# NEURAL CORRELATES OF THE ACQUISITION OF DRUG-CUE ASSOCIATIONS

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# ABSTRACT

## NEURAL CORRELATES OF THE ACQUISTION OF DRUG-CUE ASSOCIATIONS

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This thesis describes an investigation of the pattern of cellular activity displayed within the medial prefrontal cortex (mPFC), amygdala and the nucleus accumbens (Acb) during the acquisition of heroin (0.3 mg/kg SC) conditioned place preference. We measured Fos immunoreactivity (IR) in the target regions after the initial and final day of conditioning in four groups that received exposure to different aspects of condition; CS-US, CS only, US only, or noCS-noUS. After a single day of conditioning, neural activity was enhanced in the amygdala and mPFC by the administration of the drug. However, the response to heroin in the mPFC was enhanced by simultaneous exposure to the conditioning environment. In the Acb, there were equivalent increases in activity in all four groups. Importantly, group differences were only found after one day of conditioning, a conditioning period that a subsequent experiment demonstrated not to be sufficient to induce a significant place preference.

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*Introduction*

Canada is home to an estimated 100,000 injection drug users, the majority of whom use illicit opioids (Fischer et al., 2004; Fischer et al., 2005). A substantive societal morbidity and mortality burden is associated with this drug use. A recent survey indicates that only 20% of illicit drug users in Canada receive income from a legal workplace (Fischer et al., 2005). This means that most users generate income from either criminal activities and/or social support sources. A recent cost-of-illness analysis of a local sample of illicit opioid users in Toronto found a social cost burden of $45,000 per untreated user/year (Fischer et al., 2005). Furthermore, the nature of injection drug use exposes users to increased risk of contracting infectious diseases (i.e. HIV, hepatitis B, hepatitis C) and overdose (Fischer et al., 2004).

*The Problem of Relapse*

One of the greatest hurdles in facing the problem of illicit opioid use is poor treatment effectiveness. In fact, only a small portion (e.g., 25%-30%) of users of illicit opioids seek treatment (Fischer et al., 2005), and even after undergoing successful detoxification and/or methadone maintenance, a majority of patients rapidly relapse to opiate use (Amato et al., 2005).

When asked about reasons for relapse, opiate addicts describe both negative affect and drug related stimuli as precipitating factors (Rohsenow et al., 1990). Drug related stimuli are thought to wield their influence on drug-seeking via conditioned effects. These conditioned responses to environmental cues are known as cue reactivity. Environmental cues can be visual, auditory or olfactory stimuli relating directly or indirectly to drug purchase or use. Reactivity can be measured in laboratory and clinical

settings as decreases in peripheral skin temperature, decreases in galvanic skin resistance and increases in heart rate in response to the presentation of these stimuli (Rohsenow et al., 1990).

Cue reactivity is believed to enhance vulnerability to relapse even after long periods of abstinence. Although direct evidence linking reactions to drug conditioned cues and relapse to drug taking behavior in humans is limited (Meyer & Mirin, 1979), it has been found that cue reactivity can be observed in patients 30 days after detoxification (Rohsenow et al., 1990), and that pharmacological interventions that block cue reactivity also reduce the urge to use and decrease rates of relapse (Volpicelli et al., 1992; Monti et al., 1999).

In addition to the physiological component of cue reactivity as measured by autonomic changes, cognitive reactions have been identified using tasks such as the emotional Stroop task (Franken et al., 2000). In this task, abstinent heroin users and non-heroin user controls were presented with both heroin-related words and neutral words. Their task was to vocally identify the colour in which the word was presented. It was found that the response time (RT) to the drug words for the drug users, but not the control group, was significantly higher than for the neutral words. Additionally, the increased RT of the heroin users was significantly correlated with self-reported measures of drug cravings (Franken et al., 2000). It is thought that this sort of attentional bias may contribute to an addict's susceptibility to relapse, indicating that, in addition to the physiological reactivity to drug cues, cognitive biases are a component of addictive behaviour. The neural aspects of this physiological reactivity, which have been

demonstrated in both human and laboratory animal subjects (i.e. Childress et al., 1999; Shaham, et al., 2003) will be discussed later.

*Drug Research in Laboratory Animals*

Cue reactivity in patient populations can be precipitated by discrete stimuli such as drug paraphernalia or individuals associated with drug taking as well as environmental or contextual cues (Rohsenow et al., 1990; O'Brien et al., 1992; Childress et al., 1999). Studies in laboratory animals have shown that drug-conditioned stimuli play an important role in the maintenance of drug-seeking and drug-taking behaviour. This is true of discrete stimuli such as a buzzer which predicts the availability of self-administered drugs (McFarland & Ettenberg, 1997; Weiss et al., 2001) or environmental stimuli which predict the availability of drug delivery at a specific location (Bossert et al., 2004).

*Place preference methodology*

In laboratory animals, the role of environmental cues is commonly investigated using the conditioned place preference (CPP) paradigm, a model of drug seeking based on Pavlovian conditioning. In CPP, an animal receives a drug injection (the unconditioned stimulus) and is then confined to a distinct environment (the conditioned stimulus) for a short time interval (typically, 30 min). This is repeated a number of times, alternating between drug pairings and saline injections and confinement in a second environment. Then, in drug free conditions, the animal is given a choice between the two compartments and, if the drug induced a positive motivational state during conditioning, the rats will show a preference for the drug-paired environment. In this model, therefore, the conditioned response is approach and maintenance of contact with the environment

previously associated with the positive motivational effects of the drug (Bardo & Bevins, 2000).

This response can be seen specifically as a conditioned preparatory response known as Pavlovian incentive learning (Di Chiara, 2002). In contrast to conditioned consummatory responses that are similar to their corresponding unconditioned response (UCR), conditioned preparatory responses are nonspecific to a given unconditioned stimulus (US). They include responses such as approaching, exploring and orienting to the conditioned stimulus (CS) (Di Chiara, 2002). It is thought that conditioned preparatory responses are a result of excitation of a motivational system common to different US's. Therefore, in CPP, representations of the drug-specific cues acquire a connection to this common motivational system.

*Neural substrates of drug learning*

There has been much interest in using CPP and other animal models based on Pavlovian conditioning to investigate the neurobiological mechanisms of this common motivational system. This research has suggested a central role for components of the mesocorticolimbic dopamine (DA) system including dopaminergic neurons in the ventral tegmental area (VTA) and its projections to the medial prefrontal cortex (mPFC), amygdala and the nucleus accumbens (Acb) (White & Milner, 1992; Everitt et al., 1999; Schultz & Dickinson, 2000; Di Chiara, 2002; Rezayof et al., 2002; Zarrindast et al., 2003; Phillips et al., 2003; Frances et al., 2004; Hyman et al., 2006).

The mPFC has been shown to be involved in drug-related learning such that aspiration of the entire area in rats eliminates cocaine conditioned place preference (Isaac et al., 1989). This cortical region, however, has several subdivisions which may serve

different functions (Tzschentke & Schmidt, 1999; Dalley et al., 2004). Nevertheless, the anterior cingulate cortex (Cg), prelimbic cortex (PrL) and infralimbic cortex (IL) are necessary for the acquisition of drug place preference (Tzschentke & Schmidt, 1999), and both the Cg (Childress et al., 1999; Neisewander et al., 2000; Kilts et al., 2001; McClernon et al., 2005) and the PrL (Miller & Marshall, 2005a) are activated by exposure to drug conditioned cues in both humans and animal models.

The amygdala has been identified by human neuroimaging studies to be involved in responses to drug cues (Childress et al., 1999; Kilts et al., 2001). One of its subdivisions, the central amygdala (CeA), shows cellular activation following acute cocaine administration (Neisewander et al., 2000), and levels of extracellular signal-regulated kinase (ERK) are elevated in the CeA in response to cocaine conditioned cues after a period of incubation (Lu et al., 2005). ERK is a signaling molecule that has been implicated in synaptic plasticity (Thomas & Huganir, 2004) and whose phosphorylation in the CeA is required for the expression of drug-related learning (Lu et al., 2005). Another important subdivision of the amygdala, the basolateral amygdala (BLA), shows cellular activation following exposure to an environment previously paired with cocaine availability (Neisewander et al., 2000). Furthermore, lesions to the BLA block the acquisition of cocaine CPP (Fuchs et al., 2002), as well as associative learning with a cocaine-paired cue (Kruzich & See, 2001).

Finally, the Acb is well known to modulate unconditioned and conditioned responses to drugs of abuse (Wise, 1998; Di Chiara, 2002; Ito et al., 2004). For example, administration of morphine induces the expression of the immediate early gene (IEG) *c-fos* in this region (Liu et al., 1994), and intra-accumbal infusions of c-fos antisense

prevents the acquisition of heroin CPP (Tolliver et al., 2000). The Acb is composed of two subregions, the shell (AcbSh) and core (AcbC) and these two regions may have different roles in drug-related learning (Hutcheson et al., 2001). The AcbC is required for Pavlovian stimuli to exert motivational influences of instrumental behavior (Parkinson et al., 1999; Hall et al., 2001) and lesion/inactivation of this area impairs acquisition (Ito et al., 2004) and expression (Di Ciano & Everitt, 2004; Fuchs et al., 2004) of drug-seeking maintained by drug-conditioned stimuli. The AcbSh on the other hand, mediates unconditioned hedonic reactions to appetitive stimuli (Pontieri et al., 1995; Bassareo et al., 2002; Ito et al., 2004).

In spite of this evidence, it is still not clear how these brain regions are recruited during the acquisition of drug related learning. More precisely, it is not clear whether different regions respond to different aspects of conditioning. In addition, it is not clear whether different regions are differentially recruited at different stages of conditioning. Therefore, the primary objective of this research was to investigate the pattern of cellular activity displayed during the acquisition of drug-related learning within the mPFC, amygdala and the Acb. To this end, we studies expression of Fos in these regions at different stages of the acquisition of heroin place preference.

*c-fos in Drug Research*

Fos immunoreactivity (IR) is used to study neural activity because of its temporal specificity. Like all IEG's *c-fos* is expressed immediately following neural firing and the result of this expression is the protein Fos. Following depolarization Fos levels show a rapid but transient rise, returning to baseline levels within hours of neural activity (Muller et al., 1984; Morgan & Curran, 1986; Morgan & Curran, 1991; Curran & Morgan, 1995).

For this reason Fos IR is used as a metabolic marker (Dragunow & Faull, 1989) in many studies of drug learning. These include drug exposure, both acute (Liu et al., 1994; Garcia et al., 1995; Pontieri et al., 1997; Bontempi & Sharp, 1997; Frankel et al., 1999) and chronic (Pontieri et al., 1997; Erdtmann-Vourliotis et al., 1999; Frankel et al., 1999), cued classical conditioning tasks (Nordquist et al., 2003), expression of conditioned responses (Mead et al., 1999; Neisewander et al., 2000; Schroeder et al., 2000; Franklin & Druhan, 2000), enhanced stimulator properties after chronic administration (sensitization) (Pontieri et al., 1997; D'Este et al., 2002) and the return to drug seeking after a period of abstinence (reinstatement) (Shalev et al., 2003).

*Methods*

*Subjects*

Subjects were adult male Sprague-Dawley rats (Charles River, QC) weighing 250-300 g at the beginning of the experiments. Rats were single housed and maintained on a reverse light/dark cycle (8:00 am lights off; 8:00 pm lights on) with free access to food and water except during testing, which occurred during their dark cycle. All experiments were approved by the Animal Care Committee of the University of Guelph and were carried out in accordance with the recommendations of the Canadian Council on Animal Care.

*Place conditioning apparatus*

Six custom made (University of Guelph), place conditioning boxes were used in these experiments. The boxes were located in the center on the floor of a laboratory room. Each place conditioning box was made of dark gray PVC, and comprised of three

compartments: two large (30 x 40 x 26 cm) and one smaller, middle (23 x 30 x 26 cm) compartment. Removable inserts, with or without small arch-way openings (10 x 10 cm) formed the center compartment. The two large compartments differed primarily in visual cues; one large compartment was dark gray while the other had a white wall and a 10 cm white stripe painted along the top of the other walls. In addition, there were cues that provided spatial information external to the compartments, such as benches, door and lights. In this apparatus, rats do not display a significant spontaneous preference for any of the compartments. The entire apparatus was covered by black wire mesh to allow video tracking of the rats during testing. The tracking software employed was EthoVision (version 3, Noldus Information Technology, The Netherlands). This system was used to automatically record time (seconds) spent in each compartment during tests for place preference.

*Immunohistochemistry*

The c-fos immunohistochemistry protocol used was adapted from Beaule et al. (Beaule & Amir, 2001). Ninety minutes after the completion of the last conditioning session rats were anesthetized with 0.5 ml Euthanol (pentobarbital, 340 mg/ml, IP) and perfused transcardially with 300 ml of cold physiological saline (0.9% NaCl) followed by 300 ml of cold 4% paraformaldehyde in a 0.1 M phosphate buffer. Brains were removed, postfixed in 4% paraformaldehyde (4°C) overnight, and cut on a vibratome in 50 μm-thick coronal sections. Immunostaining was carried out on free-floating sections using a rabbit anti-Fos polyclonal antibody recognizing residue 4-17 of the Fos protein (Ab-5; Oncogene Science, Cambridge, MA, USA) diluted 1:100 000 with a solution of 0.3% Triton X-100 in Tris-buffered saline (TBS) with 3% normal goat serum. Sections were

incubated with the anti-Fos antibody for 48 h at 4°C, rinsed in TBS, and then transferred to a solution of 0.3% Triton X-100 in TBS containing biotinylated anti-rabbit secondary antibody (1:200, Vector Laboratories, Burlingame, CA, USA). Fos immunoreactivity was detected with a Vectastain Elite ABC kit (Vector Labs) using nickel chloride-enhanced diaminobenzidine reaction. Digitized images of Fos IR within the areas of interest were obtained using a 10x objective and QCapture software (v 2.7.3; QImaging Corporation). Labeled cell counting and area measurement was completed with software from the National Institute of Health (ImageJ). For each image, the number of immunoreactive cells was divided by the area of the region of interest to produce a density value. For each region of interest, a mean density per animal was calculated from 4 to 6 images. As mentioned in the introduction, regions of interest included: 1) three sub-divisions of the mPFC (from +3.25 to +2.75 mm from bregma) - Cg, PrL, and IL; 2) two subdivisions of the amygdala (from -2.75 to -3.00 from bregma) - CeA and BLA and 3) primary subdivisions of the Acb (from +2.00 to +1.50 from bregma) - AcbC and AcbSh (Paxinos & Watson, 2005).

*Drugs*

Diacetylmorphine HCl (heroin) was obtained from Almat Pharmachem (Concord, Ontario), dissolved in 0.9% physiological saline, and injected subcutaneously (SC) at volume of 1.0 ml/kg. The doses of heroin used for conditioning (i.e., 0.3, 1, 3 and 6 mg/kg, see below for details) were selected on the basis of previous studies performed in our laboratory (Leri & Rizos, 2005). Vehicle (0.9% physiological saline) was injected at the same volume and by the same route.

*Procedures*

*Place conditioning*

Rats were allowed 4 days to habituate to the animal facility and handled for approximately 10 minutes before the beginning of the experiment. Place conditioning consisted of habituation, conditioning, and test sessions.

Habituation session – 20 min: The purpose of this session was to allow the rats to become accustomed to the specific environment in which conditioning took place (see *Experiment 1* for details).

Conditioning sessions – 30 min each: The day after habituation, place conditioning began. Each day of conditioning consisted of two 30-min sessions, one in the morning and one in the afternoon (morning session: between 9:00 am and 12:00 pm; afternoon session: between 2:00 and 5:00 pm; minimum time between the two sessions for a given subject: 3 ½ to 4 h). Rats received one session with drug (see doses below) and the other with vehicle. The specific compartment chosen to be associated with drug was counterbalanced across rats. Also, the injection order (e.g., drug AM and vehicle PM) alternated across days of conditioning such that, on the last day, all rats received drug in the PM session. In our laboratory, this two-sessions/day procedure has been found to elicit a reliable heroin place preference (Leri & Rizos, 2005).

Test session – 20 min: Twenty-four hours following the last day of conditioning, time spent by drug-free rats in all three compartments was monitored to detect a preference.

Using this basic procedure, two experiments were carried out.

*Experiment 1:* Sixteen different groups of rats were used to study how behavior and c-Fos expression changed during the acquisition of heroin place conditioning. Table

1 provides a list of experimental groups, sample size, and the number of the figure in which the results of each analysis is represented.

As indicated in this table, animals were assigned to one of two conditioning periods: one day of conditioning (1D, n = 84 total) or four days of conditioning (4D, n = 76 total). In each period, there were four experimental groups. The primary group of interest was conditioned as described above, receiving alternating injections of vehicle and heroin (0.3 mg/kg - US, unconditioned stimulus) in a specific compartments of the apparatus (CS, conditioned stimulus), and hence "CS-US" (see Table 1). Three additional experimental groups were required to interpret the c-Fos data. The "CS" group was conditioned as described above, but received vehicle injections on all conditioning sessions (i.e., never received heroin); this group was required to measure Fos IR elicited by the conditioning apparatus. The "US" group was conditioned with the same injection schedule as the CS-US group, except that all injections were administered prior to confinement in the home cage which was placed adjacent to the conditioning apparatus; this group was required to measure Fos IR to the injections of heroin. Finally, the "noCS-noUS" group received vehicle injections on all conditioning sessions prior to confinement in the home cage which was placed adjacent to the conditioning apparatus; this group was required to control for Fos IR induced by the handling, transportation, injection and other manipulations inherent in the place conditioning procedure.

Following conditioning, animals in each experimental group were either tested for place preference ("CPP" in Table 1) or sacrificed for Fos IR ("Fos" in Table 1).

*Experiment 2:* Following completion of Experiment 1, it became necessary to verify whether a single day of conditioning using higher doses of heroin would have been

sufficient to induce a significant place preference in our conditioning apparatus. Thus, three additional groups of rats (n=10 each) received one day of conditioning with heroin at 1, 3 or 6 mg/kg. All rats in this experiment were conditioned like the "CS-US" group above.

*Statistical analyses*

In these experiments, the presence of a CPP was indicated by significant difference in times spent in the vehicle- vs the heroin-paired compartments during the test phase. In Experiment 1, relative preferences for the two compartments in the CS-US group after 1 and 4 days of conditioning were evaluated using a two-way mixed-design ANOVA. Paired t-tests were performed to verify the expected lack of preference in the groups that did not receive full heroin-compartment pairing (i.e., CS, US and noCS-noUS groups). An identical design was used to analyze CPP in Experiment 2. Data from the groups conditioned with 0 and 0.3 mg/kg heroin over 1 day of conditioning in Experiment 1 (i.e., 1D CS and 1D CS-US groups, respectively) were included in the dose-response analysis of Experiment 2. Differences in Fos immunoreactivity in the mPFC, nucleus accumbens and amygdala were evaluated using separate two-way mixed design ANOVAs. In case of significant interactions or significant main effects multiple-comparisons were performed using the Fisher's LSD procedure in order to identify individual mean differences. The alpha level was set to 0.05. All statistical analyses were performed using GB-Stat (Dynamic Microsystems, Silver Spring, USA).

*Results*

*Experiment 1*

*Behaviour*

As indicated in Figure 1, only animals that received 4 days of conditioning with 0.3 mg/kg heroin displayed a significant place preference. The two way ANOVA revealed a main effect of Days of conditioning [$F(1, 23) = 6.03$, $p < 0.05$] and of Compartment [$F(1, 23) = 4.99$, $p < 0.05$]. As expected, no CPP was observed in any other experimental group (no figure; mean ± SEM seconds spent in vehicle & heroin compartments: 1D CS group = 254.75 ± 46.26 & 273.88 ± 36.98; 1D US group = 352.55 ± 39.76 & 308.16 ± 29.63; 1D noCS-noUS group = 394.74 ± 23.86 & 403.47 ± 38.68). 4D CS group = 330.02 ± 21.30 & 307.57 ± 30.37; 4D US group = 413.92 ± 29.34 & 394.21 ± 25.16; 4D noCS-noUS group = 399.05 ± 21.90 & 420.55 ± 21.75).

*Fos-IR in the medial prefrontal cortex*

Figure 2 represents density of Fos IR in the three subdivisions of the mPFC in the noCS-noUS, CS, US and CS-US groups. In general, two patterns of results were noted in all three subdivisions: 1) Fos expression was responsive to the association between the drug and the environment; and 2) this response was found only after the initial day of conditioning. Therefore, in the anterior cingulate cortex, the two way ANOVA indicated a significant interaction between Days of conditioning and Group [$F(3, 16) = 19.13$), $p < 0.0001$], as well as significant main effects of Days of conditioning [$F(1, 16) = 5.36$, $p < 0.05$] and of Group [$F(3, 16) = 13.08$, $p < 0.0001$]. After one day of conditioning, Fos IR was significantly elevated in the CS-US group in comparison to all other groups (all $p < 0.01$). Additionally, Fos IR in the US group was significantly elevated in comparison to the CS and noCS-noUS groups ($p < 0.01$). These differences, however, were not present after 4 days of conditioning. In the prelimbic cortex, the two way ANOVA indicated a

significant interaction between Days of conditioning and Group [$F(3, 16) = 9.47$), $p < 0.0001$], as well as significant main effects of Days of conditioning [$F(1, 16) = 10.91$, $p < 0.01$] and of Group [$F(3, 16) = 6.54$, $p < 0.001$]. After one day of conditioning, Fos IR was significantly elevated in the CS-US group in comparison to all other groups (NoCS-noUS and CS: $p < 0.01$; US: $p < 0.05$). Additionally, Fos IR in the US group was significantly elevated in comparison to the CS and noCS-noUS groups ($p < 0.01$). These group differences were not present after 4 days of conditioning. Finally, in the infralimbic cortex, the two way ANOVA indicated significant a interaction between Days of conditioning and Group [$F(3, 16) = 11.55$), $p < 0.0001$], as well as a significant main effect of Group [$F(3, 16) = 7.01$, $p < 0.001$]. Again, after one day of conditioning, Fos IR was significantly elevated in the CS-US group in comparison to all other groups (NoCS-noUS and CS: $p < 0.01$; US: $p < 0.05$), and Fos IR in the US group was significantly elevated in comparison to the CS and noCS-noUS groups ($p < 0.01$). After 4 days of conditioning no group differences emerged.

*Fos-IR in the Amydgala*

Figure 3 represents density of Fos IR in the two subdivisions of amydgala in the noCS-noUS, CS, US and CS-US groups. In general, two patterns of results were noted in both subdivisions: 1) Fos expression was responsive to heroin; and 2) this response was found only after the initial day of conditioning. Therefore, in the central amygdala, the two way ANOVA indicated a significant main effects of Days of conditioning [$F(1, 16) = 28.52$, $p < 0.001$] and of Group [$F(3, 16) = 3.22$, $p < 0.05$]. After one day of conditioning, Fos IR was significantly elevated in the US and the CS-US groups in comparison to the other groups ($p < 0.05$), and these two heroin groups did not differ

from each other. After 4 days of conditioning, no group differences were found. In the basolateral amygdala, the two way ANOVA indicated significant main effects of Days of conditioning [$F(1, 16) = 19.90$, $p < 0.001$] and of Group [$F(3, 16) = 5.46$, $p < 0.01$]. After one day of conditioning, Fos IR was significantly elevated in the US and the CS-US groups in comparison to the other groups ($p < 0.05$), and these two heroin groups did not differ from each other. After 4 days of conditioning, no group differences were found.

*Fos-IR in the nucleus accumbens*

Figure 4 represents density of Fos IR in the two subdivisions of the nucleus accumbens in the noCS-noUS, CS, US and CS-US groups. The pattern of results was similar across the two subdivisions, with Fos IR being responsive to the CS, as well as the US and their combination, but only after the initial day of conditioning. In the shell, the two way ANOVA revealed a significant main effect of Days of conditioning [$F(1, 16) = 8.68$, $p < 0.05$], but no group effect. Similarly, in core, the two way ANOVA revealed a significant main effect of Days of conditioning [$F(1, 16) = 22.76$, $p < 0.001$], but no group effect.

*Experiment 2*

Rats showed no preference for the heroin paired compartment after a single day of conditioning using 0.0, 0.3, 1.0, 3.0, or 6.0 mg/kg (no figure; mean ± SEM seconds spent in vehicle & heroin compartments: 0.0 mg/kg = 253.75 ± 46.26 & 273.88 ± 65.28; 0.3 mg/kg = 257.68 ± 35.11 & 273.41 ± 36.98; 1.0 mg/kg = 251.01 ± 19.81 & 316.08 ± 30.47; 3.0 mg/kg = 237.44 ± 16.16 & 249.81 ± 31.03; 6.0 mg/kg = 194.54 ± 22.81 & 207.01 ± 19.18).

*Discussion*

This is the first study to demonstrate regional and temporal specificity of neural activity within efferents of the mesocorticolimbic dopamine system during heroin place conditioning. In fact, after a single day of conditioning, neural activity was enhanced in the amygdala by the administration of the drug, which in these experiments served as the unconditioned stimulus. In the mPFC, we also observed elevated neural activity as a result of exposure to the drug. However, this response to heroin in the mPFC was enhanced by simultaneous exposure to the conditioning environment. In the Acb, there were equivalent increases in activity in all four groups. Importantly, these group differences were only found after one day of conditioning, a conditioning period that our subsequent experiment demonstrated not to be sufficient to induce a significant place preference.

*Temporal gradient during conditioning*

We found a significant effect of the number of days of conditioning in all three regions investigated such that the elevation of Fos IR observed after one day of conditioning but not after four days. This suggests that neural activity in efferents of the mesocorticolimbic dopamine system during heroin-induced place conditioning decrease as the animal learns to attribute motivational value to the conditioned environment. This finding is consistent with accounts of learning generated by formal learning theory (Rescorla & Wagner, 1972; Rescorla, 2001), as well as with recent neurophysiological (Schultz et al., 1997; Waelti et al., 2001; Bayer & Glimcher, 2005) and neuropharmachological (Phillips et al., 2003) data.

In the Rescorla-Wagner model of learning, the change in associative strength between a US and a CS reflects the difference between the current associative strength and the actual associative strength (Rescorla & Wagner, 1972). A neutral stimulus is perceived to have no predictive value regarding a reward (or punishment). However, the environmental contingencies may be such that the stimulus is actually a very good predictor of a reward. The difference between perception and reality is called a prediction error. Thus, an initial pairing of a neutral stimulus with a reward will result in a large prediction error, while a reward perfectly predicted by a conditioned stimulus will not result in a prediction error (Schultz, 2006). Recently is has been demonstrated that this property of learning results in the rate of acquisition of associative learning being negatively accelerated such that increases in associative strength are greater during initial conditioning sessions than later in the course of conditioning (Rescorla, 2001).

Using the blocking paradigm and multiple single cell recordings from DA neurons in the mesencephalon, it was shown that the activity of individual neurons conforms to this assumption of formal learning theory (Waelti et al., 2001). The blocking paradigm is a phenomenon whereby a well conditioned stimulus blocks conditioning to a novel stimulus (Kamin, 1969). It does so due to the fact that the well conditioned stimulus, when presented in compound with the novel stimulus, is able to block the ability of the novel stimulus to elicit a prediction error (Schultz & Dickinson, 2000). Initially, during conditioning with a single neutral stimulus, midbrain DA neurons respond with a short phasic activation following reward presentation (Schultz et al., 1997). However, as conditioning progresses, DA neurons shift their phasic activation from after the reward presentation to following the stimulus presentation. This shift in

activation mirrors the extention of responding from the reward to the stimulus (Schultz et al., 1997). The blocking paradigm showed that this shift is based on prediction error such that in the absence of a prediction error there is no shift in phasic activation, and no conditioned responding to the novel stimulus (Waelti et al., 2001).

Consistent with these findings, it has been demonstrated that changes in DA levels within VTA efferents are inversely related to the number of conditioning sessions (Phillips et al., 2003). In this study, two groups of rats were conditioned; one received a reward (sucrose solution) immediately following a discrete stimulus (CS+) while the second received the reward and discrete stimulus in a non-contingent manner (CS-). Within each of these groups, there were three sub-groups that received either 1, 4 or 20 days of conditioning to represent initial, intermediate and asymptotic levels of training respectively (Phillips et al., 2003). Immediately following the final conditioning session, an immunohistochemical technique was used to measure the changes in DA levels in the amygdala, mPFC, Acb and dorsal striatum. It was found that in all regions except AcbC there were higher levels of DA IR in the CS+ than the CS- group after the earlier sessions, but not at asymptote (Phillips et al., 2003). The fact that DA neuron firing elicited by prediction error is increasing DA levels at VTA efferents in a manner consistent with predictions of formal learning theory strongly supports the involvement of the mesocorticolimbic system in the neurobiology of drug learning.

DA receptor ($D_1$) activity is related to *c-fos* expression by a variety of intracellular signaling molecules which are involved in synaptic plasticity (Kandel, 2001). These molecular cascades require increases in intracellular calcium ($Ca^{2+}$) which is primarily influenced by NMDA-type glutamate receptors (Kandel, 2001; Thomas & Huganir,

2004). In the primary cascade, the increase in intracellular $Ca^{2+}$ combined with $D_1$ activity triggers adenylyl cyclase which converts ATP to cyclic AMP (cAMP). This second messenger then acts on the cAMP-dependant protein kinase A (PKA). If this cascade does not progress further, short-term changes such as enhanced transmitter availability and release will occur (Kandel, 2001). For long-term changes to take place, gene expression must be altered. This requires the activation of a second $D_1$ dependent process (Valjent et al., 2000), the mitogen-activated protein kinase (MAPK) molecular cascade (Thomas & Everitt, 2001; Neve et al., 2004). The intracellular changes elicited by $D_1$ activity such as adenylyl cyclase activation are mediated by the receptors activation of GTP-binding proteins (G-proteins) (Neve et al., 2004). When levels of the G-protein Ras-GTP increase, the activation of the protein kinase Raf is triggered, which in turn phosphorylates the enzyme MAPK/ERK kinase (MEK). MEK then activates the extracellular signal-regulated kinase (ERK) (Thomas & Huganir, 2004). It is the combined action of ERK and PKA that allows for the phosphorylation of cAMP response element-binding protein (CREB) which in turn is involved in the transcription of IEG's such as *c-fos* (Kandel, 2001; Thomas & Everitt, 2001; Neve et al., 2004).

Many of these signaling molecules such as PKA (Gerdjikov & Beninger, 2005), ERK (Valjent et al., 2000; Gerdjikov et al., 2004; Lu et al., 2005; Valjent et al., 2006) CREB (Walters et al., 2005) and MEK (Miller & Marshall, 2005b) have been implicated in the rewarding aspects of drugs of abuse. However, our findings indicate that the intracellular activity that is associated with drug-related learning is not homogeneous throughout conditioning. Recently, evidence has indicated that signaling molecules also are active on a gradient throughout conditioning. For example, injections of calcineurin,

an enzyme that negatively regulates PKA action, into the Acb at intermediate (but not initial or asymptotic) stage of conditioning was able to produce an enhancement of an amphetamine CPP (Gerdjikov & Beninger, 2005). This result suggests that there is a gradual buildup of signaling molecule-induced change across pairing sessions that leads finally to the observation of significant learning.

It is clear from the above evidence that the rate of learning, DA neuron firing patterns and DA IR, like Fos IR, demonstrate a temporal gradient during conditioning such that greater activity is found in the initial sessions of conditioning than in the final sessions. However, preliminary evidence suggests that signaling molecules may follow the inverse pattern (Gerdjikov & Beninger, 2005). This can be explained by the fact that elevations in Fos and DA firing and release are all transient events, whereas signaling molecules do not share the rapid dynamics seen with Fos. Rather their levels accumulate with continued activity. Therefore, it is possible that these transient events represent the rate of accumulation of these signaling molecules; DA preceding and Fos following their activation. Further research will be required to confirm this relationship.

Perhaps one of the most fascinating aspects of this temporal gradient is the fact that while levels of Fos IR in our study were high after the initial conditioning day, the behavioural expression of this conditioning, a place preference, was not observed until following the fourth day of conditioning, when Fos IR did not deviate from baseline. This provides tentative neurobiological support to the notion that the acquisition of associative strength is related to, but not identical to the acquisition of behavioural performance indices (Rescorla, 2001).

*Regional specificity*

Although the temporal gradient was found in each region, the specific patterns of Fos IR were different in the amygdala, mPFC and Acb. These patterns of activity reflect each regions involvement with different aspects of conditioning.

The amygdala has been implicated in the acquisition of appetitive conditioning such as CPP. Specifically, it has been shown to be necessary for the formation of an association between a neutral stimulus and reward (White & McDonald, 1993). This has been shown using aversive Pavlovian conditioning as well (LeDoux, 2000).

A recent review, drawing from research using both appetitive and aversive conditioning, has discussed the idea that the BLA and CeA, rather than operating in a serial manner, operate in parallel and are complementary in their role in stimulus-reward associations (Balleine & Killcross, 2006). The BLA is involved in associating the CS with the sensory properties of the US, while the CeA is involved in the association of the CS with the affective properties of the US (Balleine & Killcross, 2006). In our CS-US group the drug-paired compartment is only paired with US delivery, whereas in the US group the home cage is paired with both vehicle and US delivery. However, in both groups, Fos IR was measured following drug administration and thus the formation of associations between the neutral stimulus (environmental cues) and the sensory and affective properties of the reward could occur in both CS-US and US groups. Thus, Fos IR was elevated in both the CeA and BLA following administration of heroin.

The acute effects of drug of abuse are known to be significantly modulated by the effects of the context in which they are experienced. This has been shown with regards to the psychomotor effects of amphetamines (Badiani et al., 1995), the sensitization of this psychomotor effect with cocaine, amphetamine or morphine (Badiani et al., 1995;

Browman et al., 1998a; Browman et al., 1998b) and the lethal effects of heroin (Siegel et al., 1982). A recent neurobiological investigation of this amplification of the drug effect has implicated both the mPFC and Acb (Uslaner et al., 2001). Two groups of animals were housed separately, one in stainless steel home cages and the other in white plastic cages. After 10 days, animals in both groups were administered saline, amphetamine or cocaine in the white cages, which was a novel environment for the stainless steel cage housed animals. Using *in situ hybridization*, *c-fos* expression was measured in the mPFC and Acb. They found that in the mPFC (the Acb findings will be discussed shortly) novelty alone (saline in the novel environment) was able to elicit an increase in *c-fos* expression. Additionally, both amphetamine and cocaine administered in a novel environment were able to elicit a greater response than either drug in the home cage such that the response to drug in a novel environment was greater than the response to novelty alone (Uslaner et al., 2001). The data from our study is consistent with this finding such that although both groups administered heroin expressed elevated levels of Fos in the mPFC, this response was accentuated in the animals that received heroin in the CPP compartment rather than their home cages. This implies that the more distinct or unique an environment is that is paired with a reward (drug) the greater the activity in the mPFC to the drug will be.

In the Acb Uslaner et al. (2001) found similar, but importantly different patterns than in the mPFC. Like in the mPFC, amphetamine or cocaine in a novel environment elicited a greater *c-fos* response than either drug in the home cage. However, this response to drug in a novel environment was not greater than the response to novelty alone. We observed that the elevation in Fos activity after one day of conditioning was

equal in all four groups. These data (and the data from the mPFC) imply that since the distal environment (benches, door and lights) were equally novel in all four groups, the manipulation of proximal cues can alter the novelty of the entire environment. While the mPFC is more sensitive to this manipulation than the Acb, the presence of a familiar proximal cues (the home cage) was not sufficient to attenuate responding in the Acb to the US or noCS-noUS groups.

This lack of a group difference in the Acb is particularly surprising since it has been shown using *c-fos* antisense that *c-fos* expression in the Acb is necessary for the acquisition of morphine-CPP (Tolliver et al., 2000). *c-fos* antisense is composed of the nucleotide sequence that is complementary to *c-fos* messenger RNA. Injecting this into a brain region, will effectively inactivate *c-fos* expression. This apparent contradiction between its necessity for drug-related learning and its lack of responding to the effects of the drug implies that while Fos in the Acb does not respond to the rewarding aspects of conditioning or proximal novelty, the aspects of conditioning that it does respond to are necessary for drug-related learning. If this required *c-fos* expression can be shown to be due to novelty, then future research that investigates the role of the Acb in Pavlovian conditioning will require careful analyses of the paradigms in use to ensure that they are able to differentiate the component processes (effects of novelty versus the effect of the reward) similar to what has been done in human neuroimaging literature (Meegan et al., 2004).

It appears that the three regions that we investigated are representing two aspects of conditioning. The amygdala is sensitive to the rewarding aspects of the drug, while

the Acb appears to be sensitive to the novelty of the neutral stimulus. The mPFC on the other hand appears to be sensitive in an additive manner to both aspects of conditioning.

*Future directions*

This study demonstrates that Fos IR in regions of the mesocorticolimbic system is related to reward and novelty aspects of conditioning. However, the nature of this relationship is correlational. One way to demonstrate a causal relationship would be to inject *c-fos* antisense, blocking *c-fos* expression, into the region of interest prior to acquiring a task that specifically manipulates these aspects of conditioning. These may include devaluation of food reward by satiety to manipulate the magnitude of reward, and a novel-object place preference design to measure the effect of novelty of the CS. If *c-fos* antisense blocks devaluation or novel-object place preference then it can be inferred that *c-fos* expression is a required component of these aspects of conditioning.

Although DA may be implicated in the temporal gradient that we observed, we did not specifically investigate the role of DA in these patterns of Fos IR. To address this the use of double-staining immunohistochemistry could determine the nature of the cells (e.g. pyramidal, interneuron) that are expressing Fos. Also pharmachological manipulations of DA to simulate the gradient found by Phillips et al. (2003) could reveal whether that gradient can elicit the gradient of Fos IR that we observed.

The results of this study support the notion that drug addiction is based on mechanism of 'normal' learning (Hyman et al., 2006). Our findings of a temporal gradient and regional specificity mirror those from many non-drug paradigms (Schultz et al., 1997; LeDoux, 2000; Waelti et al., 2001; Rescorla, 2001; Phillips et al., 2003; Dalley et al., 2004). Although drugs of abuse may act on the nervous system in a different

manner than many unconditioned stimuli (Di Chiara, 2002) the mechanisms that evoke compulsive drug behaviour appear to be the same as those that support other motivated behaviours (Hyman et al., 2006). Therefore, the pathological aspect of addiction is not the nature of the learning mechanism per se, but the magnitude of its activation.

If a solution for addiction is sought, then a more thorough comprehension of the acquisition and storage of associative memories are needed. The formation of these memories has been shown to undergo consolidation, the time-dependant stabilization of a long-term memory during which, the memory is in a labile state (Schafe et al., 2001; Dudai, 2004). When in this labile state, a memory is susceptible to disruption by amnesic treatments. However, following a delay, these same treatments have no effect on the memory (Dudai, 2004). Recently it has been shown that once consolidated, rather than being fixed in a stable state, a memory will re-enter this labile state following reactivation or retrieval (Nader et al., 2000). This phenomenon has been labeled reconsolidation and has been shown to be present in drug-related memories (Lee et al., 2005; Miller & Marshall, 2005b; Valjent et al., 2006; Lee et al., 2006). Interestingly, the signaling molecules being implicated in the reconsolidation of memory, such as PKA (Koh & Bernstein, 2003; Tronson et al., 2006; Kemenes et al., 2006), ERK (Miller & Marshall, 2005b; Valjent et al., 2006), and MAPK (Kelly et al., 2003) are the same as have been implicated in its consolidation (Kandel, 2001).

However, reconsolidation has not been found universally (Berman & Dudai, 2001; Milekic & Alberini, 2002). This implies that there are boundary conditions that regulate whether a memory will re-enter a labile state following consolidation (Nader, 2003). One boundary condition that has been investigated is strength of training. It was

found that stronger associations, due to overtraining, were temporarily exempt from the reconsolidation process following memory retrieval (Wang et al., 2005). Also recall that as the number of conditioning trials progress that prediction error and DA response decreases (Rescorla & Wagner, 1972; Schultz & Dickinson, 2000; Phillips et al., 2003). Thus, it is possible that the reduction in DA linked with the strengthening of an association is involved in the capacity of a memory to become labile following retrieval. By linking formal learning theory and reconsolidation research additional information regarding the dynamic nature of memory and the neural processes that underlie it will emerge. By better understanding the acquisition and maintenance of associative learning and memory, we will be better equipped to reduce the impact that drug-cue associations have on so many lives.

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*Table 1*: Summary of experimental groups

Table shows the conditioning (H= heroin; V = vehicle)and test procedure for experimental group, and the number of the figure in which the results of each analysis is represented.

| **Group** | **Conditioning** | **Test (n)** | **Figure** |
|---|---|---|---|
| 1D CS-US | H x1 | CPP (12) | 1 |
| | | Fos (7) | 2, 3 & 4 |
| 1D CS | V x1 | CPP (12) | No figure |
| | | Fos (9) | 2, 3 & 4 |
| 1D US | H x1 | CPP (12) | No figure |
| | | Fos (10) | 2, 3 & 4 |
| 1D noCS-noUS | V x1 | CPP (12) | No figure |
| | | Fos (10) | 2, 3 & 4 |
| 4D CS-US | H x 4 | CPP (12) | 1 |
| | | Fos (7) | 2, 3 & 4 |
| 4D CS | V x 4 | CPP (12) | No figure |
| | | Fos (7) | 2, 3 & 4 |
| 4D US | H x 4 | CPP (12) | No figure |
| | | Fos (7) | 2, 3 & 4 |
| 4D noCS-noUS | V x 4 | CPP (12) | No figure |
| | | Fos (7) | 2, 3 & 4 |

Figure 1: Time spent in heroin- and saline- paired compartments after 1 and 4 days of conditioning. * $p < 0.05$

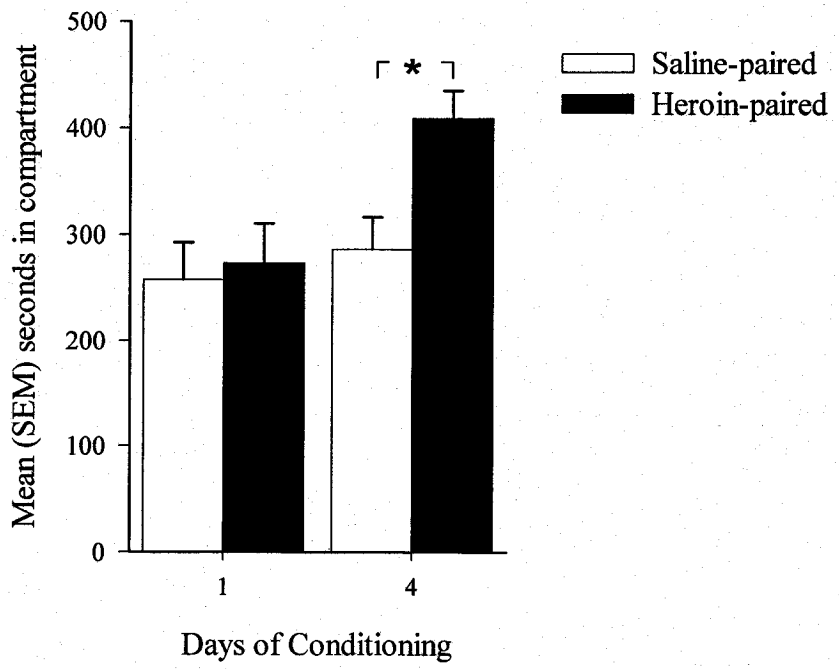

Figure 2: Density of Fos IR in the medial prefrontal cortex after 1 and 4 days of conditioning. * Significant difference from the noCS-noUS group; ** Significant difference from the CS group; † Significant difference from the US group. See text for p values.

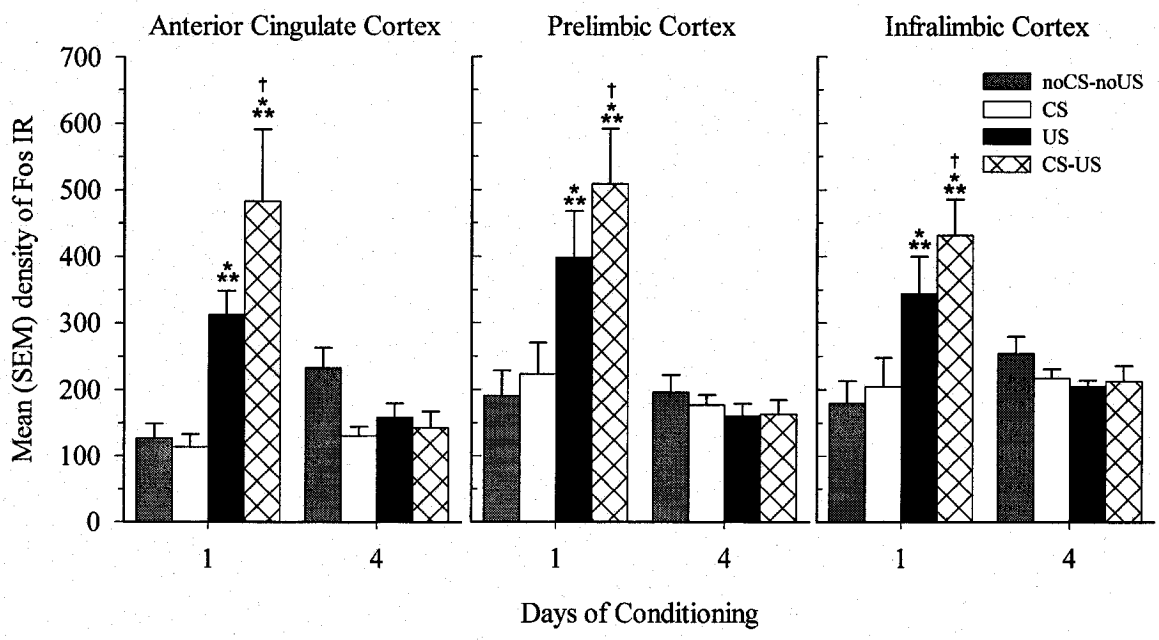

Figure 3: Density of Fos IR in the amygdala after 1 and 4 days of conditioning. * Significant difference from the noCS-noUS group; ** Significant difference from the CS group. See text for p values.

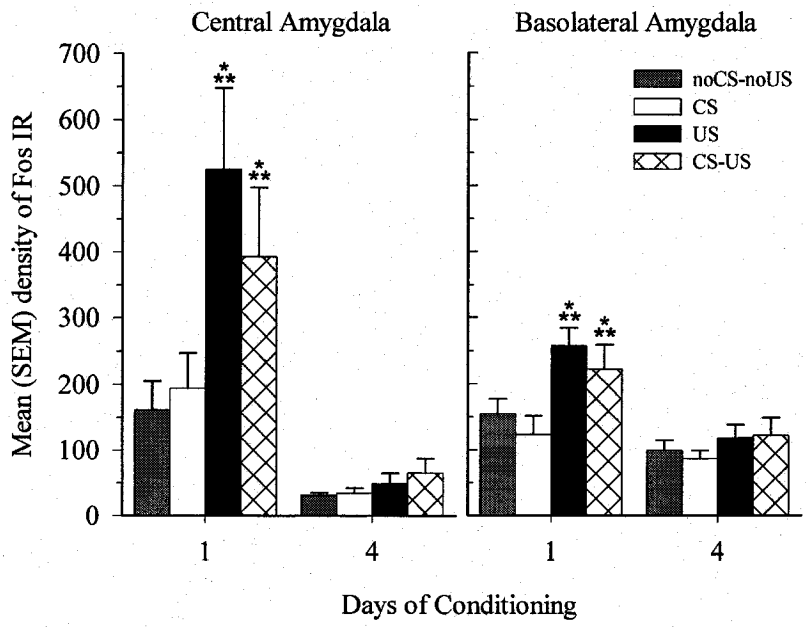

Figure 4: Density of Fos IR in the nucleus accumbens after 1 and 4 days of conditioning.