

## Heroin self-administration: II. CNS gene expression following withdrawal and cue-induced drug-seeking behavior <sup>☆</sup>

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### ABSTRACT

In the accompanying paper, we described incubation of heroin-seeking behavior in rats following 14 days of abstinence. To gain an understanding of genomic changes that accompany this behavioral observation, we measured the expression of genes previously reported to respond to drugs of abuse. Specifically, after 1 or 14 days of abstinence, mRNA expression was measured for 11 genes in the medial prefrontal cortex (mPFC) and nucleus accumbens (NAc) immediately following a single 90 min extinction session. Additionally, the role of contingency was examined in control rats that received yoked, response-independent heroin administration. Gene expression was quantified by real-time quantitative PCR. Expression of five genes (Arc, EGR1, EGR2, Fos, and Homer1b/c) was changed in the mPFC. EGR1 and EGR2 expression was increased following the 90 min extinction session in a contingency-specific manner and this increase persisted through the 14 days of abstinence. Fos expression was also increased after 1 and 14 days of abstinence, but at 14 days this increase was response-independent (i.e., it occurred in both the rats with a history of heroin self-administration and in the yoked controls). Arc expression increased following the extinction session only in rats with a history of heroin self-administration and only when tested following 1, but not 14, days of abstinence. Homer 1 b/c decreased after 14 days of enforced abstinence in rats that received non-contingent heroin. Expression of only a single gene (EGR2) was increased in the NAc. These data demonstrate that behavioral incubation is coincident with altered levels of specific transcripts and that this response is contingently-specific. Moreover, EGR1 and EGR2 are specifically upregulated in self-administering rats following extinction and this finding persists through 14 days of abstinence, suggesting that these genes are particularly associated with the incubation phenomenon. These latter observations of persistent changes in gene expression following abstinence may reflect molecular correlates of relapse liability.

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### 1. Introduction

Drug abuse (specifically heroin addiction) is a chronic relapsing disease (O'Brien, 1997). Clinically, the intent of treatment is to initiate and sustain drug abstinence. However, heroin withdrawal is characterized by intense drug craving that can trigger drug relapse. Relapse liability persists well beyond the initial withdrawal period (Krupitsky et al., 2002, 2006; O'Brien, 2005), and changes in gene expression may accompany this persistent liability. The present study examined known drug-responsive genes in rats that experienced a 90-minute extinction session following either 1 day or 14 days of abstinence from heroin self-administration. The extinction session was crucial to mimic context-induced relapse and to provide insight

into the genes whose expression is changed upon exposure to an environment previously paired with receipt of heroin.

Incubation of drug-seeking behavior is a phenomenon where drug-seeking increases as a function of increasing periods of abstinence. Incubation has been reported with both heroin (Shalev et al., 2001) and cocaine (Neisewander et al., 2000; Grimm et al., 2001) self-administration. This elevation in drug-seeking has been reported to persist during 25 days of abstinence from heroin self-administration (Shalev et al., 2001) and up to 90 days of abstinence from cocaine self-administration (Grimm et al., 2003). We have also observed incubation after 14 days of abstinence from heroin self-administration (Kuntz et al., accompanying manuscript). Tissues from the rats from that report, supplemented with tissues from yoked heroin and yoked saline control rats, were used in the present study of gene expression.

The second element addressed by this study is the role of contingency in long-lasting molecular changes that accompany heroin administration. Prior studies have demonstrated that the contingency under which a drug is administered can determine physiological responses. Differential increases in extracellular dopamine have been

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observed in the NAc between animals self-administering drug (heroin or cocaine) versus those receiving infusions administered by an experimenter (Hemby et al., 1995, 1997). Distinctive patterns of brain activity resulting from passive- versus self-administration of  $\mu$ -opioid agonists have been observed in human heroin abusers (Greenwald and Roehrs, 2005). Heroin self-administration induces a greater increase in both brain and body temperature of rats when compared with rats that receive passively administered heroin (Kiyatkin and Wise, 2002). Recently, non-contingently administered heroin has been shown to induce greater levels of stereotyped behavior than contingently administered heroin (Lecca et al., 2007). Gene expression responses to drug exposure have also been demonstrated to be contingency-dependent (Jacobs et al., 2004, 2005).

Genes examined in this study were selected for analysis because they have been previously reported by either our laboratory or other laboratories to exhibit changed expression following heroin (Jacobs et al., 2004, 2005; Koya et al., 2006), morphine (Ammon et al., 2003; Ammon-Treiber and Holt, 2005) or cocaine administration (Freeman et al., 2001, 2002a,b). Many of the genes are immediate early genes that have the potential to participate in the well-documented phenomenon of drug-induced neuroplasticity (Robinson and Kolb, 2004; Szumlanski et al., 2006, 2008; McClung and Nestler, 2008). By examining genes previously described to be responsive to heroin, morphine or cocaine, we sought to determine the relevance of these genes to the overall phenomenon of addiction; that is, we sought to identify genes that are potentially universally-responsive to drugs of abuse. Naturally, this is not a comprehensive list of genes whose expression is altered following administration of drugs of abuse. Other reports (e.g., Ahmed et al., 2005) have described a number of additional changes that are associated with synaptic plasticity.

In the present study, we examined heroin-induced changes that persist beyond the period of drug use and a 90-minute extinction session in rats either 1 day or 14 days following their final session of drug exposure. Specifically, the experiments were designed to examine gene expression under conditions in which heroin was not present in the system and rats were re-exposed to contextual cues. To gain an increased understanding of the pharmacological versus behavioral components of drug abuse in altering gene transcripts, we have included both a contingent and a dose-matched non-contingent (i.e., yoked heroin) group in this study. Gene expression in these groups was compared to each other and to that of a group of saline controls.

## 2. Methods

### 2.1. Heroin self-administration and non-contingent drug delivery

#### 2.1.1. Subjects

Male, Sprague–Dawley rats (Charles River Laboratories, Raleigh, NC) weighing 250–300 g at the start of the experiment, were individually housed in standard wire mesh cages, were maintained on a 12 hr light/dark cycle (lights on at 0700 and lights off at 1900), and were provided food and water *ad libitum* except where otherwise noted. All studies were conducted in accordance with The Pennsylvania State University Institutional Animal Care and Use Committee, strictly adhering to the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research (National Research Council, 2003).

#### 2.1.2. Surgery

Surgeries were performed 1 week after rats were introduced to their home cages. Intravenous catheters were implanted as previously described (Grigson and Twining, 2002). The catheter was coupled to a cannula that exited between the rat's shoulder blades. Rats recovered from surgery in their home cages with food and water *ad libitum* for 1 week.

#### 2.1.3. Apparatus

Behavioral procedures were performed in 12 self-administration chambers (MED Associates, St. Albans, VT) constructed as previously described (Grigson and Twining, 2002). Each chamber was equipped with two retractable sipper tubes and a stimulus light was located above each tube. A lickometer circuit was used to monitor licking. Heroin reinforcement was controlled by an electronic circuit that operated a syringe pump and programs written in the Medstate notation language (MED Associates). See Kuntz et al. (2008-this issue) for a more complete description of the apparatus.

#### 2.1.4. Habituation

During habituation, all rats were placed on a water deprivation regimen that consisted of 5 min access to water in the self-administration chamber each morning and 1 h water access in the home cage each afternoon. This habituation training occurred daily for 9 days. Water deprivation continued through the remainder of the study, with 1 h of water access in the home cage each afternoon.

#### 2.1.5. Drug-administration

Forty-eight rats were used in the study. Sixteen rats self-administered heroin using licking of an empty spout as the operant behavior. The behavior of these rats is described in the accompanying manuscript (see Kuntz et al., 2008-this issue). The thirty-two additional rats were yoked to the self-administering rats, 16 received yoked heroin infusions and 16 received yoked saline infusions. Under this paradigm, the yoked animals received infusions on the same schedule (and for the yoked heroin animals, at the same dose) as the self-administering “executive” rats. At the start of each session, two empty spouts extended into the chamber and a stimulus light was illuminated above the right (active) spout. For the self-administering rats, every 10 licks on the rightmost spout resulted in a heroin infusion (fixed ratio 10; FR10). Licks on the left (inactive) spout had no consequence. For yoked rats, licks on either spout had no consequence. Rats were trained in triads, with the yoked heroin and yoked saline group receiving infusions simultaneously each time the infusion requirement was met by the executive rat. Identical consequences occurred in all three chambers in each triad – including extinguishment of the light above the active spout, retraction of the spouts for 20 s, and onset of a house light and a 20 s tone. During this 20 s period, responses on the “active” spout had no consequence. There were 14 such daily acquisition trials.

#### 2.1.6. Forced abstinence and extinction session

Following 14 days of heroin (or saline) administration, half of the rats in each group (8 animals) received 1 day of abstinence, while the other half (8 per group) received a 2-week period of forced abstinence (i.e., the rats remained in their home cages). All rats then experienced a 90-minute extinction session during which completion of 10 licks on the active spout by the executive rat resulted in identical consequences as described during the acquisition trials, except saline, rather than heroin, was infused across the triad. During this time, rats were handled daily, food was available *ad libitum*, and 1 h of water was available in the afternoon. Catheters in all but three of the 2-week abstinence animals (one from each treatment group) remained patent throughout the extinction session.

#### 2.1.7. Behavioral measures

Rats were weighed daily. The number of licks made on both spouts were recorded during the self-administration and extinction sessions, as was the total number of infusions self-administered. In addition, the latency(s) to make the initial lick on each spout was recorded for each training session.

#### 2.1.8. Drugs

Heroin HCl was generously provided by the National Institute on Drug Abuse (Research Triangle Institute, Research Triangle Park, N.C., USA). Drug was dissolved in sterile physiological saline at a concentration of 0.3 mg/ml. Each IV-injection was a 0.06 mg/0.2 ml infusion dose delivered over 6 s.

## 2.2. Dissection and RNA isolation

### 2.2.1. Sacrifice and tissue dissection

Immediately following the 90-minute extinction session, all rats were sedated using Propofol (10 mg/kg) and decapitated. The brain was rapidly removed from the skull, placed in pre-chilled phosphate buffered saline (PBS) and then sectioned in an ice-chilled ASI brain slicer (ASI Instruments, Warren MI). The section from Bregma +4.2 to 2.2 mm was cut along the forceps minor and the cortex medial of this cut was collected (medial prefrontal cortex, mPFC). This includes the cingulate, prelimbic cortex, and medial orbital cortex. The section from +2.2 to 0.2 mm was cut 0.5 mm on each side of the midline along a line connecting the tip of the external capsule and previous cut from the tip of the external capsule and lateral ventricle, and between the ventricles. The nucleus accumbens (NAc) dissection includes core and shell. Following dissection, the tissue was placed in prechilled tubes, immediately frozen on dry ice, and then stored at  $-80^{\circ}\text{C}$ .

### 2.2.2. RNA isolation

Total cellular RNA was isolated using Tri Reagent (Molecular Research Center Inc., Cincinnati, OH) (Chomczynski and Mackey, 1995). Isolated RNA was further purified using an RNeasy Mini Kit for RNA clean-up (QIAGEN Sciences, Maryland). RNA quantity and quality were assessed using the RNA 6000 Nano Assay with an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA).

## 2.3. QRT-PCR analysis of gene expression

cdNA synthesis was performed on total RNA using Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA). 1  $\mu\text{g}$  RNA, 500 ng Oligo (dT), and 10 mM each dNTP, were incubated for 5 min at  $65^{\circ}\text{C}$  and then chilled on ice for 2 min. 5X First Strand Buffer (250 mM Tris-HCl (pH8.3), 375 mM KCl, and 15 mM  $\text{MgCl}_2$ ), 5 mM DTT (final concentration), 40 U RNaseOut, and 200 U Superscript III RT were then added. The 20  $\mu\text{l}$  reaction was incubated for 60 min at  $50^{\circ}\text{C}$  followed by a final incubation at  $70^{\circ}\text{C}$  for 15 min for termination.

Quantitative PCR was carried out on a real-time detection instrument (ABI 7900HT Sequence Detection System) in 384-well optical plates using TaqMan Universal PCR Master Mix and Assay on Demand primers and probes (Applied Biosystems, Foster City, CA) as described previously (Bowyer et al., 2007). Primer/probe sets used are listed in Table 1. All of these studies were conducted in the Functional Genomics Core Facility of the PSU Division of Shared Research Resources.

## 2.4. Statistical analysis

Behavioral data were analyzed using  $3 \times 2 \times 2$  mixed factorial ANOVAs, varying group (self-administering, yoked heroin, yoked saline), spout (active, inactive), and abstinence period (1 or 14 days).

**Table 1**

TaqMan® Gene Expression Assays were used to quantify gene expression

Abbreviation	Activity-regulated	Assay number
Arc	cytoskeletal-associated protein	Rn00571208_g1
Beta-actin	Beta-actin	Rn00667869_m1
cdk5	Cyclin-dependent kinase 5	Rn00590045_m1
Cryab	Crystalline, alpha B	Rn00564026_m1
EGR1	Early growth response 1, a.k.a. krox 24, NGFI, zif268	Rn000561138_m1
EGR2	Early growth response 2, a.k.a. krox 20	Rn00586224_m1
Fos	c-fos	Rn02396759_m1
Homer 1 b/c	Homer homolog 1, a.k.a. ania-3, HOMER1F, Vesl-1	Rn00581785_m1
Homer 1 pan	Homer homolog 1, a.k.a. ania-3, HOMER1F, Vesl-1	Rn00668905_m1
Nr4a1	Nuclear receptor subfamily 4, group A, member 1, a.k.a. NGFI-B, Nur77	Rn0057776_m1
Per2	Period homolog 2	Rn00581577_m1

The gene abbreviation, full name, and assay ID for each gene are listed in this table.

Goal-directed behavior was determined by subtracting inactive spout responses from active spout responses. Gene expression values were evaluated using  $3 \times 2$  mixed factorial ANOVAs varying group (self-administering, yoked heroin, yoked saline) and abstinence period (1 or 14 days). Gene expression data was then analyzed using one-way ANOVAs with a separate ANOVA at each time point (1 and 14 days) comparing gene expression across the three treatment groups (self-administering, yoked heroin, yoked saline). For both behavioral and gene expression data, post hoc tests were conducted using Student–Newman–Keuls tests with  $\alpha$  set at 0.05.

Analyses were performed to determine whether gene expression values correlated with self-administration (specifically, the average of values from the last 2 days of self-administration) or with drug-seeking behavior during extinction testing. These analyses were only conducted for the 16 self-administering rats because yoked rats in this study could not express contingent behavior. The correlation between behavior and gene expression was analyzed separately for each abstinence period: 1 day and 14 days abstinence. This separation was necessary because, at the time of sacrifice, the groups had undergone different experiences, and gene expression levels have the potential to be affected by the length of the abstinence period. Pearson Correlation analyses examined the relationship between active responses, goal-directed behavior, and each of the genes studied during drug-taking (acquisition) and drug-seeking (extinction) behavior.

## 3. Results

### 3.1. Body weight and water intake

Rats in all treatment groups were weighed daily throughout the study, and all rats gained weight at equal rates. Self-administering rats increased from an average weight of  $365.3 \pm 9.7$  g on the first day of self-administration to  $416.0 \pm 10.4$  g on the final day of self-administration. Weight increases for rats yoked to heroin or saline were  $380.6 \pm 6.4$  g to  $429.5 \pm 7.5$  g and  $372.3 \pm 7.2$  g to  $421.3 \pm 9.7$  g, respectively. Rats were provided 20 ml of water daily during the self-administration and abstinence periods, and all 20 ml of water were consumed daily. Detailed behavioral data are presented in the accompanying article.

### 3.2. Acquisition: heroin self-administration and non-contingent administration

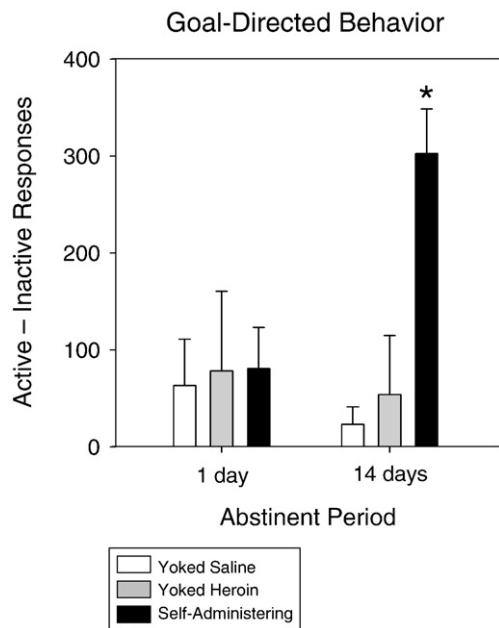
The 16 self-administering rats increased their heroin intake from an average of  $7.3 \pm 0.37$  infusions on day 1 to  $12.6 \pm 2.4$  infusions on day 14. During the terminal acquisition period, the self-administering (executive) rats, but not the yoked heroin or yoked saline controls, exhibited clear heroin self-administration behavior by making more responses on the active than the inactive spout (see Kuntz et al., 2008-this issue).

Self-administration behavior was also demonstrated by executive rats initiating licking more quickly on the active spout than on the inactive spout ( $10.77 \pm 3.6$  s vs.  $3043.0 \pm 953.2$  s, on the final day of self-administration). The latency to make initial contact with the active spout decreased on progressive days of heroin self-administration sessions and was significantly shorter for self-administering rats than the latency to contact the inactive spout (see Kuntz et al., 2008-this issue).

### 3.3. Extinction: incubation of drug-seeking behavior

#### 3.3.1. Behavioral data

Responses on both the active and inactive spouts were monitored during a 90-minute extinction session that took place either 1 day or 14 days following final access to drug. Rats that underwent a 14 day period of enforced abstinence responded more on the previously active



**Fig. 1.** Goal-directed behavior was measured by subtracting inactive responses from active responses. Goal-directed behavior in previously self-administering rats was significantly increased following 14 days of abstinence.

spout than did rats that experienced only 1 day of abstinence (see Fig. 2 in Kuntz et al. 2008–this issue). Moreover, while the executive rats with a history of heroin self-administration exhibited a similar number of licks on the active and the inactive spout after 1 day of abstinence, responding on the active spout was nearly 3 times that made on the inactive spout when the extinction test followed 14 days of abstinence. No significant differences in latency to lick were observed for the self-administering rats depending on the length of their enforced abstinence period. That is, all rats with a history of heroin self-administration initiated licking significantly more quickly on the active than the inactive spout ( $4.8 \pm 1.9$  s vs.  $8.2 \pm 2.6$  s). Finally, active vs.

inactive spout responding did not differ for either the yoked heroin or the yoked saline controls, whether tested following either 1 day or 14 days of abstinence,  $p > 0.05$ .

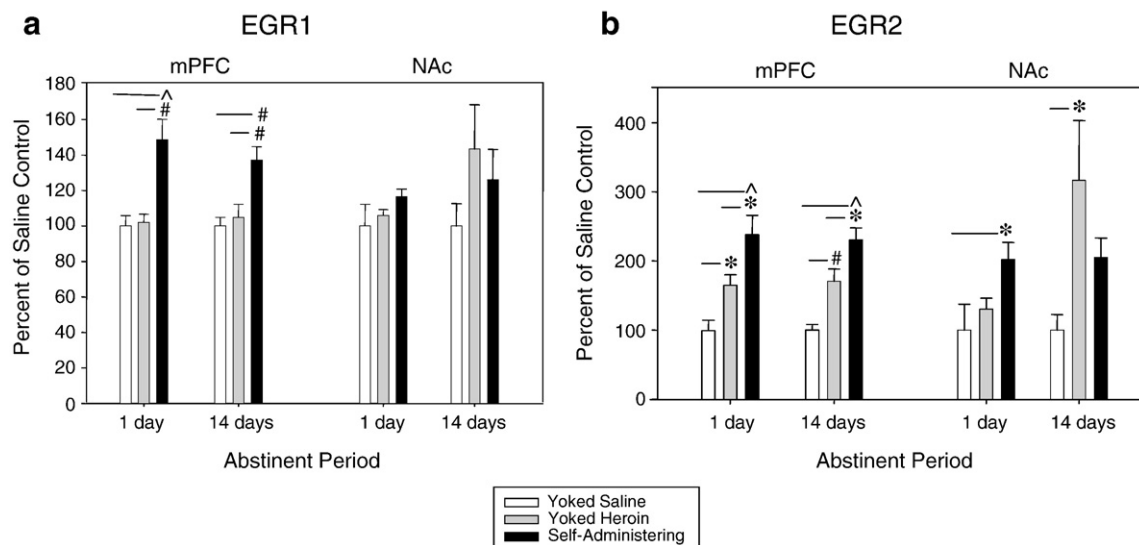
Calculations of goal-directed behavior (difference scores) indicate that goal-directed behavior increased concomitantly with incubation of heroin-seeking. No difference between responding on the two spouts was apparent when extinction occurred after only 1 day of abstinence, but executive rats responded significantly more on the active than the inactive spout when the extinction session was preceded by 14 days of abstinence (Fig. 1).

### 3.3.2. Gene expression changes that persist during withdrawal

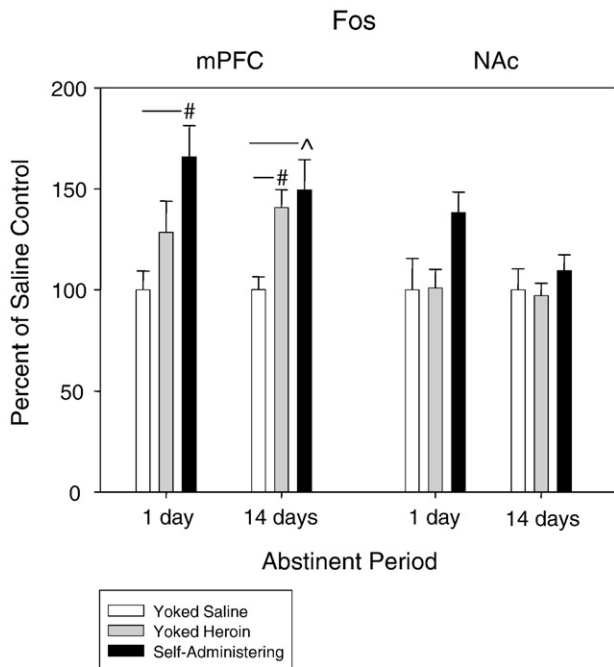
Gene expression analysis was performed in the mPFC and NAc for 11 genes previously described as responsive to morphine or cocaine administration. Gene expression was examined after the 90 min extinction session that occurred after 1 or 14 days of home cage abstinence.

One-way ANOVAs followed by Student–Newman–Keuls post hoc analysis determined that after 1 day of abstinence, EGR1 gene expression in the mPFC was significantly altered ( $F_{2,19} = 10.93$ ,  $P < 0.001$ ). Post-hoc analysis showed that expression was increased by 45% following extinction in rats with a history of heroin self-administration compared to yoked saline ( $P = 0.001$ ) and yoked heroin ( $P < 0.01$ ) rats. This differential expression persisted for the 14 day period of withdrawal ( $F_{2,18} = 10.007$ ,  $p = 0.001$ ,  $p < 0.005$  for both comparisons). (Fig. 2, panel a). No alteration in EGR1 gene expression occurred in the NAc.

EGR2 was significantly upregulated in the mPFC after the extinction session at both 1 day ( $F_{2,19} = 10.76$ ,  $p < 0.001$ ) and 14 days ( $F_{2,19} = 17.65$ ,  $p < 0.001$ ) of abstinence. Observed increases above yoked saline controls existed for self-administering rats following 1 day abstinence ( $p < 0.001$ ) and 14 days abstinence ( $p < 0.001$ ). Elevations above saline also existed for yoked heroin rats following 1 day ( $p < 0.05$ ) and 14 days ( $p < 0.005$ ) of abstinence. Importantly, levels of EGR2 gene expression were significantly higher in rats with a history of heroin self-administration compared with rats that received non-contingent administration of drug ( $p < 0.03$ ) and this effect persisted 14 days into withdrawal ( $p < 0.03$ ) (Fig. 2, panel b). NAc EGR2 gene expression increased in self-administering, but not yoked rats, after 1 day of abstinence ( $F_{2,19} = 4.18$ ,  $p < 0.05$ ). A different expression pattern was observed in the NAc after 14 days of abstinence with rats that had



**Fig. 2.** (a) EGR1 gene expression was persistently elevated above saline in the mPFC in rats that either self-administered heroin or received yoked infusions of heroin. No significance was detected in the NAc. ^ $p < 0.001$ ; # $p < 0.01$ ; (b) – EGR2 gene expression was persistently elevated above saline in the mPFC in previously self-administering rats following both 1 day and 14 days of abstinence. Gene expression in these rats was higher than expression in rats that had previously received yoked heroin infusions, although EGR2 gene expression in the yoked heroin rats was significantly higher than in yoked saline rats. ^ $p < 0.001$ ; # $p < 0.01$ ; \* $p < 0.05$ .



**Fig. 3.** Fos gene expression was persistently elevated above saline in the mPFC after 1 day of abstinence and 14 days of abstinence. Following 14 days of abstinence Fos gene expression in yoked heroin rats became significantly higher than in rats yoked to saline. No change was observed in the NAc at either time point. # $p < 0.01$ ; \* $p < 0.05$ .

received yoked infusions of heroin displaying a dramatic increase in EGR2 gene expression (300%;  $F_{2,16} = 5.37$ ,  $p < 0.02$ ) (Fig. 2, panel b).

Fos gene expression was increased in the mPFC by a history of heroin self-administration when the extinction test occurred after 1 day of abstinence ( $F_{2,19} = 5.73$ ,  $p < 0.01$ ) and remained significantly elevated compared to yoked saline controls after 14 days of abstinence ( $F_{2,19} = 6.00$ ,  $p < 0.02$ ) (Fig. 3). Fos gene expression was elevated in yoked heroin rats at both time points, although this elevation only reached significance after 14 days of abstinence ( $F_{2,19} = 6.00$ ,  $p < 0.02$ ). No significant differences in Fos expression were observed in the NAc although a trend for upregulation by heroin self-administration was evident when extinction testing occurred after 1 day of abstinence.

### 3.3.3. Changes induced by heroin that do not persist into withdrawal

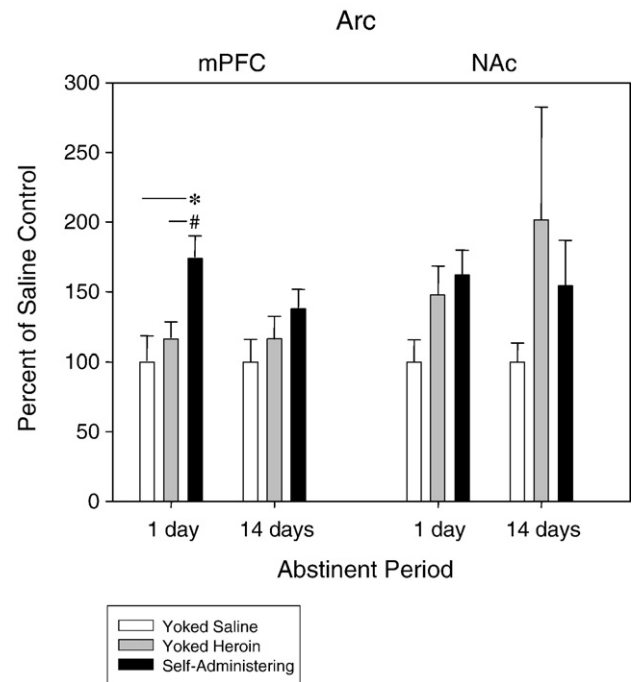
Arc was found to be upregulated in the mPFC following 1 day of abstinence ( $F_{2,19} = 7.11$ ,  $p < 0.005$ ). Gene expression in heroin self-administering rats (following extinction) was significantly higher than in rats receiving yoked heroin ( $p < 0.01$ ) and yoked saline ( $p < 0.05$ ) treatments. This change did not persist following 14 days of abstinence (Fig. 4). No significant changes were detected in the NAc.

### 3.3.4. Changes induced by heroin only evident after 2 weeks of withdrawal

Homer1 b/c gene expression was not significantly changed in the mPFC following 1 day of abstinence but, after 14 days of abstinence, rats that had formerly received yoked infusions of heroin displayed a significant decrease in Homer1 b/c ( $F_{2,19} = 3.91$ ,  $p < 0.04$ ) when compared with previously self-administering ( $p < 0.05$ ) and yoked saline ( $p < 0.05$ ) rats (Fig. 5). Homer1 b/c was not significantly changed in the NAc regardless of duration of abstinence (although a non-significant trend is evident).

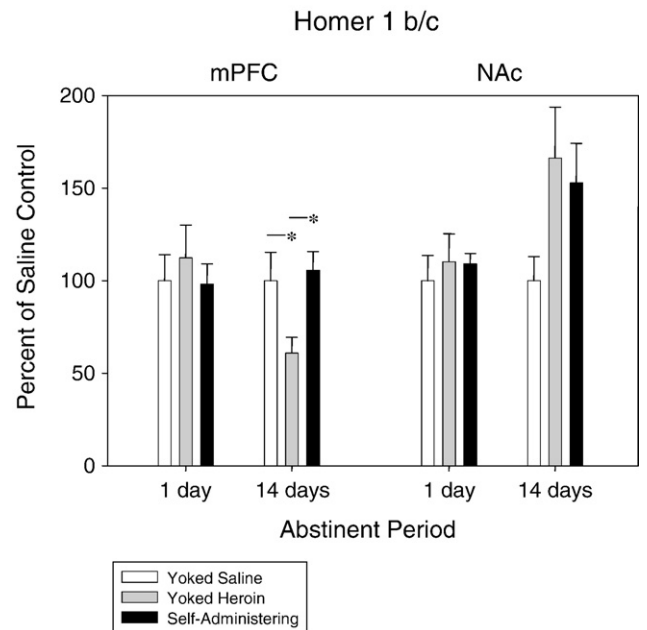
### 3.3.5. Genes unaffected by treatment

Six of the genes that were examined were not significantly changed by heroin treatment or abstinence period (Table 2). Despite the down regulation of Homer1 b/c observed in the mPFC, no significant changes were observed in either the mPFC or NAc for the pan isoform of Homer1



**Fig. 4.** Arc gene expression was elevated above saline in the mPFC in rats that had self-administered heroin. Self-administering rats experienced an increase in Arc gene expression that was significantly higher than Arc gene expression in rats that previously received yoked infusions of heroin. This elevation did not persist to 14 days of abstinence and no change in Arc gene expression was observed in the NAc. \* $p < 0.05$ ; # $p < 0.01$ .

(the primer/probe set that recognizes the a, b, and c splice variants of Homer1) and Homer1a. Expression levels of Nr4a1, Per2, Cryab, and cdk5 were also unchanged. Importantly, beta-actin was confirmed to be unchanged in all samples (data not shown). Thus, this gene served as an endogenous control to which values were normalized for comparison.



**Fig. 5.** mPFC Homer 1 b/c gene expression was unchanged after 1 day of abstinence but was significantly decreased in yoked heroin rats below expression in yoked saline and self-administering rats following 14 days of abstinence. \* $p < 0.05$ .

**Table 2**  
Six of the genes examined in this study (in addition to actin) were unchanged

	1 day abstinence			14 days abstinence		
	Yoked saline	Yoked heroin	Self-administering	Yoked saline	Yoked heroin	Self-administering
alpha B-crystallin	1.00 +/- 0.10	1.42 +/- 0.19	1.12 +/- 0.084	1.00 +/- 0.093	1.17 +/- 0.095	0.923 +/- 0.18
cdk5	1.00 +/- 0.069	1.07 +/- 0.10	1.03 +/- 0.019	1.00 +/- 0.061	0.96 +/- 0.038	0.96 +/- 0.051
Homer1 a	1.00 +/- 0.23	1.15 +/- 0.22	1.18 +/- 0.29	1.00 +/- 0.44	0.69 +/- 0.22	1.14 +/- 0.29
Homer1 pan	1.00 +/- 0.15	1.19 +/- 0.18	1.06 +/- 0.10	1.00 +/- 0.18	0.69 +/- 0.08	1.16 +/- 0.18
Nr4a1	1.00 +/- 0.19	0.67 +/- 0.12	1.14 +/- 0.12	1.00 +/- 0.14	1.07 +/- 0.18	1.50 +/- 0.26
Per 2	1.00 +/- 0.17	1.37 +/- 0.32	1.71 +/- 0.094	1.00 +/- 0.17	1.03 +/- 0.19	1.04 +/- 0.16
<i>B. NAc</i>						
alpha B-crystallin	1.00 +/- 0.13	1.14 +/- 0.064	1.13 +/- 0.060	1.00 +/- 0.069	1.26 +/- 0.16	1.09 +/- 0.12
cdk5	1.00 +/- 0.040	1.08 +/- 0.056	1.04 +/- 0.045	1.00 +/- 0.019	1.15 +/- 0.052	0.97 +/- 0.068
Homer1 a	1.00 +/- 0.18	1.27 +/- 0.28	1.13 +/- 0.19	1.00 +/- 0.42	1.30 +/- 0.22	1.10 +/- 0.44
Homer1 pan	1.00 +/- 0.053	1.17 +/- 0.093	1.20 +/- 0.091	1.00 +/- 0.17	1.34 +/- 0.16	1.21 +/- 0.15
Nr4a1	1.00 +/- 0.25	1.10 +/- 0.12	1.25 +/- 0.11	1.00 +/- 0.19	1.92 +/- 0.44	1.46 +/- 0.32
Per 2	1.00 +/- 0.092	1.02 +/- 0.019	1.13 +/- 0.048	1.00 +/- 0.073	1.20 +/- 0.12	1.13 +/- 0.037

The expression values for these genes are shown, standardized to the yoked saline expression value for that timepoint.

Similarly, levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were also confirmed to be unchanged (data not shown).

### 3.3.6. Correlational analyses

Pearson's correlation analyses detected significant correlations between gene expression values and behavior for selected genes. The two behaviors included in the analysis were average responding on the final 2 days of self-administration (drug-taking) and responding during extinction (drug-seeking). In the mPFC, gene expression that correlated with drug-taking occurred for EGR1 ( $r=0.76$ ;  $p<0.04$ ), Fos ( $r=0.84$ ;  $p<0.02$ ), and Nr4a1 ( $r=0.87$ ;  $p<0.01$ ), and these correlations only existed when evaluated after 14 days of abstinence. At this time point, Fos gene expression was negatively correlated with the goal-directed behavior ( $r=-0.83$ ;  $p<0.02$ ). Importantly, these correlations were strongly influenced by a single high-responding rat and therefore caution must be taken when noting these results. Homer 1 b/c and homer 1 pan gene expression in the mPFC was highly correlated (negative relationship) with drug-seeking behavior ( $r=-0.82$ ;  $p<0.03$  and  $r=-0.77$ ;  $p<0.05$ , respectively). Homer 1 pan gene expression in the nucleus accumbens also was negatively correlated with goal-directed responses in SA rats after 1 day of abstinence ( $r=-0.94$ ;  $p<0.001$ ). After 1 day of abstinence, Cryab was correlated with drug-seeking ( $r=0.76$ ;  $p<0.03$ ). No significant correlations were detected for Arc gene expression.

## 4. Discussion

Drug relapse is a major obstacle in overcoming addiction, and understanding the genomic contributors to incubation may assist in developing more effective therapies. Experimentally, incubation is described as a motivational process, inferred from a time-dependent increase in cue-induced drug-seeking after withdrawal from drug self-administration in rats (Gawin and Kleber, 1986; Grimm et al., 2001). In rats self-administering heroin, this increased drug-seeking behavior peaks at about 6 days of abstinence, but has been reported up to 25 days (Shalev et al., 2001). Similarly, rats that self-administer cocaine demonstrate increases in drug-seeking behavior for up to 90 days of abstinence (Grimm et al., 2003). In this study, following 14 days of abstinence from heroin self-administration, significant elevations in heroin-seeking behavior were observed when compared with rats experiencing only 1 day of abstinence (Fig. 1 and Kuntz et al., accompanying manuscript).

Spout-licking represents an operant behavior that, in this study, engendered robust responding, reliable behavior, and demonstrable incubation. Because rats were maintained on a schedule of water deprivation throughout the study, saline controls were used to verify that responding by the rats with a drug history was not due to a

history of having licked for water in the apparatus during habituation. Saline controls made significantly fewer spout licks than self-administering rats and distributed their behavior across both spouts equally. This finding indicates that the goal-directed responding of self-administering rats was focused on heroin. Furthermore, inclusion of yoked heroin rats in the present study allowed for the comparison of genomic changes resulting from the pharmacological action of heroin vs. those changes associated with contingent drug delivery. Together, analysis of gene expression of these three groups (self-administering, yoked saline control, and non-contingent heroin control) highlighted important changes associated specifically with behavioral contingency and abstinence-induced incubation.

Of the eleven genes examined (in two brain regions), several were changed following the 90 min extinction session in rats with a history of heroin self-administration and these changes remained evident even when tested following 14 days of abstinence. Three genes, all encoding transcription factors, fit this profile: EGR1, EGR2 and Fos. EGR1 is important in transcription of genes related to learning and memory. EGR1 mRNA has also been reported to increase upon exposure to cocaine-related stimuli (Thomas et al., 2003) and anti-sense inhibition of EGR1 has been demonstrated to decrease cue-induced cocaine seeking and relapse (Lee et al., 2006). In the present study, this cue-induced responsiveness persists following 14 days of abstinence, but is only manifested in the mPFC. EGR2 is highly homologous to EGR1 in its zinc finger DNA-binding domain, and recognizes the same DNA binding consensus sequence (Joseph et al., 1988; Lemaire et al., 1988; Chavrier et al., 1989, 1990). We found extinction-induced induction of EGR2 in the mPFC in both heroin self-administering and yoked, non-contingent animals. However, the effect was greater in rats with a history of heroin self-administration. This represents a case of dynamic interactions of pharmacology and behavior. Naltrexone-induced morphine withdrawal has been reported to elicit an increase in mRNA levels of EGR2 in the NAc (Bhat

**Table 3**  
Significant correlations between behavior and gene expression were detected

Gene	Behavior	Session	Abs. Pd.	Correlation coefficient	p-value
Crystallin ab	Active	Extinction	1 day	0.76	0.03
EGR1	Active	Acquisition	14 days	0.79	0.03
Fos	Active	Acquisition	14 days	0.84	0.02
Fos	Goal-dir	Acquisition	14 days	-0.83	0.02
Homer 1 b/c	Active	Extinction	14 days	-0.82	0.03
Homer 1 pan	Active	Extinction	14 days	-0.77	0.04
<i>Homer 1 pan</i>	<i>Goal-dir</i>	<i>Extinction</i>	<i>1 day</i>	<i>-0.94</i>	<i>0.001</i>
Nr4a1	Active	Acquisition	14 days	0.87	0.01

Most correlations occurred between behavior and mPFC gene expression. *Italics* indicate the correlation was detected between behavior and NAc gene expression.

et al., 1992), and the present study found EGR2 expression to be increased in the NAc following 14 days of withdrawal, but only in rats that received yoked, non-contingent, heroin infusions. Greater withdrawal (as measured by ultrasonic vocalizations) has been observed in rats that previously received non-contingent cocaine infusions compared to previously self-administering rats (Mutschler and Miczek, 1998). These distinctive contingency-dependent effects further highlight the need to discriminate behavior from pharmacology.

c-Fos is a well-characterized immediate early gene transcription factor. Fos is affected by nicotine, morphine, and cocaine treatment (Marttila et al., 2006), and multiple signaling pathways induce c-fos (Sadoshima and Izumo, 1993). Morphine either increases or decreases c-fos gene expression in striatofugal circuits, and its expression appears to be dose-dependent and directly related to the contextual novelty in which morphine is administered (Ferguson et al., 2004). c-fos mRNA expression is greater when cocaine is administered in a novel environment (Day et al., 2001; Uslaner et al., 2001a,b). Recently, Fos gene expression in both the mPFC and NAc was reported to be significantly increased in previously heroin self-administering rats only when re-exposed to the cues associated with self-administration (Koya et al., 2006). All of the rats in the present study were re-exposed during the 90-minute extinction session to the cues associated with the previous receipt of heroin. An induction in Fos expression was observed in the mPFC for self-administering animals both before and after enforced abstinence, suggesting that the response is quite persistent. An elevation in c-fos gene expression with yoked, non-contingent heroin was only present in the mPFC, and this elevation only emerged following 14 days of abstinence.

Homer1 b/c responsiveness was not immediately changed by drug self-administration or non-contingent exposure, but exhibited a decrease after 14 days of enforced abstinence. Interestingly, this change only occurred in yoked, non-contingent heroin rats. Homer1 gene expression and protein levels are reportedly decreased following chronic cocaine administration (Swanson et al., 2001; Ghasemzadeh et al., 2003), so this gene is apparently affected by multiple drugs of abuse.

Heroin exposure induces immediate behavioral changes that are accompanied by transient changes in gene expression. mPFC extinction-induced Arc mRNA expression was dramatically upregulated following 1 day of abstinence from drug administration, but returned to yoked saline levels after 14 days of abstinence. Contingency appears to play an important role in the induction of this gene because the level of Arc in the mPFC is significantly higher in self-administering rats than rats yoked to heroin. Arc is a protein involved in neuronal plasticity and memory consolidation (Guzowski et al., 2006) and therefore may be playing a role in establishing an imprint (or memory) of self-administration behavior.

The genes for which no change in expression levels were detected in this study are homer1 a, homer1 pan, Nr4a1, per2, Cryab, and cdk5. We evaluated rPer2 (rat Per2) and Cryab because they have been reported to be induced by chronic morphine administration (Ammon et al., 2003). The lack of concordance between the present findings and the previous report may be due to differences in contingency or pharmacology. While heroin is deacetylated to form morphine, it crosses the BBB more rapidly than morphine and this increased speed of action may induce unique genomic changes. Homer1 a gene expression is reportedly increased in both the heroin self-administering rats and their saline controls upon re-exposure to cues, compared with rats not exposed to cues (Koya et al., 2006). This finding suggests that Homer1 a also may have been elevated in our rats following exposure to the test chamber, but this elevation could not be detected because all rats (those with a saline or heroin history) were exposed to the test chamber and there were no home cage (i.e., no cue) controls.

The combination of behavioral and gene expression data collected by this study provides an opportunity to examine correlations between behavior and gene expression that may reveal more subtle relationships

than group means (Table 3). Given limited statistical power ( $n=8$ ), several observations were made. EGR1, Fos, and Nr4a1 gene expression is correlated with drug-taking (self-administration responding) but not drug-seeking (extinction responding), but only when evaluated after 14 days of abstinence. Thus, a history of greater heroin self-administration behavior is associated with an elevation in the expression of EGR1, Fos, and Nr4a1 in the mPFC, but only after an extended period of withdrawal (i.e., 14 days). The expression of Cryab, Homer1 b/c, and Homer1 pan in the mPFC, on the other hand, was significantly correlated with drug-seeking behavior during extinction (i.e., with responding on the previously active spout) whether tested following either 1 day or 14 days of abstinence. These findings are important in several respects. First, these data suggest that this set of genes is linked to drug-seeking, but not to drug-taking. Second, several of these genes (Cryab, Nr4a1, and Homer1 pan) did not display differential responsiveness in the aggregate, consistent with the conclusion that there are important inter-individual differences in responsiveness. Future experiments, involving no extinction session, will be required to determine the impact of abstinence period alone on gene expression. Finally, for all these studies, it is important to note that we are measuring mRNA levels and that these need not, *a priori*, reflect protein levels.

In sum, EGR1, EGR2, and Fos are elevated in the mPFC of rats when extinction testing occurs following either 1 or 14 days of abstinence and these effects are most robust in rats with a history of heroin self-administration. Moreover, the results of correlational analyses show that higher drug self-administration behavior is associated with higher levels of EGR1, Fos, and Nr4a1 gene expression in the mPFC when measured following 14 days of abstinence. Homer1 b/c was decreased in the mPFC, but only when examined after 14 days of abstinence, and only in the yoked, noncontingently administered rats. The mean expression of Homer1 pan was not altered in the mPFC, but the level of expression of Homer1 b/c and Homer1 pan varied as a function of the magnitude of the drug-seeking behavior when tested after 14 days of abstinence in rats with a history of drug self-administration. Cryab mean gene expression was also not altered in the mPFC, but varied as a function of drug self-administration when tested after 1 day of abstinence. Collectively, then, gene expression (particularly in the mPFC) tracks the contingent nature of heroin administration, the vigor of the drug-taking behavior, withdrawal period, drug-seeking behavior during extinction, as well as the intensity of this drug-seeking behavior across individual subjects.

In conclusion, the ability to model the persistent neurobiological and behavioral changes following exposure to drugs is critical to our understanding of addiction and relapse. This study combined prior knowledge of the genomic effects of drug exposure with a model of heroin self-administration and incubation. The findings of altered gene expression with heightened drug-seeking behavior mark these gene targets for future studies into their role(s) in long-term relapse liability. By addressing addiction using a molecular to behavioral approach, progress will be made towards identifying pharmacotherapeutic targets to facilitate drug abstinence and prevent relapse.

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