The Role of Dopamine in Acute Food Deprivation-induced Reinstatement of Heroin Seeking

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ABSTRACT

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Dopamine (DA) has been shown to play a significant role in drug reinforcement and the reinstatement of drug seeking due to priming or drug cue presentations. However, DA is suggested to play a limited role in stress-induced reinstatement, as modelled by footshock-induced reinstatement. Here the role of DA in acute food deprivation (FD)-induced reinstatement of heroin seeking, an alternative model of stress-induced reinstatement, is investigated. Rats were trained to self-administer heroin (0.05 mg/kg/infusion) for 10 days (days 1-5: 3 X 3 h sessions; days 6-10: 1 X 3 h session). Heroin-seeking behavior was extinguished by removing the drug. Following extinction, rats were tested for 48 h FD-induced reinstatement while pretreated with specific DA receptor antagonists. An attenuation of drug seeking was seen in rats given the high dose (10.0 μg/kg) of the DA D_1 receptor antagonist, SCH 23390; but, not the low dose of this antagonist (5.0 μg/kg) or the DA D_2 and D_3 receptor antagonists raclopride (50.0 and 100.0 μg/kg) and NGB 2904 (0.1 and 5.0 mg/kg). The lack of effect for raclopride on FD-induced reinstatement was validated by demonstrating a role for this antagonist on cue-induced reinstatement of extinguished heroin seeking. These results suggest that FD-induced reinstatement may be mediated, at least in part, by the activation of the DA D_1 receptor. Moreover, since systemic injection of SCH 23390 has previously been shown to have no effect on footshock-induced reinstatement the current study suggests that DA transmission may play a differential role in footshock and FD-induced reinstatement.

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Drug addiction is a chronic condition characterized by periods of both abstinence and relapse (O'Brien & McLellan, 1996). Sadly, relapse to drug-taking can occur months or even years after one has become abstinent (Jaffe, 1990; O'Brien & McLellan, 1996). Thus, for recovering addicts and clinicians involved in their treatment relapse poses a significant threat. Regrettably, however, the empirical study needed to investigate this phenomenon is often impossible or unethical. For example, researchers cannot expose recovering addicts to situations and stressors likely to provoke relapse. Nor, in situations in which addicts are studied immediately after a relapse is it always possible to discern the cause of such a lapse. These confounds have lead to the popularization of an animal model of drug relapse known as the reinstatement model (Epstein, Preston, Stewart, & Shaham, 2006; Shaham, Shalev, Lu, de Wit, & Stewart, 2003). Here this model is used to assess the role of dopamine (DA) in stress-induced relapse. In particular, the involvement of individual DA receptor subtypes in acute food deprivation (FD)-induced reinstatement is investigated.

The Reinstatement Model

The foundations of the reinstatement model can be traced to the 1970's research of Stretch and Gerber (Gerber & Stretch, 1975; Stretch & Gerber, 1973). These researchers demonstrated that animals trained to associate an operant task with drug reinforcement would return to this task, following extinction, if given a non-contingent injection of a previously self-administered drug. This finding was expanded upon by de Wit and Stewart (1981), who demonstrated that drug associated cues could also produce a

recommencement of previously extinguished drug-reinforced behavior. de Wit and Stewart (1981) referred to this recommencement as "reinstatement" and suggested that such behavior could be used to model human drug relapse.

Since its conception the reinstatement model has grown in popularity, due in part to its intuitive appeal. Of particular significance, is that factors which provoke relapse in humans also produce a reinstatement of drug-seeking behavior in animals, such as rodents (de Wit & Stewart, 1981) and primates (Gerber & Stretch, 1975; Stretch & Gerber, 1973). These factors include exposure to drug associated cues (de Wit & Stewart, 1981), re-exposure to self-administered or related drugs (de Wit & Stewart, 1981; 1983) and exposure to acute stress (Shaham & Stewart, 1996). Using the reinstatement model animals, particularly rats, are exposed to one of these factors, while in a drug-free state, in order to produce renewed drug seeking behavior. Manipulations, such as these, allow for the investigation of pharmacological relapse interventions (for example see: Leri, Tremblay, Sorge, & Stewart, 2004), as well as provide an opportunity to assess neuronal and environmental mechanisms associated with relapse (Bossert, Ghitza, Lu, Epstein, & Shaham, 2005; Epstein et al., 2006).

The Effect of Food Deprivation on Drug Taking

Prior to investigating the neuronal mechanisms mediating drug relapse it is important to briefly touch upon the neuronal mechanisms mediating drug use in general. Drugs of abuse are thought to exert their effects primarily by acting upon neuronal mechanisms, which evolved to encourage behaviors such as feeding and mating (Balfour, Yu, & Coolen, 2004; Kelley & Berridge, 2002; Wise & Bozarth, 1985). For this reason it is often suggested that drugs "hijack" the natural reward system (Lubman, Yucel, &

Pantelis, 2004). Drugs of abuse can alter the functioning of this system; however, the inverse is also true. Restriction or deprivation of natural rewards, such as food, can sensitize neuronal reward pathways and thus enhance the locomotor and rewarding effects of abused drugs such as amphetamine, cocaine and morphine (Bell, Stewart, Thompson, & Meisch, 1997; Deroche, Piazza, Casolini, Le Moal, & Simon, 1993). Furthermore, rats that have been food restricted show increased forebrain activation in response to amphetamine (Carr & Kutchukhidze, 2000), increased sensitivity to brain stimulation reward (BSR), and an increased drug-induced potentiation of BSR (Carr, 1996).

The above findings do not necessarily imply an increase in reward potential due to FD. Food scarcity causes numerous physiological changes which may enhance the rewarding effects of a drug without altering neuronal reward mechanisms. For example, acute FD or food restriction may alter the permeability of the blood-brain barrier and decrease plasma protien drug binding and drug metabolism (Angel, 1969; Gugler, Herold, & Dengler, 1974). Collectively these changes serve to increase drug concentration in the brain (Cabeza de Vaca & Carr, 1998). Despite this increase in concentration, acute FD or food restriction enhances drug self-administration (Carroll, France, & Meisch, 1979; Carroll & Meisch, 1979; Glick, Hinds, & Carlson, 1987). If the interaction between feeding mechanisms and drug taking were dependent upon brain drug-concentration then it would be expected that drug self-administration would decrease as the concentration of drug in the brain increased beyond an optimal dose level (Zittel-Lazarini, Cador, & Ahmed, 2007).This is not the case. Furthermore, food restriction has been shown to augment the threshold lowering effect of abused drugs

when administered systemically or intracranially (Cabeza de Vaca & Carr, 1998). If the effects of FD were due solely to a change in drug bioavailability then food restriction should have no effect on intracranially administered drugs. Moreover, food restriction has been shown to delay the suppression of drug-paired behavior during extinction, a period of time during which the drug is absent (Comer, Lac, Wyvell, Curtis, & Carroll, 1995). Collectively, these findings suggest that food restriction or acute deprivation may enhance the reinforcing properties of abused drugs. Such an effect may be mediated by a deprivation-induced sensitization of the reward pathway (Carr, 1996; Carr, 2006).

Food Deprivation and Stress-Induced Reinstatement

Among human drug abusers stress is a commonly cited reason for the initiation, maintenance and relapse to drug use (Brewer, Catalano, Haggerty, Gainey, & Fleming, 1998; Matheny & Weatherman, 1998; Sinha, 2001). Furthermore, clinical practice has shown that for recovering drug addicts one's stress coping style is strongly predictive of one's drug craving when placed in stressful situations (Grusser, Morsen, Wolfling, & Flor, 2007).

Within the reinstatement model the induction of a "stress-like" state by acute footshock (Shaham & Stewart, 1995), acute FD (Shalev, Highfield, Yap, & Shaham, 2000), yohimbine injection (Shepard, Bossert, Liu, & Shaham, 2004) and intracranial corticotropin-releasing factor (CRF) infusion (Shaham, Funk, Erb, Brown, Walker, & Stewart, 1997) has been used to trigger a relapse in drug seeking behavior. Of particular importance here is the phenomenon of acute 21 h FD-induced reinstatement of drug seeking. Contrary to previous research (see Carroll, 1985) acute FD-induced reinstatement will occur in the absence of prior exposure to food manipulation (Shalev et

al., 2000). Thus, the effect of acute FD on relapse can not be dismissed as a learned association between FD and drug reinforcement.

Rather, acute FD and other stressors may lead to a reinstatement of drug seeking by activating similar neuronal systems to those activated by the drug itself (Erb, Shaham, & Stewart, 1996; Shaham & Stewart, 1995). For instance, rats exposed, during extinction, to acute footshock or to a drug priming injection display a similar pattern of reinstatement (Shaham & Stewart, 1995). Furthermore, exposure to stressors such as footshock can cause an activation of the hypothalamic-pituitary-adrenal (HPA) axis as well as the mesocorticolimbic DA system suggesting that stress exposure can co-activate the neuronal mechanisms mediating stress reactivity and reward (Sinha, 2001). Moreover, CRF, which is traditionally thought to be involved in the behavioral response to stress (Koob & Bloom, 1985) is also released by the administration of cocaine and other abused drugs (Koob & Le Moal, 2001). Thus, extrahypothalamic CRF may have the potential to be both aversive and positively motivating. This view is supported by a recent report showing an increase in cued responding for sucrose following microinjections of CRF into the nucleus accumbens (Peciña, Schulkin & Berridge, 2006).

The Role of Dopamine in Drug Reinforcement

If true, that stress reinstates drug seeking by activating neuronal mechanisms involved in mediating the reinforcing effects of drugs and drug-associated cues, then stress-induced reinstatement, like the reinforcing effects of drugs of abuse and natural rewards, may depend upon the mesocorticolimbic DA system (Kelley & Berridge, 2002; Stewart, 1983). Activation of this pathway is thought to be crucial to the rewarding and learning aspects of addiction. Moreover, DA is thought to be involved in the detection

and prediction of reward (Schultz, 2001; Schultz, Dayan, & Montague, 1997), as well as the motivation to gain reinforcement (Berridge, 1996; Robinson & Berridge, 1993).

Given the importance of DA in motivated behavior and addiction it is important to briefly address the pharmacology of this neurotransmitter. The action of DA is mediated by at least five distinct receptor subtypes. These receptors are classified as being in one of two families: The D_1-like or the D_2-like family of receptors. The D_1-like family of receptors includes the DA D_1 and D_5 receptors; whereas, the D_2-like family of receptors includes the DA D_2, D_3 and D_4 receptor subtypes. These families are distinguished based upon the consequence of DA binding at the receptor. A broad and highly simplified distinction between DA receptor types involves the coupling of DA to adenylate cyclase. The D_1-like family of receptors are positively coupled to adenylate cyclase through an excitatory G-protein; whereas most of the D_2-like family of receptors are negatively coupled to adenylate cyclase through and inhibitory G-protein (Sokoloff & Schwartz, 1995).

The unique role of each DA receptor in drug reward and addiction can be assessed by activation or inactivation of these receptor subtypes by selective agonists or antagonist, respectively. Studies of this nature have suggested a role for both the DA D_1-like and D_2-like receptors in drug reinforcement. Rodents and primates will self-administer DA D_1- and D_2-like agonists, suggesting that activation of these receptors is reinforcing (Self, Belluzzi, Kossuth, Stein, 1996; Self & Stein, 1992; Weed & Woolverton, 1995). Moreover, systemic administration of DA D_1-and D_2-like receptor antagonists reduce cocaine reinforcement as measured by a compensatory increase in cocaine self-administration following a low and a reduction in cocaine self-administration

following a high antagonist dose injection (Britton, Curzon, Mackenzie, Kebabian, Williams, Kerkman, 1991; Koob, Le, & Cresse, 1987).

The Role of Dopamine in Food Deprivation-Induced Reinstatement

A role for DA in priming- and cue-induced reinstatement has been well established. For example, systemic administration of indirect DA agonists such as amphetamine and cocaine will reinstate drug seeking behavior in animals trained to self-administer heroin or cocaine (De Vries, Schoffelmeer, Binnekade, Mulder, & Vanderschuren, 1998; de Wit & Stewart, 1981). Moreover, localized injections of the DA D_1 and D_2 receptor antagonists SCH 23390 (Anderson, Bari, & Pierce, 2003) and sulpiride (Anderson, Schmidt, & Pierce, 2006) into the nucleus accumbens shell will cause an attenuation of cocaine priming-induced reinstatement of drug seeking; suggesting that the activation of these receptors is important for priming-induced reinstatement. As for cue-induced reinstatement, selective blockade of the DA D_1-like receptor with SCH 39166 or SCH 23390 produces an attenuation of cue-induced reinstatement and a reversal of cue-induced Fos immunoreactivity (Ciccocioppo, Sanna, & Weiss, 2001). Moreover, raclopride, a DA D_2 antagonist has been shown to completely suppress cue-induced reinstatement of cocaine seeking (Cervo, Carnovali, Stark, & Mennini, 2003). Finally, both antagonism and partial agonism of the DA D_3 receptor has been shown to attenuate cue-induced reinstatement (Cervo et al., 2003; Cervo, Cocco, Petrella, & Heidbreder, 2007; Gilbert et al., 2005; Gyertyan et al., 2007).

Despite the suggestion that cue-, priming- and stress-induced reinstatement are mediated, at least in part, by a common neuronal pathway (Kalivas & McFarland, 2003) the role of DA in stress-induced reinstatement remains unclear. Acute stress has a

definitive effect on mesocorticolimbic DA transmission; however, the nature of this effect on stress-induced reinstatement is unknown. For example, treatment with flupenthixol, a nonselective DA receptor antagonist, attenuates footshock-induced reinstatement of heroin seeking. Yet, neither the selective D_1 antagonist, SCH 23390, nor the selective D_2 antagonist, raclopride, has any affect on footshock-induced reinstatement when given systemically (Shaham & Stewart, 1996). Despite the ambiguous effects of systematic DA receptor antagonism, blockade of DA receptors in the prefrontal cortex (PFC) with the D_1 receptor antagonist SCH 23390, but not with the D_2 receptor antagonist raclopride, has been shown to produce an attenuation of footshock-induced reinstatement. Moreover, footshock-induced reinstatement is also inhibited by the DA D_3 receptor antagonist SB-277011A (Xi et al., 2004). The above findings suggest a complex role for DA in footshock-induced reinstatement.

As previously mentioned, both footshock and acute FD are models of stress-induced reinstatement. Furthermore, both footshock- and acute FD-induced reinstatement depend critically on CRF but not corticosterone (Shalev, Finnie, Quinn, Tobin, & Wahi, 2006). Thus, it may be expected that DA plays a similar role in footshock- and acute FD-induced reinstatement. However, this is not necessarily the case. Acute FD-induced reinstatement appears distinct from other forms of reinstatement in that it is attenuated by leptin, a hormone involved in the long-term regulation of energy balance (Friedman, 2002; Shalev, Yap, & Shaham, 2001).

Rationale of the Current Experiments

By using receptor-specific antagonists the following thesis will attempt to elucidate the role of DA receptor subtypes in acute FD-induced reinstatement of heroin

seeking. Generally, research into the nature of drug relapse has utilized a reinstatement model of cocaine self-administration. Unlike cocaine, which is an indirect DA agonist, heroin is an opioid receptor agonist. In the ventral tegmental area (VTA) heroin causes an inhibition of GABAeric neuronal activity by binding to μ-opioid receptors. Inhibition of GABA results in a disinhibition of VTA DA neurons and thus an increase in nucleus accumbens DA release (Pierce & Kumaresan, 2006). Additionally, there is also evidence to suggest that the reinforcing effects of heroin may be mediated through a DA-independent pathway as well. This pathway may involve the nucleus accumbens or other downstream structures (Hnasko, Sotak, & Palmiter, 2005; Koob, 1992; Pierce & Kumaresan, 2006). Consequently, DA may play a differential role in acute FD- and footshock-induced reinstatement as well as in reinstatement to cocaine or heroin seeking.

The first experiment will investigate the role of DA receptor antagonism on behavior maintained by sucrose, a non-drug reinforcer. This experiment will ensure that no suppression of motor behavior is seen with the selected doses of DA antagonists. These doses will then be used in experiments 2-4 and 5-6 to investigate the involvement of DA receptor subtypes in acute FD- and cue-induced reinstatement of heroin seeking.

EXPERIMENT 1: The effect of dopamine antagonists on sucrose-reinforced behavior

Method

Subjects

Subjects consisted of 10 male Long Evans rats (Charles River, St. Constant, QC) weighing approximately 400 g (M = 391.70, SE = 6.70) at the start of experimentation. Prior to experimentation rats were given approximately 1 week to acclimate to daily

handling as well as facility light (reversed 12:12 h light-dark cycle; lights off at 09:30 a.m.) and temperature (21 °C) conditions. Following acclimation rats were transferred to operant-training chambers where they were housed throughout the experiment. With the exception of periods of water deprivation, to ensure operant responding for sucrose, food and water were available *ad libitum.* Rats were treated in accordance with the Canadian Council on Animal Care and approval was granted by the Concordia University Animal Care Committee.

Apparatus

Rats were individually housed in one of 10 identical operant-training chambers (Coulbourn Instruments, Allentown, PA, USA; 29.0 cm x 29.0 cm x 25.5 cm) for the duration of the experiment. Each chamber was enclosed in a sound attenuating wooden compartment equipped with a fan for ventilation and external noise reduction. Each chamber had a floor composed of stainless steel rods to allow for the collection of waste into a removable tray filled with Betachip bedding. Each chamber was comprised of a front and back Plexiglas wall and two metal panel side walls. A retractable lever was mounted approximately 6 cm above the floor on each of the two side-walls (Coulbourn Instruments, Allentown, PA); one sucrose-paired or 'active' and one non-reward-paired or 'inactive.' A cuelight was located above each lever and a houselight was positioned in the top left of each chamber. The designation of the left and right levers to active or inactive was counterbalanced across the boxes. Chambers were also fitted with a liquid well which was connected via polyethylene-50 tubing (Norton Performance Plastics, Akron, OH) to a 60 ml syringe and infusion pump (Razel Scientific Instruments,

Stamford, CT). The infusion pump delivered 0.36 ml of the sucrose solution per active lever press.

Operant-training chambers were attached via Link boxes (Coulbourn Instruments, Allentown, PA, USA) to a Personal Computer equipped with Graphic State Software (Coulbourn Instruments, Allentown, PA, USA). This program was used to record the number of sucrose deliveries, active lever presses and inactive lever presses.

Sucrose & Drug

Ordinary sugar was purchased at a local supermarket and dissolved in tap water to produce a 10% sucrose solution. This solution was used to fill the 60 ml syringes.

The DA antagonist, SCH 23390 (Sigma-Aldrich, Oakville, ONT), was diluted in saline to produce a low (50 μg/kg) and high (100 μg/kg) dose. These doses where chosen based upon the work of Shaham and Stewart (1996). These authors demonstrated an attenuation of heroin-primed reinstatement with the high, but not the low antagonist dose. However, as shown below, these doses caused a suppression of sucrose-reinforced behavior and where thus reduced to 5 and 10 μg/kg. The latter doses were chosen because of their ability to attenuate cue-induced renewal of cocaine seeking (Crombag, Grimm, & Shaham, 2002).

The DA D_2 receptor antagonist, raclopride (Sigma-Aldrich, Oakville, ONT) was diluted in saline to produce a low (50 μg/kg) and a high (100 μg/kg) dose. These doses were based upon previous research demonstrating that at 100 μg/kg raclopride prevents cue-induced reinstatement (Cervo et al., 2003) and that at both 50 and 100 μg/kg raclopride attenuates context-induced renewal of drug seeking (Crombag et al., 2002).

The DA antagonist, NGB 2904 (Supplied by Dr. Amy Newman, NIDA -

Intramural Research Program, Medicinal Chemistry Section, Baltimore, MD, USA), was diluted in a vehicle of 25% hydroxypropyl beta cyclodextrin (Sigma-Aldrich, Oakville, ONT) and saline to produce a low (0.1 mg/kg) and high (5.0 mg/kg) dose. These doses failed to alter cocaine self-administration under a fixed ratio schedule of reinforcement. Yet, have been shown to reduce cue-induced reinstatement (Gilbert et al., 2005) and lower cocaine self-administration breakpoint under a progressive ratio schedule (Xi et al., 2006).

Procedure

To ensure that no suppression of motor behavior would occur, the doses for experiments 2-4 were pilot tested on rats trained to respond for sucrose. Rats were given 20 sucrose training sessions (days 1-3: one 1-h session, days 4-20: two 1-h sessions separated by 20 min). Sucrose sessions began shortly after the onset of the dark phase (10:00 a.m.). Each session started with the illumination of the houselight, a 30 s activation of the cuelight over the active lever, and the insertion of both levers. Responding on the active lever resulted in a 5 s delivery of 0.36 ml of sucrose and a 12 s timeout. During the time out period the cuelight remained on and active lever responses were not reinforced. Inactive lever responding was never reinforced. Rats that were resistant to sucrose training were water deprived overnight until they acquired the active lever-press response (typically within 3-4 days).

Beginning on training day 21 all rats were injected (s.c.) with one of three doses of the D_1 receptor antagonist, SCH 23390 (0, 50 or 100 μg/kg). Each rat was to receive all doses in a counterbalanced order. However, after the first injection a drastic reduction in responding for sucrose was observed in rats given the low and high dose of SCH

23390. Consequently, these doses were reduced to 0, 5 and 10 μg/kg. Once rats had returned to a stable level of sucrose responding, at least 2 of 3 consecutive days with less than 15% variability in active lever responding, they were injected with each of the new SCH 23390 doses, in a counterbalanced order. Following injections of the D_1 antagonist and a return to baseline responding, rats were given the D_2 antagonist, raclopride. All rats received, in a counterbalanced order, 0, 50 and 100 μg/kg (s.c.) of raclopride. Again, following a return to baseline responding, rats were injected (i.p.), in a counterbalanced order, with three doses (0.0, 0.1 and 5.0 mg/kg) of the D_3 receptor antagonist, NGB 2904. Rats were allowed to return to a baseline level (+/- 20%) of responding following each antagonist injection. For rats that did not return to this level a new baseline was calculated against which to evaluate responding while under the influence of the antagonist.

Statistical Analysis

The effect of each DA antagonist on sucrose-rewarded behavior was assessed using a series of repeated measures ANOVA's. For each DA antagonist the effect on active lever pressing, inactive lever pressing, and sucrose delivery was assessed separately. For all ANOVA's the within subjects factor was *antagonist dose* (vehicle, low, high). Significant effects of the dose condition were followed up with Fisher's Least Significant Differences comparisons. All analyses were conducted using SPSS Software v. 13 for Windows (SPSS Inc., Chicago, Illinois, USA) and all analyses were evaluated for significance at $\alpha = 0.05$.

Results

Prior to the injection of the DA D_1 antagonist one rat was removed from the experiment due to a failure to acquire sucrose responding. For the remaining 9 rats the data was analyzed for only the first hour of the 2-h (two 1 h sessions) session. It was expected that the antagonists would have the greatest behavioral effect during this time frame. Initially rats were to be injected, in a counterbalanced order, with 0, 50 and 100 μg/kg of SCH 23390. However, after the first injection a drastic reduction in active lever responding, relative to the saline injection ($n = 3$; $M = 89.00$, $SE = 29.51$), was observed in rats given the low ($n = 3$; $M = 59.00$, $SE = 19.66$) and high (n = 3; $M = 25.67$, $SE = 16.74$) antagonist doses. Following these injections an additional rat was removed from the experiment due to a failure to return to a reasonable level of responding for sucrose (when removed active lever responding had fallen to 2 responses in 1 h). Furthermore, 1 rat was excluded from the SCH 23390 condition and an additional rat was removed from the raclopride condition; both due to a failure to acquire a stable level of active lever responding. Four days after the initial SCH 23390 injections, 7 rats were re-injected with three lower antagonist doses (0, 5, and 10 μg/kg). As can be seen in Figure 1A, no significant differences were seen in sucrose delivery, $F(2, 12) = 2.11$, n.s., active lever pressing, $F(2, 12) = 1.75$, n.s., or inactive lever pressing, $F(2, 12) = 0.34$, n.s., when rats were injected with the vehicle, low or high antagonist doses.

Furthermore, raclopride injection dose (0, 50, and 100 μg/kg) had no effect on behavior. As seen in Figure 1B, active lever responding, $F(2, 12) = 0.44$, n.s., sucrose delivery, $F(2, 12) = 0.57$, n.s., and inactive lever responding, $F(2, 12) = 0.71$, n.s. was similar for all dose conditions. Finally, 8 rats were used to assess the effect of NGB 2904

on sucrose reinforced behavior (see Figure 1C). Similar to SCH 23390 and raclopride this antagonist had no significant effect on sucrose delivery, $F(2, 14) = 0.75$, n.s., responding for sucrose, $F(2, 14) = 0.84$, n.s., or generalized activity as measured by inactive lever responding, $F(2, 14) = 0.49$, n.s.

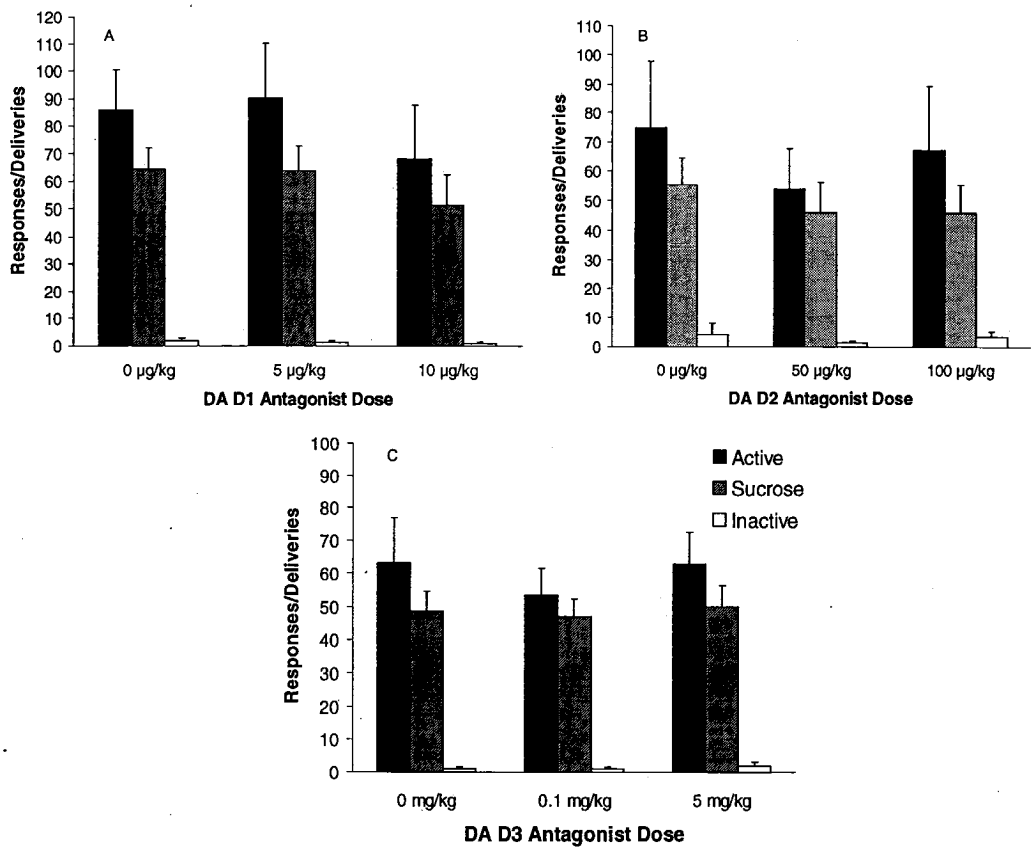

Figure 1. The effect of DA receptor antagonism on sucrose reinforced responding. Mean (+*SE*) are shown for the 7 rats that were tested with SCH 23390 (0, 5 and 10 µg/kg; A) and raclopride (0, 50 and 100 µg/kg; B) as well as the 8 rats injected with NGB 2904 (0.0, 0.1 and 5.0 mg/kg; C). No significant effects on behavior were observed.

Summary

The specific DA receptor-antagonist doses were chosen based on previous research demonstrating a behavioral effect in studies of drug taking and drug relapse. For example, the current doses of SCH 23390 and raclopride have been shown to attenuate cue-induced renewal of cocaine seeking (Crombag et al., 2002), a procedure in which

reinstatement is triggered by a return to a previously drug associated context. Furthermore, the selected doses of NGB 2904 have been shown to attenuate cue-induced reinstatement (Gilbert et al., 2005) and at the high dose to lower cocaine self-administration breakpoint under a progressive ratio schedule (Xi et al., 2006). The above sucrose results suggest that the current antagonist doses do not cause a general motor impairment and have no effect on the motivation to acquire sucrose reinforcement. Thus, any significant effect of DA antagonism, at the current doses, found in later experiments cannot be due to motor suppression or a non-specific motivational effect.

EXPERIMENTS 2-4: The role of dopamine receptors in acute food deprivation-induced reinstatement of heroin seeking

Method & Results

Subjects

A total of 110 (Exp 2: $n = 40$; Exp 3: $n = 40$; Exp 4: $n = 30$) male Long Evans rats (Charles River, St. Constant, QC) were tested in squads of 10 over a period of several months. Rats were housed in pairs in plastic shoebox cages for approximately 1 week before surgery. During this time rats were acclimated to daily handling as well as facility light (reverse 12:12 h light-dark cycle; lights off at 09:30 a.m.) and temperature (21 °C) conditions. Once the animals reached a bodyweight of approximately 350 g intravenous catheterization surgery was preformed. Following surgery, rats were given 36-48 h of recovery, during which time they were singly housed, before being transferred to self-administration chambers. With the exception of tests of acute FD-induced reinstatement, all rats had *ad libitum* access to food and water. Procedural approval was granted by the

Concordia University Animal Care Committee and animals were treated in accordance with the Canadian Council on Animal Care.

Surgery

To allow for drug self-administration rats were implanted with IV Silastic catheters (Dow Corning, Midland, MI, USA). Each catheter was cut to a length of 12 cm and a silicone bead was used to mark the bottom 3 cm of this tubing. Rats were anesthetized with a mixture of xylazine and ketamine (10 + 100 mg/kg, i.p.). Once the rat was fully anesthetized, as confirmed by the absence of a withdrawal reflex to a paw-pinch, two incisions were made: one on the skull and one on the neck (approximately 1 cm above the ventral side of the shoulder blade). Tweezers were inserted through the neck incision and the jugular vein was isolated. After isolating this vein, excess tissue was cleaned from its surface and a small incision was made. Through this incision 3 cm of the catheter was inserted; once in place the catheter was secured with three silk sutures. The remaining portion of catheter was threaded subcutaneously to the skull, where, it was attached to a modified 22-guage cannula (Plastics One Industries, Roanoke, VA). The cannula was mounted to the skull using jewelers' screws and dental cement. Prior to surgery animals were given penicillin (450,000 IU/rat) to prevent infection, and post surgery animals were given the analgesic buprenorphine (600 μg/rat; Schering-Plough Ltd., Welwyn Garden City Hertfordshire, UK) to relieve pain. Catheters were flushed, on a daily basis, throughout drug training with heparin and gentamicin (7.5 IU + 12.0 μg/rat/day) to prevent blockage.

Apparatus

After a brief recovery period animals were moved from their cages to one of 30 self-administration chambers (Coulbourn Instruments, Allentown, PA, USA; 29.0 cm x 29.0 cm x 25.5 cm). These were similar to the chambers used above; however, several modifications were made. First, the levers were raised and positioned approximately 11 cm above the floor. Additionally, the active lever was paired with drug delivery, suppression of the houselight, activation of a tone (2.9 KHz tone module, Coulbourn Instruments, Allentown, PA, USA) and illumination of a cuelight above the lever.

Drug infusions, active lever presses and inactive lever presses were recorded. For drug infusions rats were attached to a swivel (Instech Swivel Assembly, Med Associates, St. Albans, VT, USA) and drug pump via polyethylene-50 tubing, which was shielded by a metal spring. Drug pumps administered 0.13 ml of the drug solution per infusion.

Drugs

Heroin (diacetylmorphine HCL; Almat Pharma Chem, Concord, Ontario) was dissolved in sterile distilled water to produce a stock solution of 5 mg/ml. This stock was further diluted with physiological saline to produce heroin syringes with a concentration of 0.05 mg/kg/infusion. The remaining stock solution was refrigerated and used to refill syringes as necessary.

DA antagonists were prepared in the following doses; SCH 23390: 0.0, 5.0 and 10.0 μg/kg; raclopride 0.0, 50.0 and 100.0 μg/kg; NGB 2904; 0.0, 0.1 and 5.0 mg/kg, using the procedures described for experiment 1.

General Procedure

The following three experiments employed the same general procedure. The differences between these experiments lie in the number of subjects and the type of DA antagonist used. Each experiment consisted of three phases: training, extinction and reinstatement. Following a 24 h habituation period, 10 days of self-administration training were conducted. For the first 5-6 days rats had three 3-h drug self-administration sessions, which were separated by 3 h. For the last 5 days rats had only one 3-h self-administration session. The initial session of each day began shortly after the onset of the dark phase (approximately 10:00 a.m.). Sessions began with the insertion of both levers, the turning on of a houselight and the activation of a cuelight/tone complex. The cuelight and tone remained on for 30 s or until the active lever was pressed. Active lever presses resulted in a 5 s drug infusion as well as a 20 s timeout period. During the timeout period the houselight remained off, the cuelight/tone complex remained on and active lever presses were not reinforced. At the end of each session the active lever was retracted. However, to increase discrimination between the levers the inactive lever remained extended until shortly before the first session of the following day.

Following training, animals entered a period of extinction. Extinction days followed the same procedure as the last phase of training (1 3-h session/day). However, during extinction active lever presses did not result in drug delivery. To acclimate animals to the injection procedure, mock saline injections (experiments 2 & 3: s.c.; experiment 4: i.p.) were administered 15-20 min prior to the start of the session, beginning on the third day of extinction. After a minimum of 4 extinction days, animals were considered extinguished if a criterion of 15 or less active lever responses (per 3-h

extinction session) was obtained. Once this criterion was reached, rats were tested for reinstatement of drug seeking, under extinction conditions, while food deprived and while sated. Acute FD was accomplished by removal of food from the hoppers 48 h prior to the 3-h test session. While food deprived, rats were given an "off" period and no extinction sessions where run. Thus, rats remained in the self-administration chambers but the levers were not extended and the houselight remained off for 48 h prior to reinstatement testing. The food hoppers were refilled at the end of the test session. When tested under the sated condition rats were exposed to a similar "off" period, but were given free access to food during the 48 h before testing. All rats were tested twice, once while acutely food deprived and once under sated conditions. These conditions were administered in a counterbalanced order and were separated by at least one extinction day and 2 "off" days. Depending on the experiment rats were injected with SCH 23390 (s.c.; exp 2), raclopride (s.c.; exp 3) or NGB 2904 (i.p.; exp 4) 15-20 min prior to testing.

Statistical Analysis

Statistical analyses for the effect of each DA antagonist on acute FD-induced reinstatement of heroin seeking were preformed using SPSS Software v. 13 for Windows (SPSS Inc., Chicago, Illinois, USA). With this software a series of mixed factorial ANOVAs were preformed. ANOVA's investigated separately active and inactive lever pressing for each antagonist type. For each ANOVA *deprivation state* (baseline, FD, sated) was used as a within subjects factor and *antagonist dose* (vehicle, low, high) was used as a between subjects factor. For *deprivation state*, baseline was calculated as the mean of the last extinction day before each reinstatement test. Significant effects where

followed up with Fishers Least Significant Differences comparisons. All analyses were evaluated for significance at $\alpha = 0.05$.

Results

Since only minimal differences were observed between the repetitions of experiments 2-4 (see Appendix A) the data was combined to give 40 rats in experiment 2, 40 rats in experiment 3 and 30 rats in experiment 4. These numbers were reduced to 32, 27 and 24, respectively due to sickness (exp 3: 6 rats; exp 4: 1 rat), procedural errors (exp 2: 2 rats; exp 3: 1 rat; exp 4: 2 rats), statistical outliers (exp 3: 1 rat) and a failure to train (exp 2: 6 rats; exp 3: 5 rats ; exp 4: 3 rats). A failure to train was considered apparent when rats had a mean of less than 15 active lever responses per 3-h session for the last 4 days of training.

Experiment 2: The effect of the D_1 receptor antagonist, SCH 23390, on acute food deprivation-induced reinstatement of heroin seeking.

As seen in Figure 2, rats showed a preference for the active 'drug-paired' lever, relative to the inactive lever 'non-drug paired' lever throughout training (see Appendix B for daily comparisons); thus, demonstrating a learned association between the active lever and drug reinforcement. Following training rats extinguished active lever pressing in a mean of 6.38 ($SE = 0.56$) days (see Appendix C).

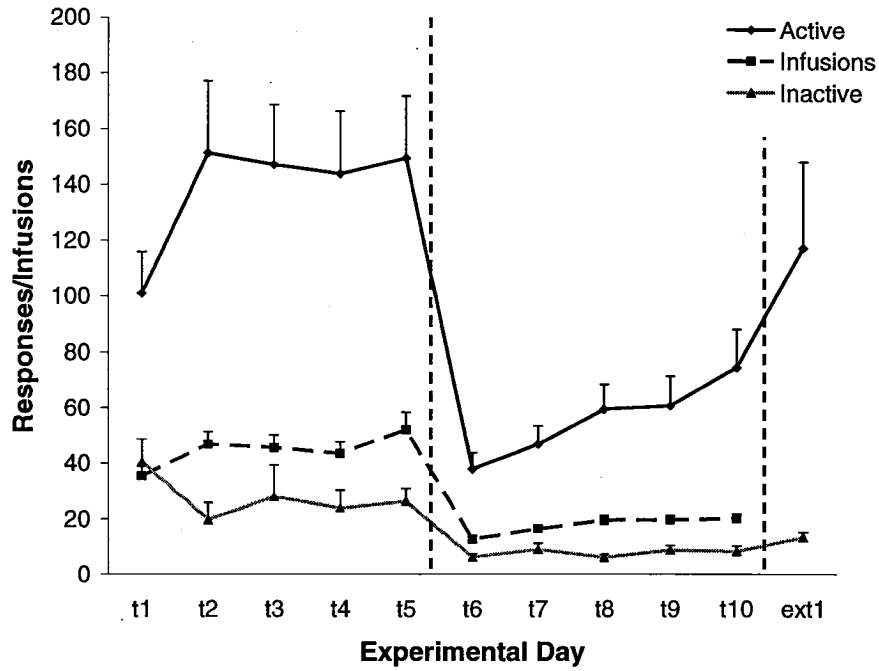

Figure 2. Heroin training phase. Mean (+ *SE*), number of active lever presses, inactive lever presses and heroin infusions (0.05 mg/kg/infusion) for the 32 rats in experiment 2 over the 10-day training period (days 1-5: three 3h sessions; days 6-10: one 3h session). Also shown is the number of active and inactive lever responses for the first day of extinction. A significant difference existed between active and inactive lever responding on all training days.

For reinstatement testing rats were assigned to one of three antagonist dose conditions (vehicle: $n = 9$; low: $n = 12$; high: $n = 11$) in such a way that there were no differences between the groups in terms of mean active lever responses ($M_{veh} = 106.52$, $SE = 31.49$; $M_{low} = 92.03$, $SE = 15.42$; $M_{high} = 95.39$, $SE = 19.53$), $F(2, 29) = 0.11$, n.s.; inactive lever responses ($M_{veh} = 17.44$, $SE = 3.29$; $M_{low} = 18.15$, $SE = 3.94$; $M_{high} = 17.50$, $SE = 3.60$), $F(2, 29) = 0.01$, n.s., and infusions ($M_{veh} = 30.56$, $SE = 4.88$; $M_{low} = 31.43$, $SE = 3.51$; $M_{high} = 31.71$, $SE = 3.67$), $F(2, 29) = 0.02$, n.s., throughout the 10 days of training. Following training and extinction rats were tested for reinstatement.

A mixed factorial ANOVA, investigating responding on the previously active lever, revealed a significant effect of *deprivation state*, $F(2, 58) = 27.11$, $p < 0.05$, and

antagonist dose, $F(2, 29) = 4.96, p < 0.05$. Furthermore, active lever pressing was also effected by a significant *deprivation state* X *antagonist dose* interaction, $F(4, 58) = 4.14$, $p < 0.05$. Upon further investigation, *deprivation state* was shown to have a significant affect on active lever responding for all antagonist dose groups; $F_{veh}(2, 24) = 12.02$, $p < 0.05$, $F_{low}(2, 33) = 6.42, p < 0.05$, $F_{high}(2, 30) = 12.96, p < 0.05$. Thus, as shown in Figure 3, acute FD caused a significant reinstatement of active lever pressing relative to baseline and sated conditions for the vehicle ($t_{base\text{-}FD}(24) = 4.37, p < 0.05$; $t_{sated\text{-}FD}(24) = 4.10, p < 0.05$), low ($t_{base\text{-}FD}(33) = 3.36, p < 0.05$; $t_{sated\text{-}FD}(33) = 2.77, p < 0.05$) and high ($t_{base\text{-}FD}(30) = 4.33, p < 0.05$; $t_{sated\text{-}FD}(30) = 4.98, p < 0.05$) antagonist groups. However, blockade of the DA D_1 receptor with SCH 23390 produced a dose dependant attenuation of acute FD-induced reinstatement of drug seeking. This attenuation is evidenced by a significantly lower reinstatement in the high-dose group relative to the vehicle group, $t(29) = 3.06, p < 0.05$. For inactive lever pressing a significant effect of *deprivation state* was found, $F(2, 58) = 9.27, p < 0.05$. Rats responded more on the inactive lever when FD than during baseline, $t(93) = 4.08, p < 0.05$, and sated, $t(93) = 2.85, p < 0.05$, conditions. It is likely that this slight increase in inactive lever responding resulted from generalized drug seeking behavior or an increase in locomotor activity. Regardless of the cause of this activity, it is clear from Figure 3 that the rats were able to discriminate between levers and preferred to respond on the previously active one. Moreover, inactive lever responding was not affected by *antagonist dose,* $F(2, 29) = 2.41$, n.s., and no significant *deprivation state* X *antagonist dose* interaction effect, $F(4, 58) = 0.77$, n.s, was observed.

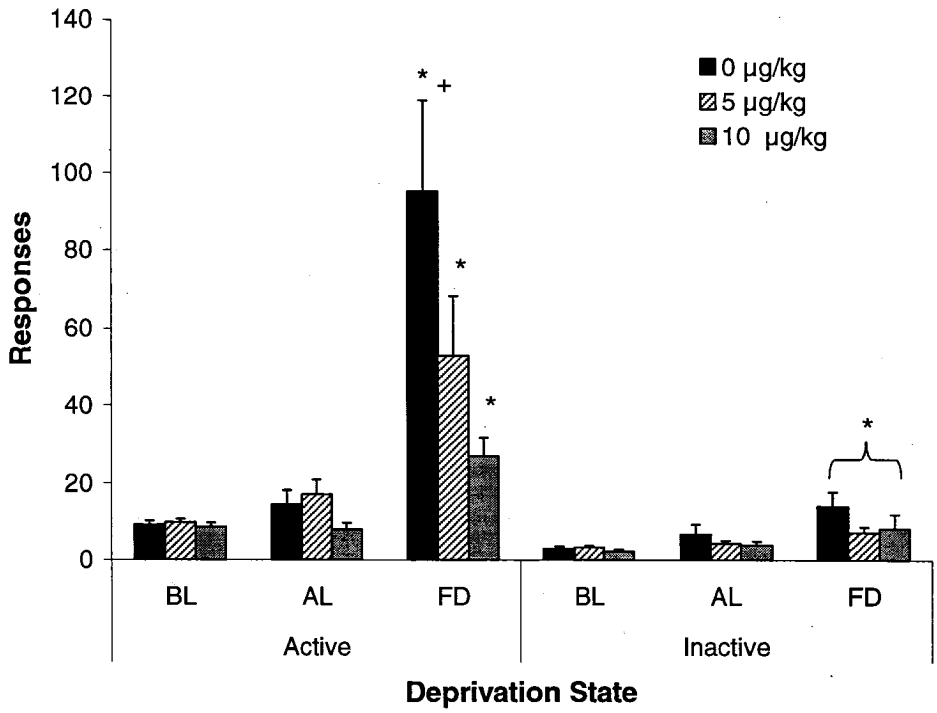

Figure 3. The effect of the DA D_1 antagonist, SCH 23390, on acute FD-induced reinstatement of heroin seeking. Data are mean (+*SE*) for active (left) and inactive (right) lever responding during baseline (BL), following 48 h FD, and sated (*ad libitum*: AL) conditions. Different groups of rats were used for each dose of SCH 23390 (0 µg/kg, $n = 9$; 5 µg/kg, $n = 12$; 10 µg/kg, $n = 11$).

* $p < 0.05$ when compared to baseline and sated conditions, + $p < 0.05$ when compared to the 10 µg group

Experiment 3: The effect of the D_2 receptor antagonist, raclopride, on acute food deprivation-induced reinstatement of heroin seeking

As seen in Figure 4, rats showed greater responding on the active lever, during training, than on the inactive lever (see Appendix B for daily statistics).

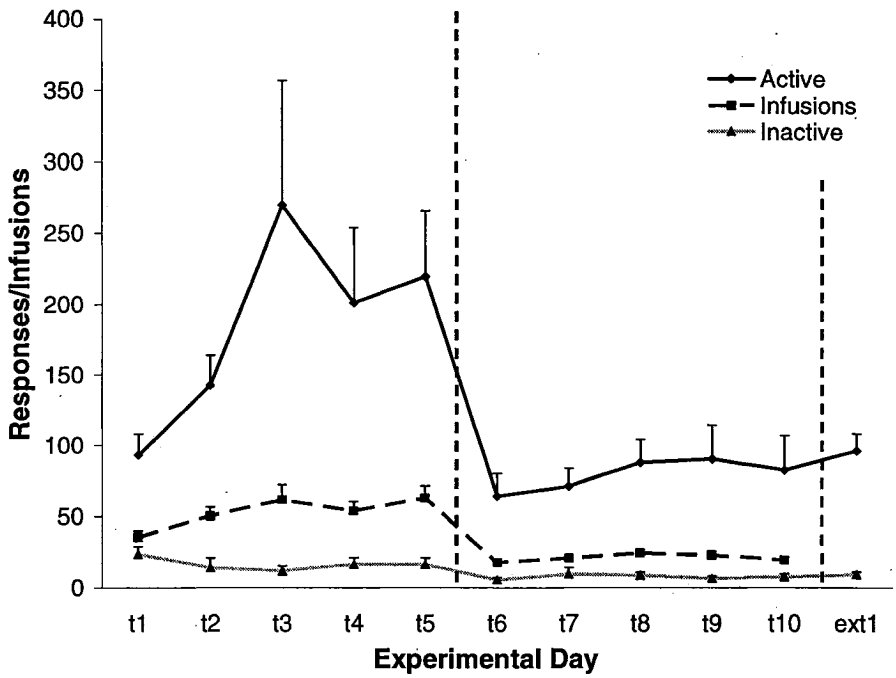

Figure 4. Heroin training phase. Mean (+*SE*), number of active lever presses, inactive lever presses and heroin infusions (0.05 mg/kg/infusion) for the 27 rats in experiment 3 over the 10-day training period (days 1-5: three 3h sessions; days 6-10: one 3h session). Also shown is the number of active and inactive lever responses for the first day of extinction. A significant difference existed between active and inactive lever responding on all training days.

Following training rats were assigned to one of three dose conditions (vehicle: n = 8; low: n = 9; high: n = 10) such that no differences existed in terms of mean active lever responses (M_{veh} = 147.13, SE = 65.21; M_{low} = 128.83, SE = 39.67; M_{high} = 123.80, SE = 32.16), $F(2, 24) = 0.07$, n.s., inactive lever responses (M_{veh} = 10.48, SE = 1.75; M_{low} = 7.73, SE = 1.92; M_{high} = 17.94, SE = 5.02), $F(2, 24) = 2.36$, n.s., and infusions (M_{veh} = 35.66, SE = 7.60; M_{low} = 40.76, SE = 8.64; M_{high} = 35.60, SE = 5.03), $F(2,24) = 0.17$, n.s during the 10 days of training.

Following training, rats were given approximately 6 days of extinction (M = 5.52, SE = 0.41; see Appendix C) prior to commencing reinstatement testing. As seen in Figure 5, *deprivation state* had a significant effect on responding directed at the

previously active lever, $F(2, 48) = 25.72$, $p < 0.05$. Acute FD significantly increased responding on this lever relative to baseline, $t(78) = 6.05$, $p < 0.05$, and sated conditions, $t(78) = 5.29$, $p < 0.05$. Raclopride dose had no significant effect on active lever pressing, $F(2, 24) = 1.73$, n.s., nor, was there any *antagonist dose* X *deprivation state* interaction, $F(4, 48) = 1.13$, n.s. Thus raclopride dose did not significantly affect acute FD-induced reinstatement.

As for inactive lever pressing, *deprivation state*, $F(2, 48) = 4.87$, $p < 0.05$, but not *antagonist dose*, $F(2, 24) = 0.06$, n.s., or *antagonist dose* X *deprivation state* interaction, $F(4, 48) = 0.34$, n.s., significantly effected responding. Acute FD caused an increase in lever pressing relative to the baseline condition, $t(78) = 2.42$, $p < 0.05$, but not relative to the sated test condition, $t(78) = 0.37$, n.s. Thus, the testing procedure itself rather than acute FD may have caused this small increase in inactive lever pressing.

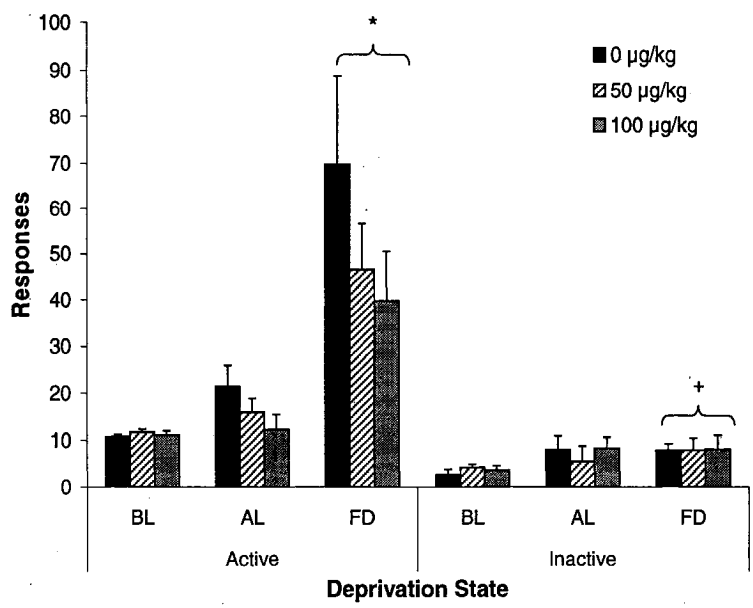

Figure 5. The effect of the DA D_2 antagonist, raclopride, on acute FD-induced reinstatement of heroin seeking. Data are mean (+*SE*) for active (left) and inactive (right) lever responding during baseline (BL), following 48 h FD, and sated (*ad libitum*: AL) conditions. Different groups were used for each dose of raclopride (0 µg/kg, $n = 8$; 50 µg/kg, $n = 9$; 100 µg/kg, $n = 10$).

* $p < 0.05$ when compared to baseline and sated conditions, + $p < 0.05$ when compared to baseline

Experiment 4: The effect of the D_3 receptor antagonist, NGB 2904, on acute food deprivation-induced reinstatement of heroin seeking

As demonstrated in experiments 2 and 3, here as well rats displayed an increased preference for the lever paired with heroin delivery throughout training (see Figure 6). With the exception of day 6, when heroin access was reduced to 3 h, rats showed significantly more active than inactive lever pressing (see Appendix B for paired samples t-test results). Moreover, specificity of lever pressing was demonstrated by a significant active ($M_{train10}$ = 34.08, SE = 4.82; M_{ext1} = 88.54; SE =14.53), $t(23) = -3.43$, $p < 0.05$, but not inactive, ($M_{train10}$ = 8.42, SE = 2.57; M_{ext1} = 10.67; SE = 1.32), $t(23) = -0.87$, n.s., lever-response burst on the first day of extinction (see Appendix C for the pattern of extinction). It took on average 5.92 (SE = 0.41) days for rats to reach the extinction criteria required for the commencement of reinstatement testing. For testing rats were assigned to one of three antagonist groups (vehicle: n = 8; low: n = 7; high: n = 9). Groups were similar in terms infusions received (M_{veh} = 27.19, SE = 3.78; M_{low} = 38.29, SE = 5.30; M_{high} = 25.34, SE = 1.62), $F(2, 21) = 2.05$, n.s., as well as active (M_{veh} = 64.15, SE = 13.11; M_{low} = 110.13, SE = 22.25; M_{high} = 61.78, SE = 9.50), $F(2, 21) = 3.15$, n.s., and inactive (M_{veh} = 16.25, SE = 13.29; M_{low} = 21.56, SE = 17.46; M_{high} = 22.18, SE = 25.43), $F(2, 21) = 0.22$, n.s., lever responses throughout the 10-day training session.

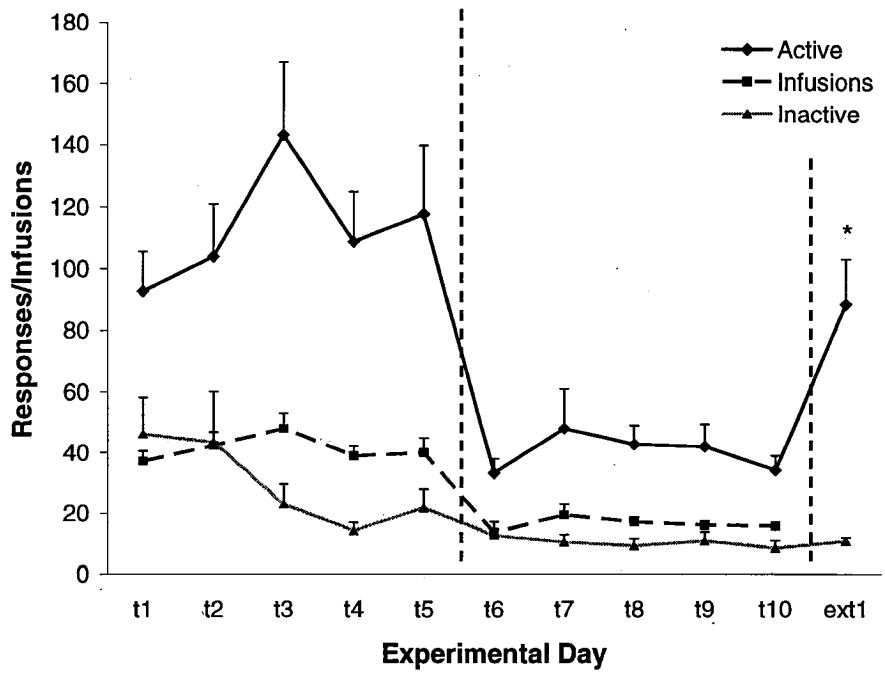

Figure 6. Heroin training phase. Mean (+*SE*), number of active lever presses, inactive lever presses and heroin infusions (0.05 mg/kg/infusion) for the 24 rats in Exp. 4 over the 10-day training period (days 1-5: three 3h sessions; days 6-10: one 3h session). Also shown is the number of active and inactive lever responses for the first day of extinction. A significant difference existed between active and inactive lever responding on all training days.

* indicates a significant ($p < .05$) increase in responding following drug removal (as compared to training day 10)

Mixed factorial ANOVA's preformed on active lever responses during reinstatement tests, revealed a significant effect of *deprivation state* on active lever pressing, $F(2, 42) = 30.07$, $p < 0.05$. Food deprived rats responded significantly more on the active lever than during baseline (see Figure 7), $t(69) = 6.71$, $p < 0.05$, or sated, $t(69) = 5.57$, $p < 0.05$, conditions. A mixed factorial ANOVA for inactive lever responding revealed a significant effect of *deprivation state*, $F(2, 42) = 3.62$, $p < 0.05$, but not *antagonist dose*, $F(2, 21) = 1.63$, n.s., or *deprivation state* X *antagonist dose* interaction, $F(4, 42) = 1.42$, n.s. Upon further investigation, using a one-way ANOVA,

no significant effect of *deprivation state* was found, $F(2, 69) = 2.97$, n.s. Thus, post-hoc comparisons were not preformed.

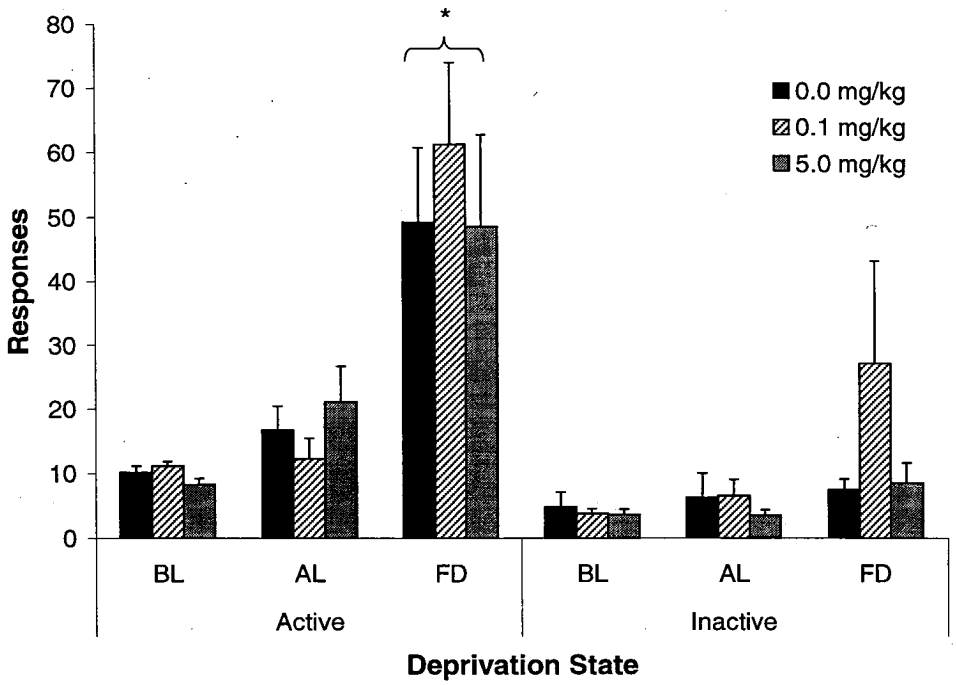

Figure 7. The effect of the DA D_3 antagonist, NGB 2904, on acute FD-induced reinstatement of heroin seeking. Data are mean (+*SE*) for active (left) and inactive (right) lever responding during baseline (BL), following 48 h FD, and sated (*ad libitum*: AL) conditions. Different groups were used for each dose of NGB 2904 (0.0 µg/kg, $n = 8$; 0.1 mg/kg, $n = 7$; 5.0 mg/kg, $n = 9$).

* $p < 0.05$ when compared to baseline and sated conditions

Summary

The above data suggest a differential role for the DA D_1, D_2 and D_3 receptor subtypes in acute FD-induced reinstatement of heroin seeking. The present experiments suggest that antagonism of D_2 and D_3 receptors with raclopride and NGB 2904, respectively, have no effect on acute FD-induced reinstatement. The only antagonist shown to have a significant effect was SCH 23390, a DA D_1 receptor antagonist. These findings suggest that acute FD-induced reinstatement is regulated, at least in part, by a neuronal mechanism which is heavily inundated by DA D_1 and not other DA receptors. Alternatively, acute FD-induced reinstatement may be mediated through the activation of

the other DA receptors investigated; but, some procedural limitations may have prevented the detection of any relevant effects. For this reason the above protocol has been adapted to assess the role of DA D_2 and DA D_3 receptors in cue-induced reinstatement of heroin seeking.

EXPERIMENTS 5-6: The effect of dopamine D_2 and D_3 receptor antagonism on cue-induced reinstatement of heroin seeking

Method & Results

Subjects

A total of 20 (Exp 5: $n = 10$; Exp 6: $n = 10$) male Long Evans rats (Charles River, St. Constant, QC) where subject to the same housing and surgical conditions as the rats in experiments 2-4.

Procedure

Following surgery rats were given 36-48 h of recovery before being placed in the same operant chambers described above. Rats were trained to self-administer heroin (0.05 mg/kg/infusion) using the procedure followed in experiments 2-4. Moreover, following training, rats were also subjected to a minimum of 4 extinction sessions. For the current experiment, however, both active and inactive lever presses were without consequence during extinction. Thus, extinction sessions consisted of a continuously illuminated houselight and two extended levers. Similar to experiments 2-4 rats were acclimated to mock saline injections (Exp 5: s.c.; Exp 4: i.p.) 15-20 min before the start of each extinction session, beginning on the third day of extinction, and were considered extinguished after reaching the same extinction criterion (< 15 active lever responses in a

3-h session). In an effort to mirror the procedure used in experiments 2-4, rats received 2 days "off" without a session prior to tests of cue-induced reinstatement. Each rat received two 3-h reinstatement tests. For these tests rats were exposed to the cues associated with heroin training. Thus, the start of each test session was marked by the insertion of both levers, the turning on of the houselight and the activation of the cuelight/tone complex. The cuelight and tone remained on for 30 s or until the active lever was pressed. Active lever presses resulted in 20 s of houselight suppression, cuelight/tone activation and drug pump activation (no drug was present). Depending upon the experiment rats were injected with raclopride (s.c.; exp 5) or NGB 2904 (i.p.; exp 6) and vehicle (exp 5: saline, exp 6: hydroxypropyl beta cyclodextrin + saline; see exp 1), in a counterbalanced order, 15-20 min prior to testing.

Drugs

Heroin and DA antagonists were prepared according to the procedures described above. During training heroin was delivered at a concentration of 0.05 mg/kg/infusion. DA antagonists were prepared in the following doses; raclopride: 100 μg/kg and NGB 2904: 5.0 mg/kg.

Statistical Analysis

Statistical analyses for the effect of raclopride or NGB 2904 on cue-induced reinstatement of heroin seeking behavior were preformed using repeated measures ANOVA's to separately investigate the effect of *antagonist state* (baseline, vehicle, antagonist) on active and inactive lever pressing during cue-induced reinstatement. Significant effects where followed up with Fisher's Least Significant Differences comparisons. All analyses were evaluated for significance at $\alpha = 0.05$.

Results

Experiment 5: The effect of dopamine D_2 receptor antagonism on cue-induced reinstatement of heroin seeking

One rat was eliminated from the current dataset due to a failure to acquire heroin self-administration (a mean of less than 15 responses on the active lever during the last 4 days of training). As seen in Figure 8, rats demonstrated a clear preference for the active lever over the inactive lever. Active lever pressing remained relatively stable throughout training. Inactive lever responding spiked on the first and fifth day of training; however, in general, responding on this lever was low.

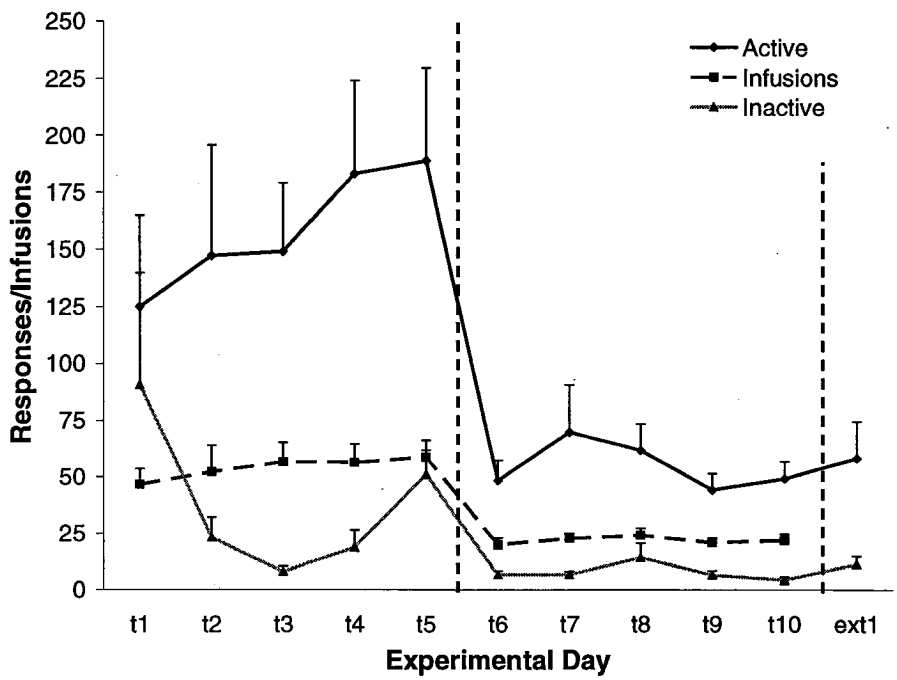

Figure 8. Heroin training phase. Mean (+*SE*), number of active lever presses, inactive lever presses and heroin infusions (0.05 mg/kg/infusion) for the 10 rats in Exp. 5 over the 10-day training period (days 1-5: three 3h sessions; days 6-10: one 3h session). Also shown is the number of active and inactive lever responses for the first day of extinction.

Following training rats received approximately 7 daily ($M = 6.89$, $SE = 0.69$) extinction sessions before being represented with the drug-associated cues. Following cue

presentation, active lever responding, $F(2, 16) = 9.32$, $p < 0.05$, but not inactive lever responding, $F(2, 16) = 1.44$, n.s., was significantly affected by *antagonist state* (see figure 9). Post-hoc analyses revealed a significant cue-induced reinstatement of active lever responding, relative to baseline responding, for the vehicle, $t(24) = 3.38$, $p < 0.05$ but not high antagonist dose condition, $t(24) = 1.18$, n.s. Moreover, active lever responding on the raclopride test day was shown to be significantly less than active lever responding on the vehicle test day, $t(24) = 2.21$, $p < 0.05$.

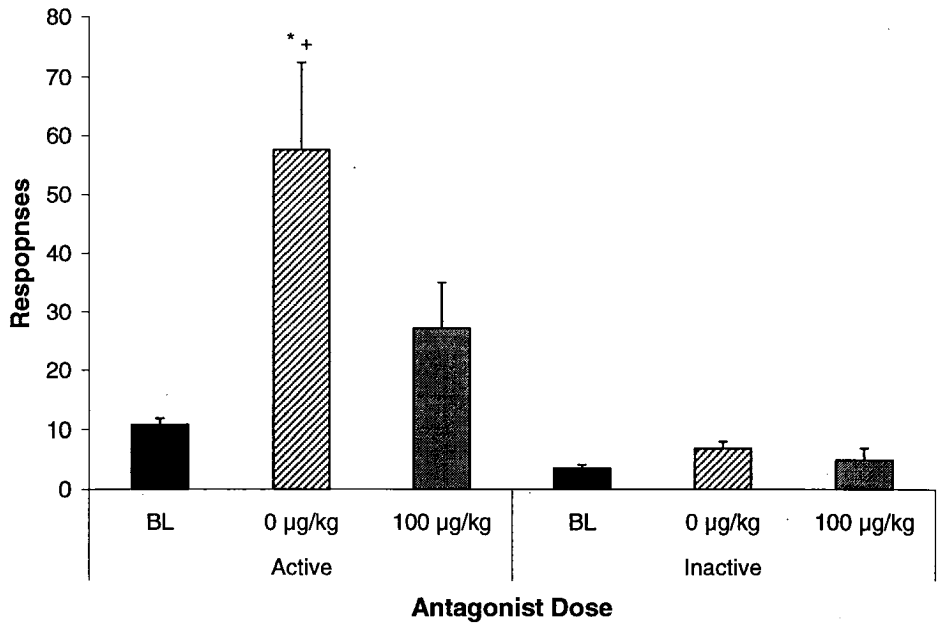

Figure 9. The effect of the DA D_2 antagonist, raclopride, on cue-induced reinstatement of heroin seeking. Data are mean (+*SE*) for active (left) and inactive (right) lever responding during baseline (BL), following cue exposure + raclopride injection (100 µg/kg, s.c.; 15-20 min before session), and cue exposure + vehicle (saline injection, s.c.) conditions; $n = 9$. The baseline (BL) condition represents mean responding on the last extinction day prior to each test session.

* $p < 0.05$ when compared to baseline, + $p < 0.05$ when compared to the raclopride condition

Experiment 6: The effect of dopamine D_3 receptor antagonism on cue-induced reinstatement of heroin seeking

One rat was removed from the current dataset due to a failure to respond for heroin. Figure 9, demonstrates the pattern of responding for the 9 remaining rats. On the first day of training, responding on the active and inactive levers was high; however, throughout training active lever responding remained high and inactive lever responding decreased. Following training rats experienced approximately 5 days (M = 5.25; SE = 0.49) of extinction before being tested for cue-induced reinstatement.

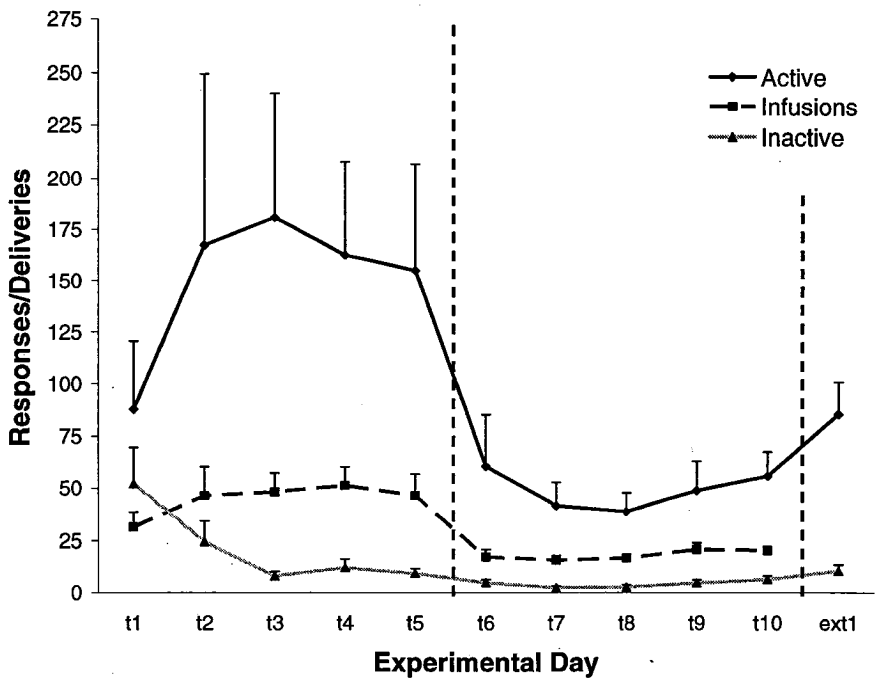

Figure 10. Heroin training phase. Mean (+*SE*), number of active lever presses, inactive lever presses and heroin infusions (0.05 mg/kg/infusion) for the 9 rats in Exp. 6 over the 10-day training period (days 1-5: three 3h sessions; days 6-10: one 3h session). Also shown is the number of active and inactive lever responses for the first day of extinction day.

A repeated measures ANOVA revealed a significant effect of *antagonist state* on active lever responding, $F(2, 16) = 5.87$, $p < 0.05$; but not inactive lever responding,

$F(2, 16) = 0.96$. n.s. Post-hoc analyses for the previously active lever revealed that rats increased lever responding, relative to the baseline condition, when pretreated with either the vehicle, $t(24) = 2.68$, $p < 0.05$, or the high antagonist dose (5 mg/kg), $t(24) = -3.23$, $p < 0.05$, prior to testing (see Figure 10). Moreover, there was no significant difference in active lever responding when rats were given the vehicle or when they were given NGB 2904 prior to reinstatement testing, $t(24) = 0.55$, n.s. For the inactive lever a repeated measures ANOVA revealed no significant effect of antagonist dose on responding, $F(2, 16) = 0.96$, n.s.

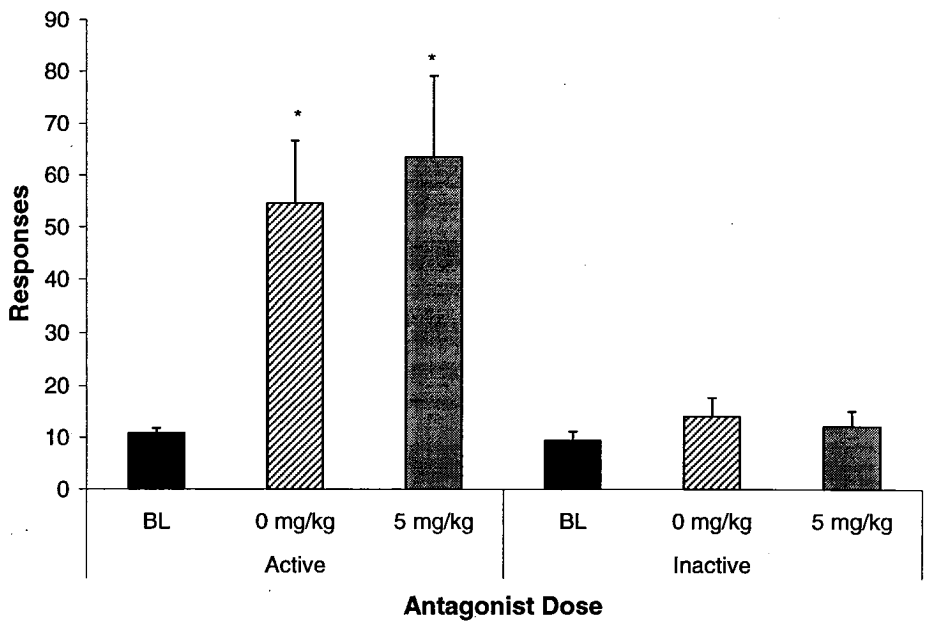

Figure 11. The effect of the DA D_3 antagonist, NGB 2904, on cue-induced reinstatement of heroin seeking. Data are mean (+*SE*) for active (left) and inactive (right) lever responding during baseline (BL), following cue exposure + NGB 2904 injection (5 mg/kg, s.c.; 15-20 min before session), and cue exposure + vehicle (25% hydroxypropyl beta cyclodextrin, i.p.) conditions; n = 9. The baseline (BL) condition represents mean responding on the last extinction day prior to each test.

* $p < 0.05$ when compared to baseline condition

Summary

The raclopride dose used in experiment 5 resulted in a clear attenuation of cue-induced reinstatement. Such attenuation of active lever responding was not seen in rats given this antagonist dose prior to tests of acute FD-induced reinstatement. Surprisingly, NGB 2904 had no effect on cue-induced reinstatement in the current experiment. This finding differs from a previous report demonstrating a role for the DA D_3 receptor in cue-induced reinstatement of cocaine seeking (Gilbert et al., 2005). Thus, it may be the case that the DA D_3 receptor plays a differential role in the reinstatement of heroin and cocaine seeking.

General Discussion

For recovering addicts exposure to stressful events constitutes a major challenge to sobriety (Hyman, Fox, Hong, Doebrick, & Sinha, 2007; Goeders, 2003). In an effort to understand the neuronal mechanisms through which stress can reinstate drug seeking, the current thesis utilized an animal model of stress-induced relapse. Generally, this model involves using inescapable footshock to trigger a behavioral reinstatement (Shaham, Erb, & Stewart, 2000). However, footshock is not a stressor likely to be experienced by recovering drug abusers. Rather, many abusers of illicit drugs may be subject to periods of food scarcity. Thus, the current thesis employed a perhaps more relevant form of stress: acute FD. The current studies suggest a potential dissociation between the neuronal mechanisms mediating acute FD- and footshock-induced reinstatement. Here, systemic injection of a DA D_1, but not D_2 or D_3, receptor antagonist produced an attenuation of reinstatement; such findings have not been demonstrated for footshock-induced reinstatement.

In an effort to validate these findings, the lack of effect for DA D_2 and D_3 receptor antagonism was further investigated using cue-induced reinstatement, an alternative model of drug relapse. At the current doses both of these antagonists have been shown to be effective in reducing cue-induced reinstatement of cocaine seeking (Cervo et al., 2003; Gilbert et al., 2005). Thus, it was expected that pretreatment with the DA D_2 antagonist, raclopride, and the D_3 receptor antagonist, NGB 2904, would attenuate cue-induced reinstatement. Contrary to this expectation, while raclopride administration showed a significant attenuating effect, NGB 2904 had no effect on cue-induced reinstatement. This result may suggest a differential involvement for the DA D_3 receptor in reinstatement depending upon the pharmacology of the previously self-administered drug.

Dopamine Receptor Blockade and Sucrose-reinforced Behavior

DA is a widespread and functionally diverse neurotransmitter. However, the current studies are only concerned with a small proportion of these effects; namely the role of DA in the reinstatement of drug seeking. In order to assess this role, the actions of DA at the D_1, D_2 and D_3 receptors were blocked by using the selective DA antagonists; SCH 23390, raclopride and NGB 2904, respectively. However, in addition to its role in drug reinforcement and addiction (Pierce & Kumaresan, 2006) DA also plays a major role in the control of motor behavior (Salamone, 1992). Hence, prior to investigating the role of DA in reinstatement the effect of DA receptor antagonism on non-drug reinforced behavior was studied (experiment 1). This experiment revealed no effect of SCH 23390, raclopride or NGB 2904 on responding for sucrose. Thus, it appears that at the current doses DA antagonism had no motor suppressing effect.

The Role of Dopamine in Food Deprivation-induced Reinstatement of Heroin Seeking

These studies expand upon previous research demonstrating a reinstatement of heroin (Shalev et al., 2000; Shalev et al., 2001; Shalev, Robarts, Shaham, &Morales, 2003; Shalev et al., 2006) and cocaine (Shalev, Marinelli, Baumann, Piazza, & Shaham, 2003) seeking following acute 21 h FD. Here a reinstatement of heroin seeking was shown with a more severe deprivation of 48 h. This procedural adjustment was made in an effort to increase the size of the reinstatement effect and is not likely to have had an affect on the neuronal systems mediating acute FD-induced reinstatement.

The results of experiments 2-4 suggest that acute FD-induced reinstatement may be mediated, at least in part, by the activation of the DA D_1 receptor. Inactivation of this receptor, with SCH 23390, prior to reinstatement testing caused a dose dependant attenuation of drug seeking behavior in FD rats (experiment 2). This result is both similar and dissimilar to findings with footshock. For footshock-induced reinstatement systemic injections of SCH 23390, even at doses 10 times greater than those used here, have no effect on reinstatement (Shaham & Stewart, 1996). In contrast, injection of SCH 23390 into the prelimbic or orbitofrontal cortex has been shown to block footshock-induced reinstatement (Capriles et al., 2003). Thus it appears that the DA D_1 receptor may play a role, albeit a different role, in both acute FD- and footshock-induced reinstatement.

The involvement of DA in acute FD-induced reinstatement seems to be limited to an activation of the DA D_1 receptor. In experiment 2 a dose-dependent attenuation of heroin seeking was observed; however, these effects were not replicated in experiments 3 or 4. Rats injected with raclopride (experiment 3) or NGB 2904 (experiment 4), prior to reinstatement testing, showed no significant reduction in drug seeking following acute

FD. These findings are similar to those demonstrated for footshock-induced reinstatement in that both forms of reinstatement are unaffected by systemic pretreatment with raclopride (Shaham & Stewart, 1996). Moreover, footshock-induced reinstatement is also unaffected by localized injection of raclopride into the prelimbic or orbitofrontal cortex (Capriles et al., 2003). Thus, it appears that DA D_2 receptor activation is not involved in stress-induced relapse, as modeled by acute FD or footshock-induced reinstatement. However, a conclusive statement to this affect can not be made. Upon further inspection of the results from experiment 3, it appears that raclopride reduced drug seeking in a dose-dependent manner. This effect is not significant; however, it is tempting to assume that at a higher dose raclopride would have significantly attenuated drug-seeking. At the current doses raclopride had no affect on responding for sucrose (experiment 1). Thus, it was assumed that these doses had no affect on motor behavior. However, prior cocaine and perhaps heroin, exposure can cause a sensitization of the cataleptic effects of raclopride (Ushijima, Mizuki, Suetsugi, Akimoto, & Yamada, 1998). Thus, rats in experiment 3 may have been more sensitive to the motor effects of raclopride than the drug naïve rats in the sucrose experiment. Furthermore, in Crombag et al's study (2002), an attenuation of drug seeking was seen in both the experimental, context renewal group, and the control group. Thus, while concluding that raclopride produced an attenuation of renewal-induced drug-seeking these authors could not rule out a potential motor suppressing effect of the antagonist.

While the DA D_2 receptor appears to play a similar role in both acute FD- and footshock-induced reinstatement, the DA D_3 receptor seems to play a disparate role in these two forms of reinstatement. Here, inactivation of the D_3 receptor, with NGB 2904,

had no effect on acute FD-induced reinstatement. Thus, it appears that this receptor is not involved in acute FD-induced reinstatement. However, inactivation of this receptor with another D_3 receptor specific antagonist, SB-277011, has been shown to attenuate footshock-induced reinstatement when injected systemically or locally into the nucleus accumbens (Xi et al., 2004). It is unclear what, if any, effect localized injection of NGB 2904 into the nucleus accumbens would have had on acute FD-induced reinstatement. As demonstrated for the DA D_1 receptor and footshock-induced reinstatement, systemic (Shaham & Stewart, 1996) and localized (Capriles et al., 2003) injections of the same antagonist may have a different effect on reinstatement.

The Role of the Dopamine D_2 and D_3 Receptors in Cue-induced Reinstatement of Heroin Seeking

The results of experiments 3 and 4 suggest that increased drug seeking following acute FD is not related to an activation of the DA D_2 or DA D_3 receptor. However, in order to validate these findings it was necessary to first demonstrate the effectiveness of the current antagonist doses in our hands. To this end, the high dose of both raclopride (experiment 5) and NGB 2904 (experiment 6) were assessed for their effectiveness in reducing cue-induced reinstatement. As previously mentioned, both raclopride and NGB 2904 have been shown effective, at the current doses, in attenuating cue-induced reinstatement of cocaine seeking in other laboratories (Cervo et al., 2003; Gilbert et al., 2005). As expected, raclopride caused a complete blockade of cue-induced reinstatement when used in experiment 5. Thus, such a dose should have adequately blocked DA D_2 receptors during acute FD-induced reinstatement. Therefore, the lack of a significant effect for raclopride in experiment 3 is most likely due to a non-involvement of this

receptor in acute FD-induced reinstatement; rather, than a peculiarity of the current research environment.

Contrary to what was expected, NGB 2904 had no effect on cue-induced reinstatement. These findings suggest one of two possibilities: 1) the NGB 2904 antagonist was ineffective in blocking the D_3 receptor in the current studies or 2) DA D_3 receptor activation is not involved in the reinstatement of heroin seeking. The first of these possibilities seems unlikely. NGB 2904 is a highly potent antagonist at the D_3 receptor. This antagonist shows a greater than 5,000 fold selectivity for the D_3 receptor over the D_1, D_4 and D_5 receptors and a greater than 800 fold selectivity for rat D_3 versus D_2 receptors in Sf9 cells. Moreover, NGB 2904 has been shown to have a 200-600 fold selectivity for the D_3 receptor over other neurotransmitter receptors such as serotonin (Newman, Cao, Bennett, Robarge, Freedman, & Luedtke, 2003; Yuan et al., 1998).

Alternatively, it may be the case that the DA D_3 receptor is not involved in the reinstatement of heroin seeking behavior. Previous studies have demonstrated a role for the D_3 receptor in nicotine-induced reinstatement of nicotine seeking (Andreoli, Tessari, Pilla, Valerio, Hagan & Heidbreder, 2003), cue-induced reinstatement of ethanol (Vengeliene et al., 2006) and cocaine seeking (Cervo et al., 2007; Xi et al., 2006) as well as footshock-induced reinstatement of cocaine seeking (Xi et al., 2004). However, to the best of my knowledge a role for the DA D_3 receptor has not been demonstrated in the reinstatement of heroin seeking.

Evidence for differential mechanisms for reinstatement of heroin and cocaine seeking can be seen in the fact that cocaine does not reliably reinstate heroin seeking (De Vries et al., 1998; Wit & Stewart, 1983). Nor, does heroin produce a reinstatement of

cocaine seeking (De Vries et al., 1998). Furthermore, heroin and cocaine seeking may be mediated by different neuronal processes. For example, priming with quinpirole, a DA D_2/D_3 receptor agonist, results in a reinstatement of drug seeking behavior in cocaine-, but not heroin-trained rats (De Vries, Schoffelmeer, Binnekade & Vanderschuren, 1999). The authors of this study suggest that DA D_2 receptor activation is involved in the motivation to seek cocaine but not heroin following prolonged withdrawal. However, this conclusion may also apply to the DA D_3 receptor, which is a member of D_2-like family of receptors, and shows higher affinity to quinpirole than the D_2 receptor (Robinson, Jarvie, & Caron, 1994).

Neuronal Substrates for Dopamine D_1 Receptor Function in Food Deprivation-induced Reinstatement

FD, like footshock causes an activation of the HPA axis and thus can be thought of as a form of stress-induced relapse (Bratt et al., 2001; Erb et al., 1998). The neuronal mechanisms mediating such reinstatement were studied by McFarland, Davidge, Lapish and Kalivas (2004) who used the DA receptor antagonist fluphenzaine (D_1 & D_2 receptor antagonist) and a combination of the $GABA_A$ and $GABA_B$ receptor agonists muscimol and baclofen to selectively inhibit a variety of neural structures during footshock-induced reinstatement. Based on the above studies these authors suggest a neuronal circuit mediating stress-induced reinstatement which involves the central extended amygdala (a limbic circuit consisting of the central nucleus of the amygdala, the ventral bed nucleus of the stria terminalis and the nucleus accumbens shell), the VTA, and a motor directing circuit involving the dorsal PFC, nucleus accumbens core and ventral pallidum. If acute FD-induced reinstatement relies upon this circuitry then it is likely that the effects of

SCH 23390 on acute FD-induced reinstatement are mediated by an inactivation of DA receptors in one of the above neural structures. The exact nature of this inactivation can not be discerned from the current study. However, it seems reasonable to assume that DA D_1 receptor inactivation may involve an area receiving VTA DAergic innervation, such as the PFC, nucleus accumbens or amygdala. Evidence to this effect comes from studies of DA D_1 antagonism in these areas with other forms of reinstatement. For example, injection of SCH 23390 into the PFC cortex has been shown to produce an attenuation of footshock-induced reinstatement (Capriles et al., 2003). Moreover, Fos expression in response to cocaine predictive cues is blocked by localized injection of SCH 23390 into the PFC or amygdala. Such injections also reduce actual drug seeking behavior (Ciccocioppo et al., 2001). Moreover, anticipatory firing of nucleus accumbens and PFC neurons prior to cocaine reinforcement is blocked by SCH 23390 (Nicola & Deadwyler, 2000).

While it is important to discern the site of action for SCH 23390 in acute FD-induced reinstatement it is also important to note that this antagonist caused an attenuation rather than a complete blockade of reinstatement. Thus DA D_1 receptor activation only partially mediates the effect of acute FD-induced reinstatement. This is similar to footshock-induced reinstatement where DA is thought to play a limited role. Rather, footshock-induced reinstatement is suggested to be mediated by central noradrenaline and extrahypothalamic CRF activation (Shaham et al., 1997). Similar to footshock, acute FD-induced reinstatement is dose dependently blocked by α-helical CRF, a CRF receptor antagonist (Shalev et al., 2006). Recently, a role for the DA D_1 receptor in the regulation of CRF has been demonstrated. When injected with SCH 23390

at doses of 0.05 mg/kg and 0.10 mg/kg rats demonstrate a significant reduction in CRF-enhanced startle response (Meloni, Gerety, Knoll, Cohen, & Carlezon, 2006). Thus it may be the case that DA D_1 receptor antagonism indirectly attenuates drug seeking by reducing CRF transmission.

Also, highly relevant to the current studies is the demonstration that leptin, a hormone involved in the long-term regulation of energy balance (Halaas et al., 1995), will completely suppress acute FD-induced reinstatement while having no effect on footshock-induced reinstatement (Shalev et al., 2001). The attenuating effect of leptin on acute FD-induced reinstatement of drug seeking may be mediated through DA. Leptin receptor mRNA expression can be seen in numerous brain areas (Elmquist, Maratos-Flier, Saper & Flier, 1998). For example, leptin receptor protein has been localized on DA cells in the VTA (Figlewicz, Evans, Murphy, Hoen & Baskin, 2003; Fulton, Pissios, Manchon, Stiles, Frank, Pothos et al., 2006; Hommel et al., 2006). Moreover, electrophysiological studies have revealed that leptin can reduce VTA DA cell firing, both *in vivo* and in slice preparations (Hommel et al., 2006).

Summary

The results of the present study suggest that acute FD-induced reinstatement may involve DA D_1, but not D_2 or D_3, receptor mediated transmission. Since antagonist injections were given systemically one can only hypothesize, based on previous research, about the mechanism of action for the DA D_1 antagonist. It may be the case that SCH23390 inactivates D_1 receptors in the PFC, the nucleus accumbens or the amygdala all of which receive DA innervation from the VTA, and are critically involved in the reinstatement of drug seeking behavior. Furthermore, since SCH 23390 attenuated, rather

than blocked acute FD-induced reinstatement it can be assumed that acute FD-induced reinstatement is not fully mediated by DA transmission.

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APPENDICES

APPENDIX A: Experimental Repetitions

Table 1-A.

Results from each of four experimental repetitions assessing the role of the DA D_1 receptor in acute FD-induced reinstatement.

	Repetition	***N***	**Mean (SE)**	**Statistic**
Average active lever responding (train 1-10)	1	9	90.63 (21.09)	$F(3, 28) = 0.12, p = 0.95$
	2	6	112.53 (26.15)	
	3	8	94.16 (20.40)	
	4	9	96.47 (30.77)	
Average inactive lever responding (train 1-10)	1	9	15.28 (3.69)	$F(3, 28) = 0.17, p = 0.92$
	2	6	19.02 (5.55)	
	3	8	18.73 (3.24)	
	4	9	18.43 (4.85)	
Infusions	1	9	30.00 (3.91)	$F(3, 28) = 0.71, p = 0.55$
	2	6	37.88 (6.21)	
	3	8	28.53 (3.77)	
	4	9	30.61 (4.46)	
Baseline active lever responding	1	9	11.22 (0.69)	$F(3, 28) = 3.12, p = 0.04$*
	2	6	9.08 (1.33)	
	3	8	7.25 (0.77)	
	4	9	8.89 (1.07)	
FD active lever responding	1	9	36.33 (10.85)	$F(3, 28) = 1.65, p = 0.20$
	2	6	37.00 (5.47)	
	3	8	56.50 (20.26)	
	4	9	87.56 (26.50)	
Sated active lever responding	1	9	11.78 (1.13)	$F(3, 28) = 0.38, p = 0.77$
	2	6	12.83 (6.31)	
	3	8	16.88 (5.42)	
	4	9	11.89 (2.77)	
Baseline inactive lever responding	1	9	2.39 (0.52)	$F(3, 28) = 0.29, p = 0.83$
	2	6	3.00 (0.82)	
	3	8	2.63 (0.61)	
	4	9	3.17 (0.77)	
FD inactive lever responding	1	9	9.00 (1.91)	$F(3, 28) = 0.66, p = 0.58$
	2	6	4.67 (1.23)	
	3	8	12.13 (4.80)	
	4	9	10.67 (4.33)	
Sated inactive lever responding	1	9	4.56 (1.11)	$F(3, 28) = 1.14, p = 0.35$
	2	6	2.83 (0.95)	
	3	8	7.25 (2.82)	
	4	9	4.11 (0.92)	

* significant difference between repetitions

Table 2-A.

Results from each of four experimental repetitions assessing the role of the DA D_2 receptor in acute FD-induced reinstatement.

	Repetition	*N*	Mean (SE)	Statistic
Average active lever responding (train 1-10)	1	4	225.00 (82.07)	$F(3, 23) = 2.40, p = 0.09$
	2	9	82.38 (16.70)	
	3	9	182.37 (57.10)	
	4	5	58.36 (15.50)	
Average inactive lever responding (train 1-10)	1	4	5.73 (1.56)	$F(3, 23) = 1.50, p = 0.24$
	2	9	13.41 (4.25)	
	3	9	9.92 (2.01)	
	4	5	19.98 (7.38)	
Infusions	1	4	56.48 (16.78)	$F(3, 23) = 2.70, p = 0.07$
	2	9	30.13 (3.81)	
	3	9	42.58 (7.00)	
	4	5	25.56 (3.43)	
Baseline active lever responding	1	4	10.25 (0.92)	$F(3, 23) = 0.73, p = 0.55$
	2	9	11.61 (0.75)	
	3	9	11.72 (0.67)	
	4	5	10.40 (1.23)	
FD active lever responding	1	4	54.00 (20.42)	$F(3, 23) = 0.04, p = 0.99$
	2	9	50.00 (12.81)	
	3	9	53.22 (18.01)	
	4	5	46.00 (9.02)	
Sated active lever responding	1	4	20.00 (6.46)	$F(3, 23) = 0.90, p = 0.46$
	2	9	16.33 (3.32)	
	3	9	18.33 (4.58)	
	4	5	9.40 (0.93)	
Baseline inactive lever responding	1	4	1.50 (0.20)	$F(3, 23) = 2.60, p = 0.08$
	2	9	5.06 (1.04)	
	3	9	2.50 (0.61)	
	4	5	4.40 (1.49)	
FD inactive lever responding	1	4	8.00 (2.56)	$F(3, 23) = 0.82, p = 0.50$
	2	9	8.22 (1.77)	
	3	9	5.44 (2.42)	
	4	5	12.00 (5.39)	
Sated inactive lever responding	1	4	4.50 (2.18)	$F(3, 23) = 0.55, p = 0.65$
	2	9	9.89 (3.75)	
	3	9	5.56 (2.75)	
	4	5	8.00 (1.95)	

Table 3-A.

Results from each of three experimental repetitions assessing the role of the DA D_3 receptor in acute FD-induced reinstatement.

	Repetition	***N***	**Mean (SE)**	***Statistic***
Average active lever responding (train 1-10)	1 2 3	9 9 6	63.00 (12.56) 92.12 (18.27) 74.00 (17.12)	*$F(2, 21) = 0.92, p = 0.41$*
Average inactive lever responding (train 1-10)	1 2 3	9 9 6	17.01 (6.60) 24.38 (6.18) 18.00 (8.66)	*$F(2, 21) = 0.36, p = 0.70$*
Infusions	1 2 3	9 9 6	26.43 (3.15) 31.66 (4.05) 28.30 (4.35)	*$F(2, 21)) = 0.53, p = 0.59$*
Baseline active lever responding	1 2 3	9 9 6	8.44 (0.92) 11.78 (0.61) 8.75 (1.02)	*$F(2, 21) = 5.16, p = 0.02$**
FD active lever responding	1 2 3	9 9 6	44.89 (10.91) 61.67 (14.43) 50.17 (12.57)	*$F(2, 21) = 0.49, p = 0.62$*
Sated active lever responding	1 2 3	9 9 6	11.67 (3.09) 24.00 (5.52) 14.83 (2.09)	*$F(2, 21) = 2.53, p = 0.10$*
Baseline inactive lever responding	1 2 3	9 9 6	3.50 (0.97) 5.61(1.89) 2.75 (0.28)	*$F(2, 21)) = 1.11, p = 0.35$*
FD inactive lever responding	1 2 3	9 9 6	8.00 (2.80) 24.11 (12.51) 6.33 (1.09)	*$F(2, 21) = 1.41, p = 0.27$*
Sated inactive lever responding	1 2 3	9 9 6	4.56 (1.73) 7.78 (3.30) 2.83 (1.38)	*$F(2, 21) = 0.96, p = 0.40$*

* significant difference between repetitions

APPENDIX B: Paired Samples t-tests

Paired t-tests demonstrating the disparity between active (Act) and inactive (Inact) lever responding throughout training (days 1-5: 3 X 3h; days 6-10: 3h) for rats in experiments 2 (n = 32; top), 3 (n = 27; middle) and 4 (n = 24; bottom). All tests are significant.

Comparison	***Act Mean (SE)***	***Inact Mean (SE)***	***Significance***
Act1 v Inact1	101.03 (15.07)	40.38 (8.47)	$t(31) = 3.54, p < .05$
Act2 v Inact2	151.28 (25.91)	19.78 (6.08)	$t(31) = 4.96, p < .05$
Act3 v Inact3	147.03 (21.80)	27.97 (11.45)	$t(31) = 4.51, p < .05$
Act4 v Inact4	1443.72 (22.68)	23.97 (6.64)	$t(31) = 5.09, p < .05$
Act5 v Inact5	149.44 (22.37)	26.28 (4.88)	$t(31) = 5.28, p < .05$
Act6 v Inact6	38.06 (5.80)	6.28 (1.33)	$t(31) = 5.24, p < .05$
Act7 v Inact7	46.97 (6.64)	9.09 (2.29)	$t(31) = 4.96, p < .05$
Act8 v Inact8	59.56 (8.99)	6.22 (1.17)	$t(31) = 5.55, p < .05$
Act9 v Inact9	60.88 (10.70)	8.84 (1.75)	$t(31) = 4.57, p < .05$
Act10 v Inact10	74.66 (13.58)	8.47 (1.93)	$t(31) = 4.85, p < .05$

Comparison	***Act Mean (SE)***	***Inact Mean (SE)***	***Significance***
Act1 v Inact1	93.26 (14.94)	24.11 (5.61)	$t(27) = 3.61, p < 0.05$
Act2 v Inact2	142.74(21.22)	14.37 (7.36)	$t(27) = 4.37, p < 0.05$
Act3 v Inact3	269.81 (86.90)	12.22 (3.65)	$t(27) = 3.24, p < 0.05$
Act4 v Inact4	201.33 (52.77)	16.70 (4.83)	$t(27) = 3.68, p < 0.05$
Act5 v Inact5	219.93 (45.93)	16.52 (5.06)	$t(27) = 2.16, p < 0.05$
Act6 v Inact6	64.44 (15.86)	5.85 (1.62)	$t(27) = 3.62, p < 0.05$
Act7 v Inact7	71.48 (12.41)	9.78 (4.94)	$t(27) = -0.22, p < 0.05$
Act8 v Inact8	88.00 (16.33)	8.89 (2.17)	$t(27) = 2.87, p < 0.05$
Act9 v Inact9	90.44 (23.96)	6.85 (1.72)	$t(27) = 3.66, p < 0.05$
Act10 v Inact10	82.44 (24.66)	7.96 (2.25)	$t(27) = 3.25, p < 0.05$

Comparison	***Act Mean (SE)***	***Inact Mean (SE)***	***Significance***
Act1 v Inact1	92.83 (12.78)	46.25 (11.91)	$t(23) = 3.14, p < 0.05$
Act2 v Inact2	103.96 (16.98)	43.50 (16.65)	$t(23) = 4.17, p < 0.05$
Act3 v Inact3	143.13 (23.78)	22.96 (6.62)	$t(23) = 4.80, p < 0.05$
Act4 v Inact4	108.83 (16.03)	14.21 (2.62)	$t(23) = 6.09, p < 0.05$
Act5 v Inact5	117.83 (21.88)	21.92 (6.07)	$t(23) = 4.22, p < 0.05$
Act6 v Inact6	33.21 (4.77)	12.71(4.43)	$t(23) = 2.89, p = 0.83$
Act7 v Inact7	48.13 (12.86)	10.33 (2.70)	$t(23) = 2.82, p < 0.05$
Act8 v Inact8	42.17 (6.38)	9.13 (2.39)	$t(23) = 4.84, p < 0.05$
Act9 v Inact9	42.00 (7.52)	10.79 (3.05)	$t(23) = 3.97, p < 0.05$
Act10 v Inact10	34.08 (4.82)	8.42 (2.57)	$t(23) = 4.72, p < 0.05$

APPENDIX C: Rate of Extinction

Table 1-C.

The number of days required for rats in experiment 2 to reach the extinction criteria required for reinstatement testing.

Rat	**Extinction days before first off period**	**Extinction days before second off period**
1	5	9
2	4	11
3	11	15
4	7	11
5	8	12
6	4	10
7	5	8
8	4	9
9	4	8
10	7	14
11	6	10
12	5	9
13	8	12
14	5	11
15	4	8
16	11	14
17	6	10
18	13	12
19	10	14
20	10	16
21	17	20
22	6	10
23	6	10
24	4	8
25	4	8
26	5	11
27	4	8
28	4	8
29	4	8
30	4	8
31	5	9
32	4	8
Mean	**6.38**	**10.59**
Standard Error	**0.56**	**0.51**

Table 2-C.

The number of days required for rats in experiment 3 to reach the extinction criteria required for reinstatement testing.

Rat	Extinction days before first off period	Extinction days before second off period
1	5	9
2	7	11
3	5	9
4	4	9
5	4	8
6	4	11
7	7	11
8	6	10
9	9	14
10	4	8
11	6	10
12	4	9
13	5	9
14	4	8
15	5	11
16	7	11
17	4	9
18	4	9
19	4	8
20	8	14
21	6	11
22	9	17
23	7	13
24	6	10
25	5	9
26	6	11
27	4	10
Mean	**5.52**	**10.33**
Standard Error	**0.30**	**0.41**

Table 3-C.

The number of days required for rats in experiment 4 to reach the extinction criteria required for reinstatement testing.

Rat	**Extinction days before first off period**	**Extinction days before second off period**
1	10	16
2	4	8
3	4	9
4	8	12
5	5	9
6	4	8
7	4	8
8	6	10
9	4	8
10	8	12
11	9	14
12	6	10
13	8	13
14	5	10
15	4	9
16	8	15
17	4	12
18	4	12
19	7	17
20	9	17
21	7	11
22	4	9
23	6	10
24	4	18
Mean	**5.92**	**11.54**
Standard Error	**0.41**	**0.64**

Table 4-C.

The number of days required for rats in experiment 5 to reach the extinction criteria required for reinstatement testing.

Rat	Extinction days before first off period	Extinction days before second off period
1	11	17
2	6	10
3	4	9
4	8	12
5	8	12
6	6	10
7	6	14
8	7	20
9	6	12
Mean	**6.89**	**12.89**
Standard Error	**0.65**	**1.20**

Table 5-C.

The number of days required for rats in experiment 6 to reach the extinction criteria required for reinstatement testing.

Rat	Extinction days before first off period	Extinction days before second off period
1	8	26
2	4	8
3	6	17
4	5	11
5	6	13
6	4	9
7	4	8
8	5	12
9	4	11
Mean	**5.25**	**13.00**
Standard Error	**0.49**	**2.14**

APPENDIX D: Antagonist Group Assignment

Experiment 2

Active Lever Responding During Training

Source	**Sum of Squares**	***df***	**Mean Square**	***F*-value**	**Sign. Value**
Between Groups	1138.34	2	569.17	0.11	0.89
Error	144747.59	29	4991.30		

Inactive Lever Responding During Training

Source	**Sum of Squares**	***df***	**Mean Square**	***F*-value**	**Sign. Value**
Between Groups	3.43	2	1.72	0.01	0.99
Error	4262.35	29	146.98		

Infusions During Training

Source	**Sum of Squares**	***df***	**Mean Square**	***F*-value**	**Sign. Value**
Between Groups	7.03	2	3.52	0.02	0.98
Error	4820.78	29	166.23		

Experiment 3

Active Lever Responding During Training

Source	**Sum of Squares**	***df***	**Mean Square**	***F*-value**	**Sign. Value**
Between Groups	2588.69	2	1294.35	0.07	0.93
Error	444550.34	24	18522.93		

Inactive Lever Responding During Training

Source	**Sum of Squares**	***df***	**Mean Square**	***F*-value**	**Sign. Value**
Between Groups	532.41	2	266.21	2.36	0.12
Error	2709.44	24	112.891		

Infusions During Training

Source	**Sum of Squares**	***df***	**Mean Square**	***F*-value**	**Sign. Value**
Between Groups	157.78	2	78.89	0.17	0.84
Error	10887.66	24	453.65		

Experiment 4

Active Lever Responding During Training

Source	**Sum of Squares**	***df***	**Mean Square**	***F*-value**	**Sign. Value**
Between Groups	11086.34	2	5543.17	3.15	0.07
Error	36980.17	21	1760.96		

Inactive Lever Responding During Training

Source	**Sum of Squares**	***df***	**Mean Square**	***F*-value**	**Sign. Value**
Between Groups	172.15	2	86.07	0.22	0.81
Error	8237.49	21	392.26		

Infusions During Training

Source	**Sum of Squares**	***df***	**Mean Square**	***F*-value**	**Sign. Value**
Between Groups	422.64	2	211.32	2.05	0.15
Error	2168.62	21	103.27		

APPENDIX E: Reinstatement Testing

Experiment 1

Dopamine D_1 Antagonist (Repeated Measures ANOVA)

Active Lever Responding

Source	Sum of Squares	*df*	Mean Square	*F*-value	Sign. Value
Antagonist Dose	1878.00	2	939.00	1.75	0.22
Error	6430.00	12	535.83		

Inactive Lever Responding

Source	Sum of Squares	*df*	Mean Square	*F*-value	Sign. Value
Antagonist Dose	2.95	2	1.48	0.34	0.71
Error	51.71	12	2.95		

Sucrose Delivery

Source	Sum of Squares	*df*	Mean Square	*F*-value	Sign. Value
Antagonist Dose	764.67	2	382.33	2.11	0.17
Error	2179.33	12	181.61		

Dopamine D_2 Antagonist (Repeated Measures ANOVA)

Active Lever Responding

Source	Sum of Squares	*df*	Mean Square	*F*-value	Sign. Value
Antagonist Dose	1579.71	2	789.86	0.44	0.65
Error	21466.95	12	1788.91		

Inactive Lever Responding

Source	Sum of Squares	*df*	Mean Square	*F*-value	Sign. Value
Antagonist Dose	33.43	2	16.71	0.35	0.71
Error	577.91	12	48.16		

Sucrose Delivery

Source	Sum of Squares	*df*	Mean Square	*F*-value	Sign. Value
Antagonist Dose	396.29	2	198.14	0.57	0.58
Error	4155.05	12	346.25		

Dopamine D_3 Antagonist (Repeated Measures ANOVA)

Active Lever Responding

Source	Sum of Squares	*df*	Mean Square	*F*-value	Sign. Value
Antagonist Dose	507.25	2	253.63	0.87	0.45
Error	4245.42	14	303.24		

Inactive Lever Responding

Source	Sum of Squares	*df*	Mean Square	*F*-value	Sign. Value
Antagonist Dose	3.58	2	1.79	0.49	0.63
Error	51.75	14	3.70		

Sucrose Deliveries

Source	Sum of Squares	*df*	Mean Square	*F*-value	Sign. Value
Antagonist Dose	30.33	2	15.17	0.30	0.75
Error	705.67	14	50.41		

Experiment 2

Dopamine D_1 Antagonist (Repeated Measures ANOVA)

Active Lever

Within Subjects Effects

Source	Sum of Squares	*df*	Mean Square	F-value	Sign. Value
Deprivation State	47077.08	2	23538.54	27.11	0.00*
Interaction	14380.37	4	3595.09	4.14	0.01*
Error	50361.42	58	868.30		

Between Subjects Effects

Source	Sum of Squares	*df*	Mean Square	*F*-value	Sign. Value
Antagonist Dose	9343.97	2	4671.98	4.96	0.01*
Error	27333.74	29	942.54		

Inactive Lever

Within Subjects Effects

Source	Sum of Squares	*df*	Mean Square	*F*-value	Sign. Value
Deprivation State	813.15	2	406.58	9.27	0.00*
Interaction	135.73	4	33.93	0.77	0.55
Error	2544.75	58	43.88		

Between Subjects Effects

Source	Sum of Squares	*df*	Mean Square	*F*-value	Sign. Value
Antagonist Dose	182.80	2	91.40	2.41	0.11
Error	1101.03	29	37.97		

Experiment 3

Dopamine D_2 Antagonist (Repeated Measures ANOVA)

Active Lever

Within Subjects Effects

Source	Sum of Squares	*df*	Mean Square	*F*-value	Sign. Value
Deprivation State	26341.78	2	13170.89	25.72	0.00*
Interaction	2311.14	4	577.79	1.13	0.35
Error	24585.06	48	512.19		

Between Subjects Effects

Source	Sum of Squares	*df*	Mean Square	*F*-value	Sign. Value
Antagonist Dose	2314.22	2	1157.11	1.73	0.20
Error	16066.79	24	669.45		

Inactive Lever

Within Subjects Effects

Source	Sum of Squares	*df*	Mean Square	*F*-value	Sign. Value
Deprivation State	303.04	2	151.52	4.87	0.01*
Interaction	41.74	4	10.44	0.34	0.85
Error	1495.03	48	31.15		

Between Subjects Effects

Source	Sum of Squares	*df*	Mean Square	*F*-value	Sign. Value
Antagonist	9.13	2	4.57	0.06	0.95
Error	1915.03	24	79.79		

Experiment 4

Dopamine D_3 Antagonist (Repeated Measures ANOVA)

Active Lever

Within Subjects Effects

Source	Sum of Squares	*df*	Mean Square	*F*-value	Sign. Value
Deprivation State	25538.81	2	12769.40	30.07	0.00*
Interaction	1007.36	4	251.84	0.59	0.67
Error	17837.05	42	424.69		

Between Subjects Effects

Source	Sum of Squares	*df*	Mean Square	*F*-value	Sign. Value
Antagonist Dose	101.54	2	50.77	0.07	0.93
Error	14592.87	21	694.90		

Inactive Lever

Within Subjects Effects

Source	Sum of Squares	*df*	Mean Square	*F*-value	Sign. Value
Deprivation State	1485.44	2	742.72	3.62	0.04*
Interaction	1165.55	4	291.39	1.42	0.24
Error	8613.68	42	205.09		

Between Subjects Effects

Source	Sum of Squares	*df*	Mean Square	*F*-value	Sign. Value
Antagonist	698.08	2	349.04	1.63	0.22
Error	4501.64	21	214.36		

Experiment 5

Cue Induced Reinstatement and Dopamine D_2 Antagonist (Repeated Measures ANOVA)

Active Lever

Within Subjects Effects

Source	Sum of Squares	*df*	Mean Square	*F*-value	Sign. Value
Antagonist Dose	10124.39	2	5062.19	9.32	0.00*
Error	8690.44	16	543.15		

Inactive Lever

Within Subjects Effects

Source	Sum of Squares	*df*	Mean Square	*F*-value	Sign. Value
Antagonist Dose	48.72	2	24.36	1.46	0.27
Error	271.44	16	16.97		

Experiment 6

Cue Induced Reinstatement and Dopamine D_3 Antagonist (Repeated Measures ANOVA)

Active Lever

Within Subjects Effects

Source	**Sum of Squares**	***df***	**Mean Square**	***F*-value**	**Sign. Value**
Antagonist Dose	14220.52	2	7110.269	5.87	0.01*
Error	19387.82	16	1211.74		

Inactive Lever

Within Subjects Effects

Source	**Sum of Squares**	***df***	**Mean Square**	***F*-value**	**Sign. Value**
Antagonist Dose	96.72	2	48.36	0.96	0.41
Error	809.11	16	50.57		