVA, Holm-Sidak). Comparison of the post CS prime responses between the A6 and A30 groups shows that the drug seeking behavior in response to the non-contingent CS prime does not incubate as a

function of the additional 24 day withdrawal period, as they did not significantly differ in their reinstatement response ($p=.615$, one way ANOVA). This is in contrast to the findings above concerning the diffuse contextual drug cues.

3.5. Reinstatement response to the non-contingent drug prime:

The A6 and A30 groups were also tested for their response to a single, non-contingent drug prime (cocaine i.v. at a dose 0.5mg/kg) on the second reinstatement test day. Figure 6.2C represents the active and inactive lever presses for 30 minutes before ("pre") and after the drug prime ("post"). The post prime responses on the active lever in both the A6 and A30 groups were significantly greater than their respective pre prime responses (one way ANOVA, $p<.05$ for A6 and $p<.01$ for A30 groups). Similar to the results with the CS primed reinstatement test, a comparison of drug prime response between the A6 and A30 groups indicates that the drug seeking behavior in response to the non-contingent cocaine prime does not incubate over the additional 24 day withdrawal period in these abstinent animals, as they did not significantly differ in their reinstatement response (one way ANOVA, $p>.05$). Again, this is distinct from the findings above concerning the diffuse contextual drug cues.

3.6. Experiment 2: Drug-Seeking Response in Extinguished Rats

Three different groups of animals were given extinction training for 90 minute extinction sessions for a period of 5 days and then tested for reinstatement of drug-seeking behavior at different time points from their last extinction session (Fig 6.3). One group of animals were extinguished beginning 1 day after their last self-administration session, this group was then tested for reinstatement responses 1 day after the last extinction session. Thus, this "early extinction" group "tested 1" day later for reinstatement was designated group "EET1". A second group was also extinguished beginning 1 day after self-administration, but reinstatement testing

was not initiated until 24 days after the last extinction session. This "early extinction" group was "tested 24" days later for reinstatement and was designated as group "EET24". A third group was not extinguished until 24 days after the last self-administration session, this group was then tested for reinstatement responses 1 day after the last extinction session. This "late extinction" group was "tested 1" day later for reinstatement and was designated as group "LET1". The rate of extinction over the 5-day extinction training period was similar among all three groups and the amount of active lever responding by last day of extinction training was not significantly different (data not shown).

3.7. Response to the diffuse contextual cues:

All three extinguished groups were tested for their drug seeking behavior in response to the contextual drug stimuli (Fig 6.3A) as described above. The EET1 and LET1 groups were tested on the first day after their last extinction session. Regardless of whether extinction was experienced 1 day after the last self-administration session (EET1) or whether the extinction was experienced 24 days after the last self-administration session (LET1), these animals showed a similar level of responding. However, we observed a significant increase in the contextual response from the animals extinguished 1 day after the last self-administration session, but not tested for reinstatement until 24 days since last extinction session (EET24). Analyses by one way ANOVA showed a significantly higher responding on the active lever in this group ($p<.001$) as compared to the groups tested on the next day after their last extinction session.

3.8. Response to the non-contingent CS:

The extinguished animals were tested for their response to the non-contingent CS during the first reinstatement test session. Figure 6.3B illustrates the active and inactive lever presses for both the 10 minutes before ("pre") and 10 minutes after ("post") delivery of the compound CS

for the EET1,LET1 and the EET24 groups. In all the 3 groups the post prime response on the active lever was significantly greater compared to their pre prime response ($p<.05$, one way ANOVA, Holm-Sidak). The comparison of the post prime responses between these extinguished groups showed no significant difference (one way ANOVA, $p>.05$).

3.9. Response to the non-contingent drug prime:

The three extinguished groups were also tested for their response to a single, non-contingent drug prime (cocaine i.v. at a dose 0.5mg/kg) on the second reinstatement test day. Figure 6.3C illustrates the active and inactive lever presses for 30 minutes before ("pre") and after the drug prime ("post"). The post prime responses on the active lever in the EET1, EET24 and the LET1 groups were significantly greater than their respective pre prime responses (one way ANOVA, $p<.01$ for all groups). The comparison of the post prime responses between these extinguished groups showed a significantly greater reinstatement response for EET24 as compared to EET1 ($p<.01$, one way ANOVA).

3.10. Comparison between the extinguished and abstinent animals:

In this study, we were interested in characterizing both the persistence of the effects of extinction training as well as exploring the temporal component for the initiation of such training following the end of drug self-administration. To fully evaluate these parameters, we needed to compare the effects of extinction training to those of equivalent periods of abstinence. We have previously reported that extinction training is effective in reducing reinstatement responding to contextual cues, non contingent CS, and non contingent drug prime events (Kelamangalath, et al., 2007), as illustrated between Figs 2.2&2.3 (compare A6 vs. EET1). In the current study, regarding the reinstatement response elicited by exposure to diffuse contextual cues (Figs 6.2A&6.3A), we have found that both the EET24 and LET1 groups still significantly reduced

reinstatement responding ($p<.001$, one way ANOVA, Holm-Sidak) compared with their abstinent time-matched controls (vs. A30). These findings demonstrate that the effects of extinction training persist for more than 3 weeks concerning this form of reinstatement, and that such a delay in initiating extinction training does not prevent a significant reduction in responding.

With respect to the reinstatement response elicited by exposure to CS cues (Figs 6.2B & 6.3B), we found that compared with their respective time-matched abstinent controls, only the EET1 group significantly ($p<.05$, two way ANOVA, Holm-Sidak) reduced reinstatement responding. These results suggest that in the case of the EET24 group, the effectiveness of extinction training to reduce CS reinstatement does not persist over this time period and in the case of the LET1 group, the effectiveness of extinction training to reduce CS reinstatement is diminished if training is delayed following the final day of self-administration. Finally, when the reinstatement response was elicited by exposure to drug prime (Figs6.2C&6.3C), we found that both the EET1 and the LET1 groups significantly ($p<.05$, two way ANOVA, Holm-Sidak) reduced reinstatement responding as compared with their respective abstinent control groups (A6 & A30). These results suggest that in the case of the EET24 group, the effectiveness of extinction training to reduce US reinstatement decays over this 24 day time period following the final day of self-administration.

4. Discussion

In this study we investigated the time-dependent increase in the drug seeking behavior induced by the different withdrawal periods in animals having previously self-administered cocaine. A between subject design was utilized in which different groups of animals were tested at different time points. We found that the drug seeking behavior in response to the diffuse

contextual cues of the self-administration chamber increase in a time dependent manner, such that animals tested after a withdrawal period of 30 days exhibited an increased response than those tested after a withdrawal period 6 days. In contrast, we did not observe a time dependent increase in the noncontingent cue-induced reinstatement response nor the noncontingent drug-induced reinstatement response in the animals tested at these same withdrawal time points. In the second part of this study our aim was to investigate two aspects of practical importance regarding the effects of extinction on reinstatement of drug seeking behavior: The first was to assess the persistence of the effects of extinction on reinstatement. The second was to determine whether there was evidence for a critical time period within which extinction training should be initiated for it to be effective in reducing the reinstatement response. Details concerning these results and their implications are outlined below.

Our previous findings indicate that the extinction training protocol we have employed is effective in reducing the reinstatement response to diffuse contextual cues, non-contingent cue prime and non-contingent drug prime (Kelamangalath, et al., 2007). These results were obtained from initiating the extinction training the next day following 15 days of cocaine self-administration (illustrated herein as the EET1 group). In comparison, the data from the reinstatement tests for the EET24 group suggests that the effects of extinction to reduce the reinstatement levels in response to certain stimuli such as the diffuse contextual cues and the drug prime may not be persistent while it might be still effective in reducing the reinstatement response to the non-contingent cue prime. The reinstatement data from the LET1 group suggest that the extinction still remains effective in reducing the reinstatement response even if there is a delay in the initiation of extinction training.

4.1. Time-dependent effects on drug seeking behavior in the abstinent animals

The present data shows a time dependent increase in the drug seeking behavior in response to the diffuse contextual cues of the drug taking environment among the abstinent animals as a function of the withdrawal period. We observed incubation like effect in response to the diffuse contextual cues when the abstinent animals were introduced to the operant chamber environment for the first time after 30 days of withdrawal. Here our results agree with the findings of other investigators where they found a resistance to extinction among animals kept as abstinent for longer withdrawal periods than the ones kept as abstinent for shorter withdrawal periods (Tran-Nguyen et al,1998; Grimm et al,2001,2003). Concerning the cue induced reinstatement study, these investigators had used a contingent method where the discrete cues associated with the drug availability were presented in a response contingent manner and they observed either modest time dependent changes (Tran-Nguyen et al, 1998; Neiswander et al, 2000; Semenova and Markou ,2003) or no changes (Di Ciano and Everitt,2002; Deroche- Gamonet et al,2003 and Marinelli et al,2003). In contrast others have found a time dependent increase in the drug seeking behavior using this paradigm of cued reinstatement study (Grimm et al, 2001, 2003 and Lu et al, 2004). Though the cocaine seeking behavior is operationally defined as the non-reinforced lever pressing behavior, the contingent method of cued reinstatement study utilizes the reinforced manner of lever pressing where the animal's lever pressing behavior is reinforced by the response contingent presentation of the cue. Because of this issue, the validity of this method of cued reinstatement protocol is questionable in modeling the human condition of relapse in which self reported craving can be triggered by the passive exposure to these cues. As craving in humans can be induced via the non-contingent presentation of these cues, it has been suggested that the CS reinstatement protocol should employ this form of priming as well (Katz and

Higgins, 2003). Here in our study we have utilized a single non-contingent passive presentation of the CS to study the cue induced reinstatement response. With our protocol we did not observe an incubation of the cued reinstatement response.

Several recent studies have investigated the incubation like effect on the drug induced reinstatement of drug seeking behavior. It is common for addicts to experience a lapse in abstinence and such re-exposure to drugs can quickly reinstate the drug seeking behavior. Hence it is important to study the degree of vulnerability to drug induced relapse among animals kept as abstinent for longer withdrawal periods so as to determine whether this follows the incubation pattern. Three studies assessed the time dependent changes in the drug seeking behavior induced by cocaine priming and found that the drug seeking behavior either increases over the first month of withdrawal (Tran-Nguyen et al, 1998 using i.p. cocaine 15mg/kg) or decreases (Deroche-Gamonet et al, 2003 using a range of doses (0.2-1.6mg/kg IV using a within subject design) or not change (Lu et al, 2004 using i.p. cocaine at 2.5,5 and 15mg/kg). Thus, it is not clearly proven whether the cocaine induced drug seeking behavior follows the similar pattern as the contextual responding among the abstinent animals with a between subject design using cocaine prime at the same dose and route as the self-administration. In our study we have used a between subject design and the animals were primed with a single dose of cocaine at a dose of 0.5mg/kg IV (same as the self administered dose and route) and our results show that the cocaine prime induced drug seeking behavior does not increase in a time dependent manner over the one month withdrawal period being assessed.

4.2. Time-dependent effects on drug seeking behavior in the extinguished animals

In this part of our study we had two aims, one was to study the persistence of the effects of extinction and the other was to study whether extinction remains effective in reducing the drug

seeking behavior even if there is a delay in the initiation of the extinction training protocol. Both these aspects of the effects of extinction on the reinstatement of drug seeking behavior are equally important when it comes to the extinction therapy in human addicts. It has been explained in the literature that the effects of extinction go away with the passage of time and the responding might come back to the pre-extinction level. This phenomenon is described as spontaneous recovery by Bouton (2005). So far, there has not been a clear representation of this effect of spontaneous recovery in cocaine self administered and extinguished rats in the addiction literature, though the renewal effect has been shown previously (Crombag and Shaham, 2002). One study reported the effect of spontaneous recovery in rats self administered nicotine and extinguished thereafter. These rats were tested after 21 days of last exposure to the self-administration boxes and they noticed that the rats reliably reinstated their response to nicotine when tested (Shaham et al., 1997). Another study reported that stress can reliably reinstate the extinguished responding in cocaine self administered rats after 4-6 weeks of drug free period (Erb et al., 1996). In both these studies, the investigators utilized a with-in subject design and the same animals were tested immediately after extinction and after the passage of a certain time period after extinction. In the present study we found that the effects of extinction on the reinstatement of drug seeking behavior is not persistent with some kinds of priming stimuli (e.g. context or drug itself), but may be persistent with the the non-contingent cue prime. Meil and See (1997) studied the effect of inactivation of the BLA on the cue induced reinstatement tested a group of extinguished animals 21 days after their last extinction experience and found that their contextual response was increased to the pre-extinction levels which can be described as the spontaneous recovery of responding to the context, and they described context as one of the strongest stimuli that can influence responding. We observed that in addition to effects of

extinction on the contextual responding, the effect of extinction on drug induced reinstatement also diminish with the passage of time, making the animals more sensitive to reinstatement. These findings support the necessity of having booster trials of extinction or spaced extinction trials to maintain extinction over time. Rescorla (1997) found that when animals are extinguished in the presence of cues, the extinguished cues returned in one group of rats tested 8 days post extinction compared to those tested immediately after extinction. Bouton (1993) has shown that the spontaneous recovery among extinguished animals is inevitable if extinction trials are distributed in relatively isolated temporal or spatial pockets. Usually in human addiction therapy, the treatment sessions are conducted primarily for several consecutive sessions which then cease. Our results from the current study reinforce the idea of having the spaced extinction trials as reminder sessions and research in other areas has shown that when blocks of extinction trials are spaced out, the spontaneous recovery is greatly attenuated

In another group of animals in which the initiation of extinction was delayed for a period 3+ weeks, we found that the extinction training is still effective in reducing the drug seeking behavior induced by all kinds of priming stimuli. This finding is promising in the field of addiction therapy because it demonstrates that extinction therapy can still be effective in reducing the chances of relapse, even if there is a delay in initiating it.

Altogether our present results provide an insight into the time dependent effect of abstinence and extinction on the reinstatement of drug seeking behavior. We have found that among the abstinent animals (over the one month of withdrawal period), the non-contingent cue primed drug seeking and the cocaine induced drug seeking does not follow the same pattern as the context induced drug seeking. Among the extinguished animals we have shown the spontaneous recovery of responding to the diffuse contextual stimuli of the drug taking

environment and the cocaine prime but not to the non-contingent cue prime. We have also found out that the extinction training remains effective in reducing the reinstatement response levels to all sorts of priming stimuli employed in our tests even if the training is initiated after a period of abstinence. Considered together, our findings suggest that extinction therapy can be practically employed as a method of behavioral modification for the treatment of substance abuse.

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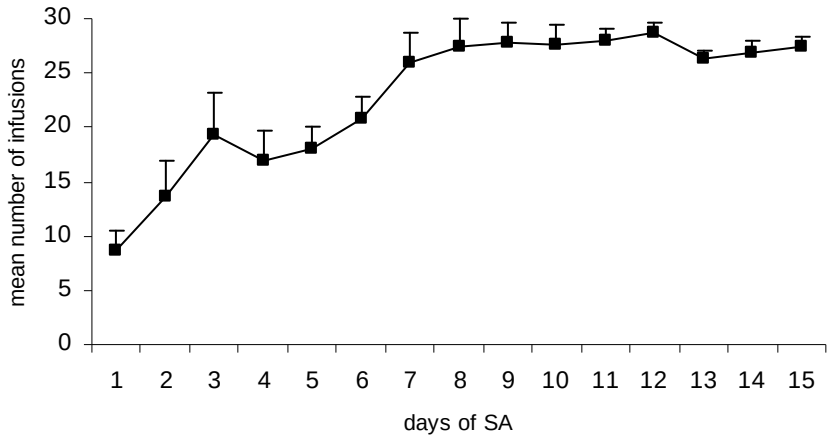
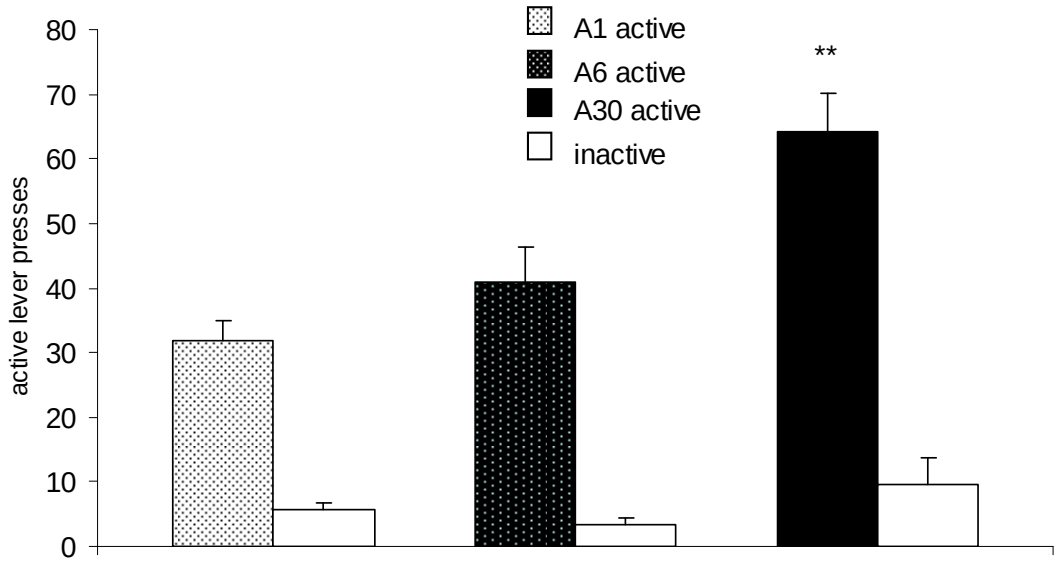


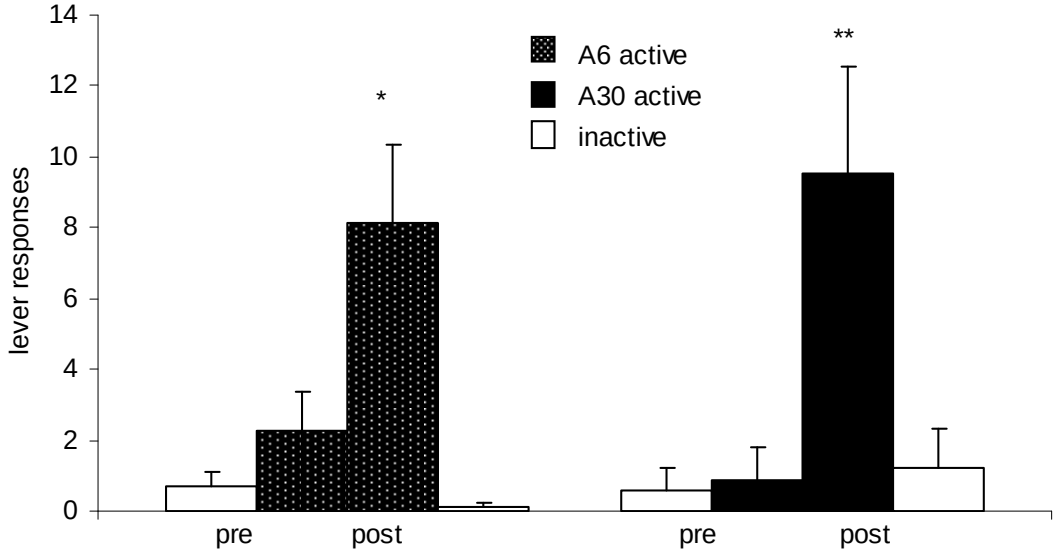
Figure 6.1 Cocaine self-administration training:

The results illustrate the average number of cocaine infusions earned daily during the 90 minute sessions of self-administration phase (days1-15). Transition to FR3 schedule on day 13 did not significantly alter the number of earned infusions, and the treatment groups tested in this study did not significantly differ in the mean number of cocaine infusions earned per session nor the total amount of cocaine ingested.

A


A1 active
A6 active
A30 active
inactive
active lever presses
80
70
60
50
40
30
20
10
0
**

B


A6 active
A30 active
inactive
lever responses
14
12
10
8
6
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pre
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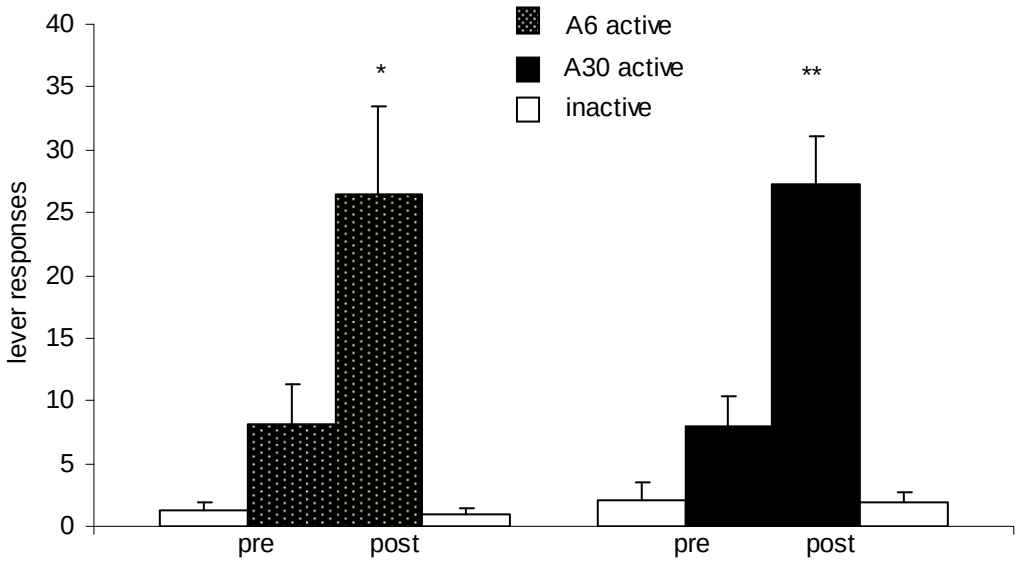


Figure 6.2 Exposure to the contextual cues or the priming with either CS or US evoked drug seeking behavior among the abstinent animals:

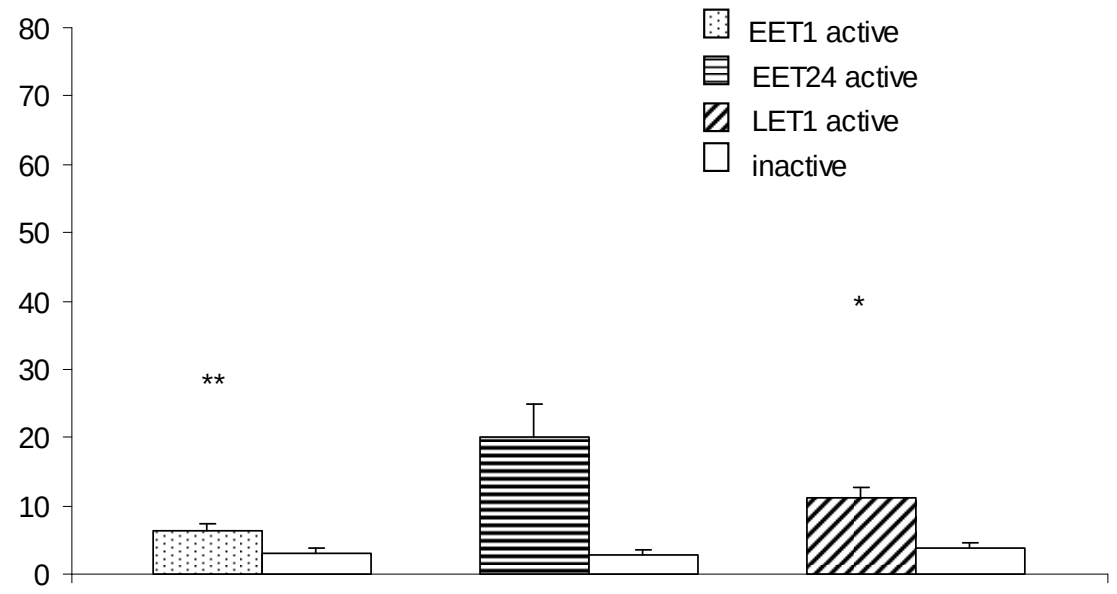
(A) Incubation effect in the enforced abstinent animals shown as a response to the diffuse contextual cues on the first test day: Data show the mean± SEM of the lever presses for the initial10 minutes on the first reinstatement test day in 1 day (n=32, derived from the EET1&EET24 extinction groups), 6 days (n=14) and 30 days (n=9) abstinent animals when the animals were placed back in the operant chamber after the respective withdrawal periods. Filled bars represent the active and the empty bars represent the inactive lever presses for each group. Drug seeking behavior in 30 days abstinent animals in response to the diffuse contextual cues was significantly higher as compared to the 1 day (A1) and 6 days (A6) abstinent group (**p<.01).

(B) Data show the mean±SEM of the active (filled bars) and inactive (empty bars) lever presses 10 minutes before and after the single non-contingent CS prime for the 6 days abstinent (A6,n=14) and 30 days abstinent (A30,n=9) group. Filled bars on the left represent the pre prime

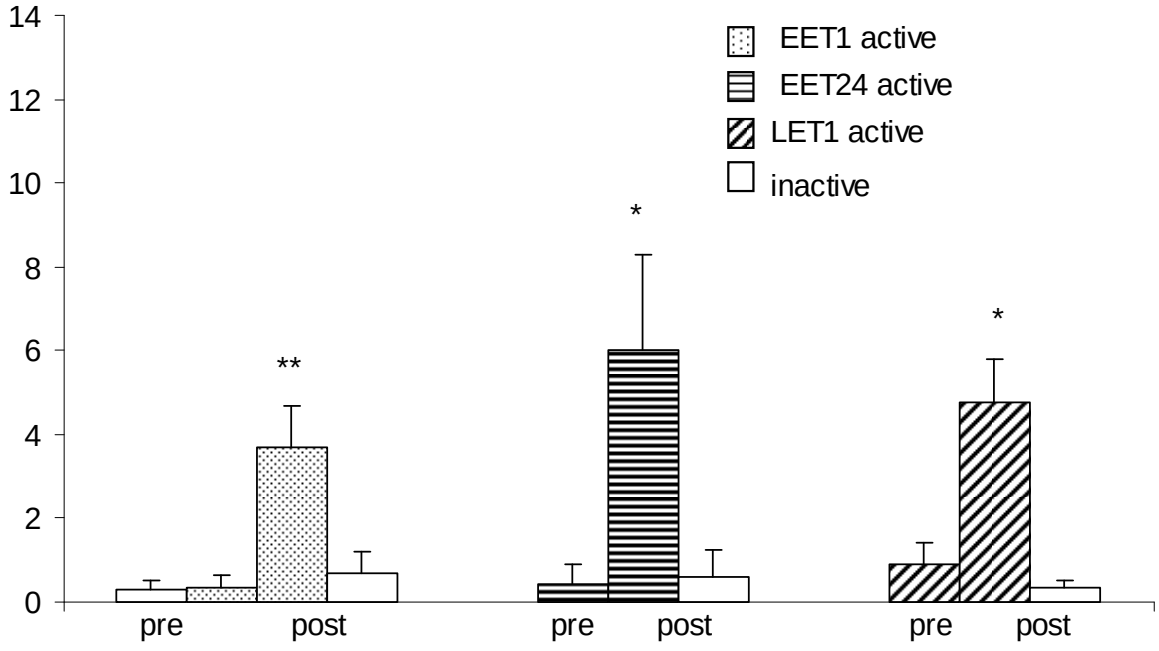
response (30-40min) and on the right represent the post prime response (40-50min) on the active lever for each group. Similarly the empty bars on the left represent the inactive lever presses for the pre-prime period and on the right represent the post prime response for each group. Lever pressing responses during the pre-prime period was very minimal and the delivery of the single non-contingent CS prime specifically reinstated the response on the active lever. Post prime response on the active lever was significantly higher for the A30 group as compared to the pre prime response (one way anova, Holm-Sidak, **p<.01).

(C) Data represent the mean ± SEM of the lever presses for the 30 minute window before and after the delivery of the single non-contingent intravenous drug prime at a dose of 0.5mg/kg at time =40 minute of the 90 minute test session on the second test day. Filled bars represent the active and the empty bars represent the inactive lever presses. In each group, the filled bars on the left show the active lever presses for the pre prime period (10-40 min) and those on the right show the same for the post prime period (40-70min). Similarly the empty bars on the left represent the inactive lever presses for the pre prime period and those on the right show the same for the post prime period. For both the groups (A6, n=14 and A30, n=10) the post prime response on the active lever was significantly greater than the pre prime response (* p<.05, one way anova, Holm-Sidak).

A


80
70
60
50
40
30
20
10
0
EET1 active
EET24 active
LET1 active
inactive
**
*

B


14
12
10
8
6
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2
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EET1 active
EET24 active
LET1 active
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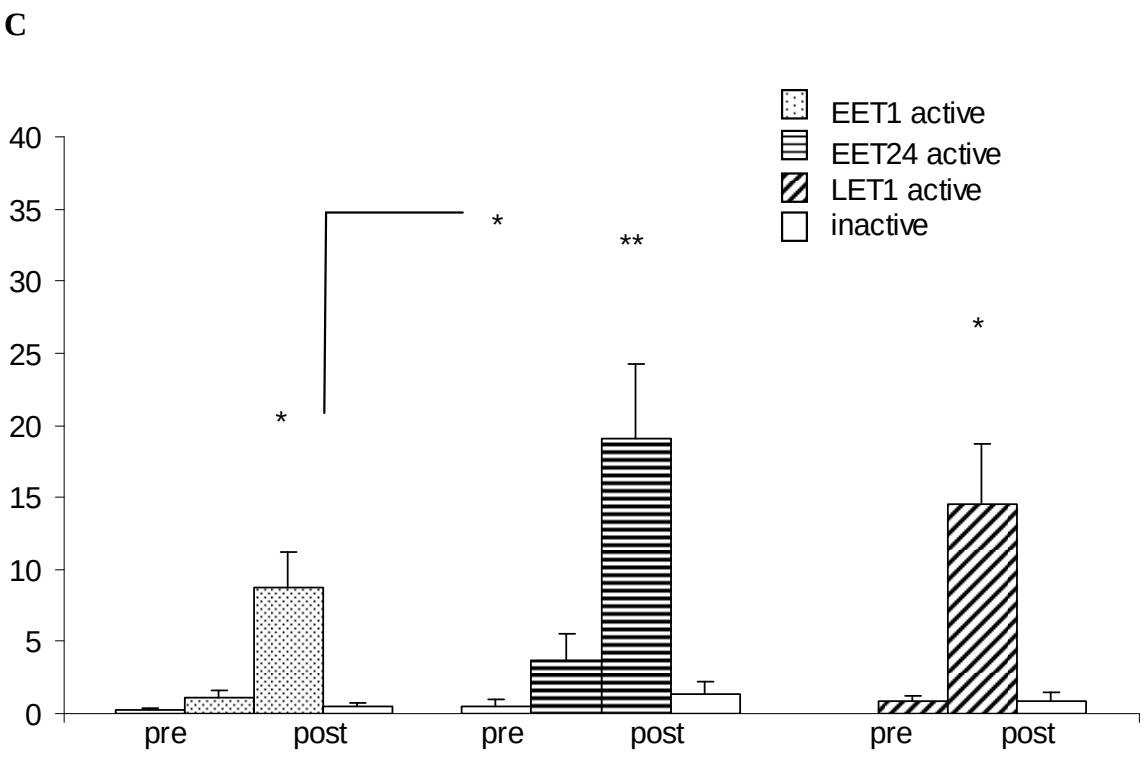

Figure 6.3 Exposure to the contextual cues or the priming with either CS or US evoked drug seeking behavior among the extinguished animal:

(A) Data show the mean± SEM of the lever presses for the initial10 minutes on the first reinstatement test day in the group EET1 (n=22), EET24 (n=10) and LET1 (n=9) when they were exposed to the diffuse contextual cues of the operant chamber environment. Filled bars represent the active lever responding and the empty bars represent the inactive lever responding. The group EET24 showed a significant renewal of contextual responding on the active lever (**p<.001, *p<.05, one way anova, Holm-Sidak) when tested 24 days after their last extinction experience than the extinguished animals tested 24 hrs after (groups EET1 and LET1) the last extinction experience.

(B) Data show the mean±SEM of the active (filled bars) and inactive (empty bars) lever presses 10 minutes before and after the single non-contingent CS prime for the groups EET1 (n=22), EET24 (n=9) and LET1 (n=9). Filled bars on the left represent the pre prime response (30-

40min) and on the right represent the post prime response (40-50min) on the active lever for each group. Similarly the empty bars on the left represent the inactive lever presses for the pre-prime period and on the right represent the post prime response for each group. Lever pressing responses during the pre-prime period was very minimal and the delivery of the single non-contingent CS prime (light and tone) specifically reinstated the response on the active lever. Post prime response on the active lever was significantly higher for the EET1 and EET24 groups as compared to the pre prime response (one way anova, Holm-Sidak, **p<.001, *p<.01).

(C) Data represent the mean ± SEM of the lever presses for the 30 minute window before and after the delivery of the single non-contingent intravenous drug prime at a dose of 0.5mg/kg at time =40 minute of the 90 minute test session on the second test day. Filled bars represent the active and the empty bars represent the inactive lever presses. In each group, the filled bars on the left show the active lever presses for the pre prime period (10-40 min) and those on the right show the same for the post prime period (40-70min). Similarly the empty bars on the left represent the inactive lever presses for the pre prime period and those on the right show the same for the post prime period. For all the three extinguished groups, EET1 (n=20), EET24 (n=8) and LET1 (n=9), the delivery of the cocaine prime reinstated the drug seeking behavior specifically on the active lever (**p<.001,*p<.01, one way anova, Holm-Sidak). The post prime response of the group EET24 was significantly greater (*p<.01, one way anova, Holm Sidak) than the post prime response of the group EET1.

CHAPTER 7

SUMMARY AND CONCLUSIONS

Drug addiction is a major socio economic problem in United States due to the increase in criminal activity, impaired health in drug abusers and the loss of productivity from these resulting in significant economic loss. Cocaine addiction is defined as a process that generally starts with recreational use and deteriorates over time into a compulsive and chronically relapsing drug taking disorder. The studies recognize that relapse is one of the major challenges in the treatment of drug addiction. Animal self-administration and reinstatement model is a well accepted model to study the relapse. An extinction protocol is usually included in the animal reinstatement paradigm before the animals are tested for reinstatement after self-administration of cocaine. Extinction refers to discontinuing the reinforcement, and here the animals learn new meaning about the drug taking context and the instrumental action of lever pressing. The lever presses are not reinforced with conditioned stimulus (light and tone) and the unconditioned stimulus (cocaine) during extinction. Under these conditions animals extinguish their lever pressing behavior. As the animals are learning new meaning about the previously drug available context in extinction, extinction is proposed to involve a new learning process. The objectives of this study were based on the central hypothesis that extinction involves new learning and N-methyl D-aspartate receptor dependent (NMDAR dependent) synaptic plasticity mechanisms are recruited during this new learning process.

The objectives of the present study were to (1) evaluate the involvement of NMDAR mediated mechanisms in the extinction learning process and in the effects of extinction on

reinstatement, (2) Investigate whether facilitation of NMDAR activity during extinction can enhance the effects of extinction on reinstatement in animals trained to self-administer cocaine in a short access protocol, (3) Investigate whether facilitation of NMDAR activity during extinction can enhance the effects of extinction on reinstatement in animals trained to self-administer cocaine in a long access protocol and (4) Study whether activation of NMDAR mediated mechanisms in the ventral hippocampus during extinction are necessary for the extinction to be effective in reducing the reinstatement. The last study described in chapter 6 investigates the persistence of the effects of extinction on reinstatement and studies whether the training latency for extinction training matters for the extinction to be effective.

It is well accepted that extinction reduces reinstatement response as compared to non-extinction or simple enforced abstinence. However, the molecular mechanisms underlying the effects of extinction on reinstatement of drug seeking are relatively understudied. The effectiveness of extinction to reduce the reinstatement is always compared to the reinstatement response of an abstinent group, kept abstinent for an equivalent period of time. The first study described in chapter 2 confirms the role of NMDAR mediated mechanisms in the effects of extinction on reinstatement. We could show that 5 days of extinction training significantly reduces the reinstatement evoked in response to the contextual drug stimuli, the conditioned stimulus and the cocaine prime as compared to enforced abstinence. Then, to investigate the role of NMDAR mediated mechanisms in the effects of extinction on reinstatement, 2 groups of animals were treated with either (±) CPP (a competitive antagonist of NMDAR) at 5 mg/kg i.p. or with D-serine (a full agonist of NMDAR at the strychnine insensitive glycine site of the NMDAR) at 100mg/kg i.p. before the animals were subjected to extinction training. Extinction

and reinstatement data of the treatment groups were compared to those of the saline treated control extinguished group.

Blocking the NMDAR activity during extinction (in the (±) CPP group) did not alter the extinction learning process, but facilitating the NMDAR activity (in the D-serine group) enhanced the extinction learning process to some extent, although all the extinguished animals reached a similar floor level of extinction by the day 5 of training. After the 5 days of extinction training, the animals were tested for reinstatement from the next day and the animals were drug free on these test days. Though the D-serine treatment showed some facilitation of extinction, the advantage of this effect was not observed in the reinstatement response of these animals as compared to the controls, possibly because of the overtraining effect of extinction. In the (±) CPP treated group, extinction training remained effective in reducing the reinstatement response to the contextual drug stimuli and the non-contingent CS prime compared to the control extinguished group. In contrast to this finding, the (±) CPP group showed a significantly greater reinstatement to the cocaine prime and the response of the (±) CPP group was almost similar to that of the non extinguished (abstinent) group. The findings from this study suggested that the activation of NMDAR mediated mechanisms during extinction was necessary for the effects of extinction on drug induced reinstatement.

Once the activation of NMDARs during extinction was demonstrated to be necessary for the effects of extinction on drug induced reinstatement, we were interested to investigate whether facilitation of NMDAR activity during extinction can enhance the effects of extinction on reinstatement. It was essential to avoid the overtraining effect of extinction in this study to make a distinction of the effect of treatment compared to controls since this effect will be revealed only with incomplete extinction. Hence, the second study described in chapter 3 utilizes a sub-

maximal extinction training protocol and the number of extinction training days was reduced from 5 to 1. On the extinction training day, two groups were treated with D-serine at a dose of 100mg/kg, one prior to extinction and another group immediately after extinction and the results were compared to that of the saline treated control extinguished group. The reinstatement in response to the contextual drug stimuli and the CS prime was not influenced by the D-serine treatment during extinction. However, the levels of cocaine induced reinstatement in both the D-serine treated groups (pre and post extinction) were significantly lower as compared to saline treated controls. The lack of the effect of D-serine pre- treatment on extinction learning process and the enhancement of the effects of extinction on drug induced reinstatement in the D-serine pre and post extinction group suggests that D-serine is responsible for this effect by helping in the consolidation of the memory of extinction once the extinction learning has taken place. The reinstatement response of the D-serine treated abstinent group compared to the saline treated control abstinent group confirmed that the effect of D-serine on reinstatement is dependent on extinction. Extinction is widely used as a behavioral modification therapy in drug addicts and if this finding could be translated effectively into the extinction therapy in humans, D-serine treatment during extinction could emerge as an effective adjunct pharmacotherapy to psychotherapy of extinction.

Studies described in chapter 4 utilized the long access protocol of self-administration which is proposed to model the binge taking of drugs in drug addiction. The reinstatement in response to the contextual drug stimuli and the non-contingent CS prime remained unaltered in the D-serine treated group. The reinstatement response to the cocaine prime in the animals treated with D-serine during extinction was significantly lower compared to the saline treated control extinguished group. This study suggested that the facilitation of the NMDAR activity

during extinction can be considered as a promising adjunct pharmacotherapy to enhance the effects of extinction and thus to reduce the chances of drug induced relapse.

Since the hippocampus is involved in the formation of new memories and ventral hippocampus is implicated in drug induced reinstatement, we were interested to study whether the NMDAR mediated mechanisms in the ventral hippocampus is necessary for the effects of extinction on reinstatement. For this purpose, competitive antagonist of NMDAR, (±) CPP was infused intra hippocampally to the ventral hippocampus at 100 and 200 ng doses prior to extinction training. Nothing could be concluded about the effects of (±) CPP treatment from the results of the CS and cocaine primed reinstatement as the response from the control group showed a very low level of responding to these priming stimuli. The contextual response among the (±) CPP treated group was significantly greater than the saline infused control group and this effect was not an expected one since the ventral hippocampus is not yet implicated in contextual reinstatement. This study has to be repeated with more number of animals to reach any solid conclusion about the requirement of the NMDAR mediated mechanisms in the ventral hippocampus for extinction to be effective in reducing the reinstatement.

The last study investigates the persistence of the effects of extinction and studies whether delaying the initiation of the extinction training impairs the effectiveness of extinction in reducing the reinstatement response. We found that the abstinent animals incubate their drug seeking behavior in response to the contextual cues of the drug environment, but not to the conditioned cues and to the non-contingent drug prime. When the animals undergone extinction training were tested after 3 weeks of their last exposure to the extinction context, they showed the effect of spontaneous recovery in response to the contextual drug stimuli and to the non-contingent cocaine prime but not to the non-contingent CS prime. Another group of animals

extinguished after 3 weeks of enforced abstinence since last day of self-administration when tested after the extinction, showed that the extinction still remained effective in reducing the reinstatement response compared to enforced abstinence. The study shows that there is not a critical time period for extinction training to be performed for that to be effective in reducing reinstatement and this implies that extinction therapy can be performed in human drug addicts anytime during abstinence as extinction always is advantageous to prevent the chances of relapse compared to enforced abstinence.