INCUBATION OF HEROIN-SEEKING BEHAVIOR
AND ACCOMPANYING MOLECULAR CHANGES

A Dissertation in
Neuroscience
By
Kara L. Kuntz-Melcavage

Submitted in Partial Fulfillment
of the Requirements
for the Degree of
Doctor of Philosophy

May 2009
The dissertation of Kara L. Kuntz-Melcavage was reviewed and approved* by the following:

Kent E. Vrana
Elliot S. Vesell Professor of Pharmacology
Dissertation Adviser
Chair of Committee

Patricia S. Grigson
Professor of Neural and Behavioral Sciences

Jong K. Yun
Associate Professor of Pharmacology

Patricia J. McLaughlin
Professor of Neural and Behavioral Sciences

Willard M. Freeman
Assistant Professor of Pharmacology

Robert J. Milner
Professor of Neural and Behavioral Sciences
Director, Graduate Program in Neuroscience

*Signatures are on file in the Graduate School
Abstract

The field of neuroscience provides an opportunity to combine psychological and biological studies with the ultimate goal of gaining a better understanding of physiological systems. The research presented in this dissertation has used this combinatorial approach to make a contribution to the field of drug abuse, specifically relapse to heroin use. The notion that a handful of genes are particularly important to relapse has guided these studies, expanding on the knowledge that currently exists for cocaine relapse. Incubation of drug-seeking (increased drug-seeking with extended periods of abstinence) is a behavioral model of relapse that has been well-characterized following cocaine self-administration. Data presented in this dissertation demonstrates that incubation also occurs for heroin-seeking behavior and, furthermore, incubation is not limited to drug-seeking behavior, but also extends to goal-directed behavior.

Following the behavioral studies, gene expression was assessed in the medial prefrontal cortex and nucleus accumbens using quantitative real-time PCR (QRT-PCR). This study included controls that received non-contingent infusions of heroin at the same time and same dose as the self-administering rats. That is, the rats in this group had no control over the administration of the drug. Additional controls received saline infusions at the same time as self-administering rats received infusions of heroin. The genes that were examined were selected because they had previously been reported to have altered expression following administration of cocaine, morphine, or heroin (in a different
behavioral model of heroin use). Prior to sacrifice, rats were reintroduced to the self-administration chambers and allowed to seek drug (although no drug was infused) for 90-minutes. Therefore, the observed gene expression levels were potentially impacted by both heroin abstinence and contextual re-exposure. Five of the twelve genes that were examined were found to be significantly changed, and changes were altered in a time-specific and region-specific manner. Furthermore, in some cases, gene expression was affected differently in rats that self-administered heroin compared to rats that received the same dose and schedule of heroin exposure, but did not self-administer the drug.

Examination of gene expression in the medial prefrontal cortex following the 90-min drug-seeking session in the animal model of incubation of heroin-seeking behavior was continued in a microarray experiment. Following a whole genome screen of nearly 41,000 genes, analysis focused on genes that are involved in behavior and neuroplasticity. QRT-PCR was performed for 22 genes identified by the arrays to be significantly changed between the self-administering and yoked saline rats, and 7 of the changes were confirmed. Additionally, 2 other genes that were identified as changed in the microarray experiment have been confirmed in the gene expression work presented elsewhere in this dissertation and in work published by another laboratory.

The research in this dissertation demonstrates the similarities between heroin and other drugs of abuse, but also highlights important differences. This material adds to the current knowledge of relapse to drug use by demonstrating that some genes are universally affected following use of either cocaine or
heroin. Also, the importance of contingency (i.e., active or passive administration of drug) on gene expression has been demonstrated, a finding that evokes reconsideration of previously published studies. The added understanding of changes in gene expression that occur in a behavioral model of relapse to heroin-seeking behavior helps to narrow the focus of possible “relapse genes” and should be applicable to studies of the broader problem of addiction.
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ACKNOWLEDGEMENTS

Achieving the completion of my doctoral studies is something that has been made possible with the help and encouragement of countless people. My parents, Thomas and Joann Kuntz, have provided me untold support and encouragement throughout my life. Pete Melcavage, my husband, has offered abundant support during my final graduate years, patiently listening to my many frustrations and helping to rejoice in my celebrations. My decision to pursue a Ph.D. was inspired by Michelle Duffourc, a phenomenal teacher (and person) who showed me that scientific research could be fun. Donald Hoover and Mary Howell also were essential in my scientific development. Kent Vrana has been a memorable graduate advisor who has taught me a lot and provided an environment that has been optimal for my development of independence. Members of my committee, especially Sue Grigson, have truly enhanced my graduate education and motivated me to have a thoughtful approach to scientific problems. Members of the Vrana laboratory, including Nurgul and Ugur Salli, Miguel Barthelary, Mindy Lull, and Amritha Jaishankar have been helpful in discussing techniques and helping to troubleshoot experimental problems. Lastly, there are numerous fellow graduate students to whom I am very thankful for the thoughtful conversations. Bob Twining, especially, provided many discussions that helped me gain an appreciation for behavioral research and intellectual approaches to problems. As I prepare to enter a new stage in my life, it is my hope to always remember the many people who have been positive contributors to my accomplishments.
Chapter 1

CNS Genes Implicated in Relapse

Kara L. Kuntz-Melcavage, Willard M. Freeman, Kent E. Vrana

Invited review published in *Substance Abuse: Research and Treatment* 2: 1-12 (2008). The text (and formatting) represents the final, copy-edited version of the journal article (with the exception of pages 24 and 25). K.L.K. and K.E.V. developed the central theme of this review. K.L.K performed all of the writing and constructed the figures. K.E.V. provided intellectual and editorial input into the manuscript. W.M.F. provided intellectual and editorial input into the manuscript.
Abstract

Drug abuse is a condition that impacts not only the individual drug user, but society as a whole. Although prevention of initial drug use is the most effective way to prevent addiction, avoiding relapse is a crucial component of drug addiction recovery. Recent studies suggest that there is a set of genes whose expression is robustly and stably altered following drug use and ensuing abstinence. Such stable changes in gene expression correlate with ultrastructural changes in brain as well as alterations in behavior. As persistent molecular changes, these genes may provide targets for the development of therapeutics. Developing a list of well-characterized candidate genes and examining the effect of manipulating these genes will contribute to the ultimate goal of developing effective treatments to prevent relapse to drug use.
Introduction

Relapse to drug use is a major barrier to overcoming addiction (Stewart 2008; Leshner 1996). Identifying changes in brain gene expression that underlie relapse liability will ultimately lead to a better understanding of what drives recovering addicts to relapse to drug use. Such an understanding will subsequently provide molecular targets on which pharmacological interventions to prevent relapse can be focused. Drug use alters the individual on multiple levels: behavior, neurochemistry, electrophysiology, and microanatomy, but underlying all these changes are gene and protein expression modifications (for review see Nestler 2001 and Falcon and McClung 2008). This review briefly describes several behavioral models of relapse, identifies a list of genes that are commonly found to be involved in relapse, and highlights epigenetic studies that are delving further into the role of genomic contributors to relapse. Studies that combine behavioral and molecular techniques to examine the relevance of certain genes to relapse are discussed, as are future directions of study to advance development of treatments for relapse.
Models of Relapse

Human relapse to drug use is an overarching problem addressed by studies of addictive drugs. For humans, relapse to drug use can occur even after substantial treatment for addiction and prolonged abstinence (DeJong 1994; O’Brien 2003). The difficulty in modeling relapse to drug use in animals has presented a challenge to researchers interested in the genomic underpinnings of addiction. Clearly, an effective model of relapse will replicate the human experience of drug use and relapse (Figure 1A). Currently, a few behavioral relapse models exist, providing an effective way to study relapse in the controlled laboratory environment. Although these models differ slightly from one another, all adhere to the central experimental approach of drug self-administration followed by a period of abstinence (Figure 1B).

![Figure 1 – Schematics of Relapse](image)

(A) Human drug use ends in either successful sustained abstinence or relapse. (B) Drug relapse is modeled in animals by cue-induced or stress-induced resumption of drug self-administration following an abstinent period. Categories of gene changes following drug use are depicted in ovals.
Incubation is the most recently recognized model of relapse. Occurring in response to both stimulants and opiates, incubation is described as a time-dependent increase in cue-induced operant responding after withdrawal from self-administration (Grimm et al. 2001; Lu et al. 2006). Incubation occurs following self-administration of cocaine (Freeman et al. 2008), methamphetamine (Shepard et al. 2004), and heroin (Shalev et al. 2001; Kuntz et al. 2008a); therefore, it may represent a generalizable phenomenon applicable across addictive drugs.

For both animals and humans, reinstatement of drug seeking behavior can be triggered by re-exposure to the previously self-administered drug, the context in which it was taken, or specific environmental cues. In the animal model of extinction-reinstatement, drug seeking behavior is extinguished by multiple sessions during which no drug is received following responses on a previously drug-paired operant. In this paradigm, the drug reinforcer is disassociated from the behavior. Following extinction of the drug-seeking behavior, a stimulus such as an injection of the drug, re-exposure to the environment in which drug was received, or a stressful event can trigger resumption of the drug-seeking behavior. Extinction-reinstatement is known to occur for both cocaine-seeking behavior and heroin-seeking behavior (Crombag et al. 2008; Rogers et al. 2008). Extinction-reinstatement has been a widely used animal model for studying relapse, although the applicability of this model to human drug relapse has been debated (Katz and Higgins 2003; Epstein et al. 2006). Extinction of drug-seeking is a crucial component of the extinction-reinstatement model in animals; however
an analogous situation does not occur in humans. Therefore, the experience of animals that undergo the extinction-reinstatement procedure is quite different from that of human relapsing drug users.

Sensitization to a drug involves an increased drug response to the same amount of a drug following its repeated administration (Feldman et al. 1997; Kalivas et al. 1998a; Kalivas et al. 1998b; Smith et al. 2008). While not a faithful behavioral model of relapse, sensitization provides a highly visible demonstration of how responses to drugs remain altered following a period of abstinence from drug use (Vezina 2007). Although sensitization occurs for a variety of drugs, it is best expressed following administration of psychomotor stimulants. In psychomotor sensitization, an animal receives injections of a psychomotor stimulant (e.g., cocaine) for a period of time and eventually becomes hyper-responsive to an injection of the stimulant. Both incubation and sensitization provide models of enduring behavioral changes that persist past cessation of drug administration.

Human studies of relapse can provide important insights but are confounded by numerous variables, such as polydrug use, different drug doses, and variations in lifestyle. Because of the confounding variables presented by human studies, animal relapse models provide the opportunity to study relapse in a controlled environment where animals can be manipulated by genetic, behavioral, and pharmacological methods.
Genes Implicated in Relapse

The animal models of relapse described above have been used to identify genes that are significantly altered following drug use and abstinence. The progression from initial drug use to dependence and addiction is characterized by multiple behavioral and physiological changes. Alterations in gene expression are believed to underlie these broader changes and have been demonstrated to alter behavior (Nestler et al. 2001; McClung and Nestler 2003). Following drug use and abstinence, genomic changes can be divided into three broad categories (Figure 2). Expression of particular genes can be increased or decreased during the period of drug-administration and arrive at a new steady-state of expression that is maintained throughout a period of drug abstinence. In this case, the physiologic condition produced by the new levels of gene expression can drive a former drug-user to relapse. In a second scenario, drug-administration again induces a change in gene transcript expression. However, as the time of
abstinence from the drug increases, transcripts return to their pre-drug level of expression. These genes are less likely to be responsible for long-term relapse to drug use, but they may be necessary initial factors for more enduring secondary expression changes. A third group of genomic changes appear during the abstinent period following drug use. In animal studies, it is known that some of these changes result from re-exposure to a previously drug-paired context. In each of the described scenarios, drug use results in a homeostatic imbalance that is ultimately manifested in an individual’s behavior. Given the multitude of genes present within a genome, when examining a particular behavior (such as relapse), it is useful to focus on a subset of genes believed to be involved in that behavior. The number of genes believed to be associated with drug use exceeds 100 (Worst et al. 2005; Kreek et al. 2005; Hemby 2006; Yano and Steiner 2007). However, the majority of these gene expression changes are present only during drug administration (Figure 2, middle panel). Therefore, far fewer genes remain altered following a period of abstinence. A list of several genes that have been found to be altered in a variety of relapse models is provided in Table 1. Many of these genes are transcription factors, while others are involved in dopamine and G-protein signaling. The external factor of drug abuse is able to induce intranuclear changes that eventually affect entire cells, neighboring cells, and ultimately the physiology of an entire organism (Figure 3). Genes identified in this article range from transcription factors that exist within the nucleus to genes encoding proteins that act intracellularly to those found within the plasma membrane to secreted factors.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Full Name</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGR1</td>
<td>Early growth response 1</td>
<td>Kuntz et al, 2008; Schmidt et al, 2005; Hellemans et al, 2006; Covington et al, 2005; Freeman et al, 2008; Lee et al, 2006</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
<td>Graham et al, 2007; Pu et al, 2006; Liu et al, 2005 &amp; 2008; Filip et al, 2006; Itoh et al, 2005; Le et al, 2005</td>
</tr>
<tr>
<td>DRD2</td>
<td>Dopamine receptor D2</td>
<td>Li et al, 2006; Shao et al, 2006; David et al, 2007 &amp; 2008</td>
</tr>
<tr>
<td>DRD4</td>
<td>Dopamine receptor D4</td>
<td>Li et al, 2006; Shao et al, 2006; David et al, 2007 &amp; 2008</td>
</tr>
<tr>
<td>RGS9</td>
<td>Regulator of G-protein signaling 9</td>
<td>Zachariou et al, 2003; Stanwood et al, 2006</td>
</tr>
<tr>
<td>GluR1</td>
<td>Glutamate receptor subunit GluR1</td>
<td>Backtell et al, 2008</td>
</tr>
<tr>
<td>c-fos</td>
<td>Fos</td>
<td>Ostrandar et al, 2003</td>
</tr>
<tr>
<td>GAD67</td>
<td>Glutamic acid decarboxylase</td>
<td>Carta et al, 2008</td>
</tr>
</tbody>
</table>

Several genes have been identified to be changed in models of relapse.
One well-known addiction-related gene is early growth response 1 (EGR1, a.k.a., zif268, NGFI-A, and krox 24). We have recently shown EGR1 gene expression to be increased in the medial prefrontal cortex (mPFC) of rats that exhibit incubation of heroin-seeking behavior following 14 days of abstinence from heroin self-administration (Kuntz et al. 2008b) and EGR1 has been repeatedly implicated in drug relapse by other laboratories (For recent examples, Schmidt et al. 2005; Lee et al. 2005; Covington et al. 2005, although a wealth of additional studies exist). In an illustration of how specific genes are affected in relapse models, either increased or decreased in expression depending on the specific drug previously used, expression of EGR1 is decreased in the mPFC of rats that have been abstinent from cocaine self-administration for either 1, 10, or 100 days (Freeman et al. 2008), while it is increased following abstinence from heroin (Kuntz et al. 2008b). The divergent directions of changes between the

![Figure 3 – Cellular location of relapse-related genes](image)

Figure 3 – Cellular location of relapse-related genes. Drug abuse affects transcription of genes that ultimately have intranuclear, cytoplasmic, and plasma membrane actions.
heroin and cocaine studies raises the question of whether these differences can be attributed to the depressive verses stimulant properties of heroin and cocaine, respectively, or variations in the psychodependence and physical dependence induced by different drugs. However, another study of relapse to cocaine seeking has reported mPFC EGR1 gene expression to be significantly increased rather than decreased following cocaine abstinence and contextual re-exposure. In this study, genes that were upregulated upon exposure to an environment in which cocaine was previously administered are not upregulated when rats are re-exposed to an environment in which cocaine was not previously available (Hearing et al. 2008a). Hearing’s study included contextual re-exposure while the samples in Freeman’s study were from rats that had not experienced contextual re-exposure. Re-exposure to a drug-related context is known to impact gene expression (Badiani et al. 1998; Ferguson et al. 2003; Badiani and Robinson 2004) and offers a plausible explanation for the between-study differences. Variations in the cocaine dose (0.6 mg/kg verses 1.5 mg/kg), operant training method (discrete trial model versus fixed ratio), duration of training sessions (6h verses 2h), and RNA quantitation techniques are all possible reasons for the variations in results. Region-specific upregulation of EGR1 has also been observed in rats that experience an amphetamine sensitization challenge (Guitart-Masip et al. 2008). Subregions of the amygdala and striatum varyingly display increases or decreases in EGR1 gene expression, with the direction of change varying depending on the inbred characteristics of
the strain and the specific brain region. For most of the subregions examined, EGR1 expression increased in rats that experienced an amphetamine challenge.

Brain-derived neurotrophic factor (BDNF) has been implicated in relapse to drug-seeking behavior. An upstream regulator of the immediate early genes Fos and Arc, BDNF promotes cocaine-taking and relapse behavior in rats (Graham et al. 2007). Interestingly, in the ventral tegmental area (VTA) BDNF protein levels have been found to be increased 10-15 days following withdrawal from cocaine injections, but not 1 day following withdrawal (Pu et al. 2006). Acute, but not chronic, cocaine administration increases striatal BDNF mRNA in rats (Liu et al. 2008). Thus, it is possible that an initial rise in BDNF mRNA levels is followed by subsequent increase in protein levels before the BDNF gene expression returns to a baseline. Because BDNF controls expression of other genes that are implicated in relapse, such as Fos and Arc, BDNF may be a crucial “switch” that activates numerous relapse-related intracellular cascades. Following cocaine withdrawal, levels of BDNF mRNA are reportedly increased in the hippocampus (Filip et al. 2006). Gene expression levels of trkB, the receptor for BDNF, were decreased in this study. Perhaps the decrease in receptors is indicative of a physiological response to counteract the increased levels of BDNF expression. Taken together, these findings suggest that changes in BDNF gene expression are both time and contingency-dependent. The suggested importance of BDNF to drug abuse has prompted studies investigating human polymorphisms of the BDNF gene; however, currently only modest associations between BDNF gene variants and substance abusers have been identified (Itoh
Levels of BDNF mRNA are increased following a single injection with cocaine (Le et al. 2005), as are levels of the dopamine D3 receptor (DRD3). BDNF is known to control DRD3 mRNA expression (Guillin et al. 2001), and this finding of concurrent increases in expression of these genes provides support for an interaction between these two genes. A reasonable hypothesis is that increased BDNF gene expression levels are responsible for increased DRD3 gene expression levels.

Increased dopamine signaling is an essential component of drug abuse (DiChiara and Bassareo 2007). Five different subtypes of receptors exist for dopamine: D1, D2, D3, D4, and D5. Expression levels of the genes encoding these receptors have been examined in both human and animal studies of relapse, with expression of specific DRD alleles being prevalent in humans who exhibit addictions to smoking and gambling (Comings et al. 1997). The precise role of DRD and dopamine in addictive behaviors remains to be elucidated, but the generalization of DRD across a range of addictive behaviors suggests this gene may be relevant to addiction beyond the specific area of drug abuse. The mesolimbic dopaminergic system, often referred to as the reward pathway, is important for the appetitive drive to engage in a variety of goal-directed behaviors, regardless of whether they involve drugs (Alacro et al. 2007). Increasing DRD2 expression in the nucleus accumbens reduces ethanol consumption in rats (Thanos et al. 2004), while DRD2 knock-out mice that are treated with DRD2 vectors suggest that DRD2 levels play a complex role in regulating the amount of alcohol consumed (Thanos et al. 2005). Human heroin
abusers possessing a polymorphism in the DRD4 or DRD2 genes report significantly higher levels of heroin craving than counterparts who do not possess either of these polymorphisms (Li et al. 2006; Shao et al. 2006). Polymorphisms in the DRD2 and DRD4 genes are believed to be predictive of how successful a person will be at smoking cessation (David et al. 2007; David et al. 2008). Although these studies examine polymorphisms rather than changes in gene expression, the implication is the same as with expression studies: aberrances in dopamine receptor genes are suspected to be involved in drug use.

Regulators of G-protein signaling are intracellular proteins that terminate G-protein signaling by accelerating GTPase activity (Krumins et al. 2004; Xie and Palmer 2005; Abramow-Newerly et al. 2006). Several genes exist in this protein family, and while the full extent of their involvement in intracellular functions remains to be elucidated, signaling by dopamine receptors and opioid receptors are known to involve RGS molecules. Messenger RNA for RGS2 and RGS4 is increased following opiate withdrawal (Gold et al. 2003). RGS4 mRNA in the prefrontal cortex and dorsolateral striatum is significantly decreased 21 days following either contingent or non-contingent cocaine administration. Interestingly, in both brain regions, re-exposure to the context in which cocaine was administered prior to the abstinence period restored RGS4 gene expression levels (Schwendt et al. 2007). Acute morphine increases RGS9 protein, while chronic exposure decreases the protein levels (Zachariou et al. 2003). RGS expression can be affected by dopamine signaling, and expression of RGS2, RGS4, and RGS9 are significantly decreased in dopamine D1 receptor knock-out
mice (Stanwood et al. 2006). Cocaine induces an increase in RGS4 gene expression through D1 receptors (Zhang et al. 2005). Acute amphetamine decreases RGS4 mRNA expression in rat forebrain (Schwendt et al. 2006). Again, induction or reduction of mRNA expression is seen for RGS4 depending on whether the drug being studied is an opiate or a stimulant. Unfortunately, variables in drug-taking schedules and abstinent periods necessitate the design of a specific study to assess whether the different directions of change in RGS4 gene expression are solely the result of the drugs studied.

AMPA receptors are composed of multiple glutamate receptor subunits, and expression of these glutamate receptor subunits is altered during withdrawal from cocaine (Lu et al. 2005) and heroin (Zhong et al. 2006; Bossert et al. 2006). Glutamic acid decarboxylase, isoform 67 (GAD67) is a GABA-synthesizing enzyme whose expression is known to be changed following drug use. After exposure to schedules of either morphine, amphetamine, or nicotine that induce behavioral sensitization, GAD67 mRNA expression is significantly increased in the central amygdala (Carta et al. 2008). This increase is evident following an initial drug treatment period and remains following sensitization, rather than a change specifically induced by the sensitization procedure. In hippocampal neurons, GAD67 gene expression is induced by EGR1 (Luo et al. 2008), suggesting a possible pathway through which some of the observed drug-induced changes in gene expression are coordinated (Figure 4).

Additional genes whose expression is reportedly increased following abstinence from heroin and decreased following abstinence from cocaine are Arc
(activity-regulated cytoskeletal protein) and c-fos. Studies of relapse to cocaine-seeking report EGR1, Arc, and c-fos gene expression to be increased following cocaine abstinence and contextual re-exposure (Klebaur et al. 2002; Ostrander et al. 2003; Zavala et al. 2008; Hearing et al. 2008b). Changes in Arc and c-fos gene expression also exist in the amygdala following re-exposure to an environment paired with opiate withdrawal, although the direction and magnitude of change is variable between sub-nuclei regions (Lucas et al. 2008). Comparing the basolateral amygdala (BLA), intercalated cell masses (ITC), and central nucleus of the amygdala (CeA) following re-exposure to a drug-paired environment, both Arc and c-fos increased more in cells that were also positive for GAD67 when examined in the ITC and CeA. However, in the BLA, expression of both genes was higher in GAD67 negative neurons.

**Figure 4 – Gene interaction pathway.** Genes discussed in this review can be placed into a hypothetical pathway of interaction. References depicted provide documentation of the intermolecular interactions. Clearly, the presence of all these relationships in a single cell is purely conjectural at this time.
Contextual cues play a crucial role in neuronal gene expression changes that are associated with drug use. Several immediate early genes are reportedly upregulated when rats are reintroduced to an environment in which they previously self-administered cocaine, and neuronal regions including the nucleus accumbens (Fuchs et al. 2008), dorsal striatum (See et al. 2007), prefrontal cortex, amygdala, and hippocampus (Fuchs et al. 2005). Thus, the context of drug-taking affects gene expression.

**Epigenetics**

The field of epigenetics examines how intranuclear changes in the structure of DNA and chromatin can produce changes in gene transcription (Colvis et al. 2005). Changes in gene expression are the endpoint of complex interactions of histones and chromatin that determine which genes will be transcribed (Renthal and Nestler 2008). Histone acetylation, histone methylation, histone phosphorylation, and DNA methylation combine to determine gene expression levels, and thus these are the mechanisms by which drugs actually elicit genomic effects. DNA is normally tightly wound around histones, forming the basis for chromatin structure. Transcription factors gain access to DNA that is not tightly wound around histones, and therefore epigenetic changes are central to determining which genes will be transcribed, and ultimately result in alterations in gene expression levels. In the nucleus accumbens, histone acetylation is induced by both acute and chronic cocaine administration (Kumar et al. 2005), although acute administration is associated with acetylation of H4 while chronic
administration results in acetylation of H3. Acute administration results in deacetylation that promotes c-fos gene expression, while chronic administration accompanies histone deacetylation that promotes BDNF and cdk5 gene expression. In the mPFC, decreased levels of H3 acetylation are seen both 1 and 100 days after cocaine self-administration (Freeman et al. 2008). In addition to the cocaine administration, cocaine-induced sensitization has also been associated with chromatin remodeling (Schroeder et al. 2008). In this case, changes in BDNF and D1 dopamine receptor gene expression were identified. Histone deacetylases remove acetyl groups from histones to change chromatin structure, and changes in expression of these enzymes could be responsible for drug-induced acetylation changes. Function of histone deacetylase 5 (HDAC5) is decreased in the NAc after chronic cocaine exposure (Renthal et al. 2007), and this decreased function alters the transcription pattern for several genes. Histone deacetylase 1 (HDAC1) affects c-fos gene expression following amphetamine exposure (Renthal et al. 2008). Expression of HDAC1 is normally chronically repressed by ΔfosB, but when it is expressed, HDAC1 allows chromatin remodeling that promotes c-fos gene expression.

Changes in mRNA levels that are correlated with changes in the chromatin modification of their respective genes have been documented. Thus, the role of chromatin structure in drug-induced changes in gene expression is a field which is rich in potential discoveries. Epigenetic studies are destined to provide a deeper understanding of the detailed mechanisms behind changes in gene expression.
Gene Intervention Behavioral Studies

Although many genes can be identified as being changed following drug use and relapse, the relevance of these genes to relapse must be assessed through behavioral studies. Fortunately, technologies such as siRNA, antisense oligonucleotides, and genetic knockouts allow the importance of specific genes, in specific neuronal areas, to be assessed. Infusion of EGR1 antisense RNA into the amygdala 90 min prior to a session of memory reactivation abolishes cue-induced reinstatement of cue-induced cocaine-seeking (Lee et al. 2005; Lee et al. 2006). Because drug-seeking behavior was reduced when EGR1 gene expression was reduced immediately prior to reconsolidation, the role of EGR1 in relapse appears to be closely tied to memory formation. This finding suggests EGR1 is an important gene for relapse, but because EGR1 functions as a transcription factor, the changes in expression of several genes transcribed by EGR1 is likely important to relapse. In fact, this is the authors’ conclusion and points to the importance of further studies to gain a broad scope of genomic changes. Genetic manipulation, combined with behavioral relapse models, provides a powerful approach for investigating the relevance of particular genes to relapse. Intracranial infusions of antisense oligodeoxynucleotides (against EGR1) into rat amygdala prior to reactivation and reconsolidation of drug-seeking memory reduces seeking behavior for both cocaine and heroin (Hellemans et al. 2006; Lee et al. 2006). The gene-intervention studies discussed in this paper utilize antisense oligomers. However, oligos often have toxicity and it is necessary to confirm there is no damage to physiological functions or regions
other than the one targeted. Therefore, models in which suppression of gene expression does not require introduction of a foreign agent are preferable for examining the role of specific genes.

**Future Directions**

*Dynamic database*

The studies discussed thus far provide a summary of what we currently know about genes whose expression levels are changed in models of relapse, but further steps must be taken to ensure a continuing increase in knowledge about relapse to drug abuse. An excellent start will be the development of a dynamic database to report genes that have been found to be implicated in relapse. A database similar to this was initially produced for cocaine-responsive genes (Freeman et al. 2002), but newer, web-based approaches, such as the Allen Brain Atlas ([www.brain-map.org](http://www.brain-map.org)) could combine drug, behavioral, anatomical, and gene expression data. A central repository for relapse information will accelerate the progress that can be made in further experiments investigating relapse to drug use.

*Temporally and spatially-selective knock-outs*

The relevance of specific genes to addiction can be studied using transgenic mice. Mice that are heterozygous or homozygous mutants for the EGR1 immediate early gene show behavioral differences from their wild type counterparts. Specifically, cocaine-induced locomotor sensitization is
significantly lower in EGR1-/- and EGR1+/- mice than in EGR1+/+ mice (Valjent et al. 2006). To ensure that EGR1 was the only transcription factor affected by the knock-in, the researchers in this particular study examined pathways upstream from EGR1 and parallel signaling pathways. Because EGR1 is a transcription factor, a valid concern is the possibility that another (or several other) genes whose transcription is regulated by EGR1 are affected. An ideal experiment to examine the relevance of particular genes to relapse is to use mice in which gene knockouts are both temporally and spatially specific. Transgenic mice, such as CRE-LacZ reporter mice (Shaw-Lutchman et al. 2003), provide ideal models in which such studies can be conducted. Combining measurements of transcription with and without expression of a particular gene with drug exposure provides insight about whether a particular gene is affected by that drug (Green et al. 2006). This approach can be used in models of relapse to drug-seeking to establish the importance of particular genes to the relapse phenomenon. Of course, the difficulty in this approach is the paucity of relapse mouse models. Therefore, further developments in either murine models of relapse or rat genetic manipulation (such as viral delivery of shRNA) will be required before the power of this approach is fully harnessed. The importance of cAMP-response element (CRE) to opiate withdrawal behavior has been determined using a transgenic mouse model (Shaw-Lutchman et al. 2002; Han et al. 2006). Region-specific changes in CRE-mediated transcription occur during opiate withdrawal, and CRE activity increases opiate withdrawal while decreasing CRE activity decreases opiate withdrawal.
The importance of glutamate receptors to sensitization to cocaine is apparent through a study using viral-mediated gene transfer of wild-type or pore-dead Glutamate receptor 1 (GluR1) into rat NAc (Backtell et al. 2008). Infusions of wt-GluR1 viral vectors result in a decrease in cocaine sensitization. Cocaine-seeking during extinction and cocaine-induced reinstatement were also reduced in rats in which GluR1 was overexpressed. Further studies will be needed to elucidate how GluR1 overexpression prevents these relapse measures in animal models, but it is likely that many of the genes highlighted in this review are involved in the mechanism.

Drug discovery

An ultimate goal of drug abuse research is to design pharmaceutical treatments to prevent relapse. Identification of target genes is the first step in drug discovery, and a database of genes suspected to be related to relapse could be valuable to making the drug discovery process more efficient. Now that research has determined that intervention with EGR1 gene expression decreases relapse, the search for pharmaceutical agents that affect EGR1 gene expression and downstream targets of EGR1 in specific neuronal regions should begin. Future relapse prevention research should occur on multiple levels, ranging from molecular to behavioral, and combining different fields as often as possible.
Conclusion

Knowledge of the gene expression patterns that exist following prolonged drug use and abstinence is useful in understanding the neurobiological drive to relapse despite the ensuing adverse consequences. How does one progress from taking a drug because it elicits rewarding effects to engaging in drug use as a necessity? The answer to this question may lie in the intricate molecular changes occurring in neurons during drug use and abstinence. These molecular changes, however, will be intricately intertwined with learning and memory. Although the focus of this article has been on drug addiction, the contribution of some of these genes to relapse may be through their role in memory formation and retrieval. Multiple hypotheses exist to explain addiction (Robinson and Berridge, 2003; Thomas et al. 2008; Koob and Le Moal 2008a; Koob and Le Moal 2008b). Whether addiction and relapse stem from the avoidance of unpleasant physical states, motivational systems that drive compulsive behavior, or memories that refuse to be silenced, is a topic of debate, and components of each theory are likely necessary for a complete understanding of addiction. The genomic profile of former addicts who are highly susceptible to relapse represents a combination of memory and drug-induced changes, and knowledge of this pattern of gene expression will be critical for designing the most effective pharmacological treatments for relapse.
Rationale, Hypotheses, and Brief Summary of Dissertation Studies

The idea that certain genes are important for relapse expands upon the well-accepted belief that the expression of specific genes is affected by drug use. Relatively little is currently known about gene expression patterns that exist at the time of relapse; previous studies have primarily focused on changes in gene expression that exist immediately after drug use. Furthermore, many studies have been conducted using animals that receive non-contingent drug infusions (administered to the study subject by an investigator).

With the goal of studying an individualized niche in drug abuse, I chose to focus on gene expression changes that exist at the time of relapse to heroin use. A behavioral relapse model was required prior to commencing molecular studies, and while incubation (defined as increased drug-seeking behavior with extended periods of abstinence) is currently well-documented for cocaine, reports of behavioral incubation are less prevalent in heroin literature. Additionally, the operant behavior of spout-licking is rarely documented in drug-abuse literature. Therefore, it was necessary to determine whether a behavioral model of incubation of heroin-seeking behavior could be produced using this tool (Chapter 2). The hypotheses that incubation of heroin-seeking, rather than cocaine-seeking, behavior would be observed and that incubation would be observed using spout-licking as the operant behavior were examined. Rats successfully acquired heroin self-administration behavior and, indeed, exhibited incubation of heroin-seeking behavior following 14 days of abstinence. Additionally, the incubation of goal-directed behavior was reported for heroin.
Following successful creation of a model of heroin relapse, studies of neuronal gene expression occurred, with the regions of focus being the medial prefrontal cortex and ventral striatum (nucleus accumbens). These two regions are largely involved in behavior and reward, respectively. Initially, several genes that were previously documented to express changed expression following drug exposure were investigated. This approach provided knowledge about whether genes that have been previously suggested to be affected by drug use are also affected in the heroin incubation relapse model (Chapter 3). *It was hypothesized that many, but not all, of the genes implicated in other models of drug exposure would exhibit changed expression following incubation of heroin-seeking.* Of the 11 genes examined, 5 were found to have significant changes in expression, and the changes were found to be both time-specific and region-specific.

Moving beyond examination of known genes suspected to display expression changes, a microarray analysis highlighted genes whose changed expression might historically have gone uncharacterized (Chapter 4). *The hypothesis guiding the microarray study was that genes that have a function in neuroplasticity would be identified to have changed expression.* Brain-derived neurotropic factor (BDNF) was confirmed to have altered gene expression, as well as several other genes that were previously not associated with heroin abuse.

This body of work demonstrates how different scientific fields can combine to yield comprehensive investigations of problems. Additionally, as detailed in the discussion (Chapter 5), the gene expression work may provide a foundation
for future treatments targeted not only at drug addiction, but also at psychiatric
disorders involving learning and memory.
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Chapter 2

Incubation of Goal-directed Heroin-seeking Behavior in Rats

Kara L. Kuntz, Robert C. Twining, Anne E. Baldwin, Kent E. Vrana, Patricia S. Grigson

Published in Pharmacology, Biochemistry, and Behavior 90: 344-348 (2008). K.L.K., K.E.V., and P.S.G. designed the study described in this article. K.L.K performed all of the writing and constructed the figures. P.S.G. and K.E.V. provided intellectual and editorial input into the manuscript. R.C.T. performed catheter implantation surgeries and provided advice on data analysis. A.E.B. assisted with the behavioral procedures and data entry.
Abstract

This study used heroin self-administration to investigate incubation of goal-directed heroin-seeking behavior following abstinence. Male Sprague-Dawley rats self-administered heroin on a fixed ratio 10 (FR10) schedule of reinforcement with licking of an empty spout serving as the operant behavior during 14 daily 3 h sessions. After this acquisition period, all rats received a 90 min extinction session following either 1 d or 14 d of home cage abstinence. When the extinction session occurred after only 1 d of home cage abstinence, rats with a history of heroin self-administration divided their responses equally between the previously “active” and “inactive” spouts. However, when the extinction session occurred following 14 d of home cage abstinence, the rats exhibited marked goal-directed heroin-seeking behavior by licking more on the previously “active” than “inactive” spout. These findings demonstrate that heroin-seeking behavior incubates over time, resulting in goal-directed heroin-seeking behavior in rats following 14 d but not 1 d of abstinence. Moreover, this facilitatory effect occurred in response to a different training schedule, a lower total drug intake, and after shorter periods of daily access than previously reported with heroin.

Keywords: incubation, heroin, self-administration, goal-directed behavior
Introduction

Heroin is a highly rewarding drug with strong abuse potential. Use of the drug is currently on the rise, in part due to higher purity, which allows for intranasal rather than intravenous administration (NIDA Research Report Series – Heroin: Abuse and Addiction, 2005). In addition to the potent rewarding properties of the drug, the problem of addiction is further complicated by the fact that nearly 90% of all addicted individuals will relapse to drug use following even prolonged periods of abstinence (DeJong 1994). Addiction, then, is recognized as a brain disease of chronic relapse (Leshner 1996; Koob et al. 1998) and continued research is essential to understand the neurobiological basis of the disease.

Recent work has demonstrated that craving and relapse increase with increasing periods of abstinence. This phenomenon has been termed “incubation” and occurs in rats (Tran-Nguyen et al. 1998; Grimm et al. 2003; Lu et al. 2005) and in humans (Gawin and Kleber 1986). Operationally, incubation is defined as an increase in drug-seeking as a function of the time since the last drug exposure. This increase in drug-seeking is positively correlated with the length of the withdrawal period. In rats, the strength of the correlation continues through an arbitrary period of abstinence, after which there is a gradual decrease in drug-seeking (Shalev et al. 2001).

Incubation has been observed in rats in several studies of cocaine self-administration (Grimm et al. 2001; Lu et al. 2004b; Lu et al. 2005), but in only one report involving heroin self-administration (Shalev et al. 2001). In that report, rats
were trained to self-administer heroin (0.1 mg/kg, iv) for 9 h/day for 10 days. Rats were then withdrawn from heroin for varying numbers of days. All rats were then tested in repeated 60-min extinction sessions, spaced 5 – 10 min apart until they reached the extinction criterion (less than 15 responses/h). Heroin-seeking behavior during extinction was greater following 6, 12, or 25 days of withdrawal, than it was after just one day of withdrawal. In the present work, we re-examined this initial report using a lower dose of heroin (0.06 vs. 0.1 mg/kg iv), shorter daily access periods (3h vs. 9h), and a different operant behavior (spout licking vs. lever pressing) that supports a relatively high level of responding. Spout-licking is an effective operant behavior for self-administration with the notable benefit that nearly all rats take drug during the first drug self-administration session (Jones et al. 2002; Grigson and Twining 2002; Liu and Grigson 2005). Moreover, we also measured responding on both an active and an inactive spout during acquisition training and during extinction testing in an effort to gauge the change in goal-directed heroin-taking and heroin-seeking behavior over time.
Methods

Subjects – The subjects were 32 male, Sprague-Dawley rats (Charles River Laboratories, Raleigh, NC) weighing 250-300g at the start of the experiment. They were individually housed in standard wire mesh cages in a colony room with controlled temperature, humidity, and ventilation. The rats were housed 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. Tissues obtained from these subjects are analyzed in the companion paper that describes the effect of heroin administration (and the contingency of its delivery) on gene expression following 1 or 14 d of abstinence. 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).

Surgery – Following one week acclimation to the colony room, intravenous catheters were implanted as previously described (Grigson and Twining 2002). Briefly, each rat was anesthetized with the intramuscular administration of a ketamine (70mg/kg)-xylazine (16mg/kg) mixture. A chronic indwelling Silastic catheter was implanted into the right jugular vein and secured with a suture. The catheter was coupled to a cannula that exited between the rat’s shoulder blades. Rats received one week to recover in their home cages with food and water available ad libitum.
**Apparatus** – Behavioral testing was conducted 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 that entered the chamber through holes spaced 16.4 cm apart (center to center). A stimulus light was located above each tube. A lickometer circuit was used to monitor licking on both active and inactive spouts. Each chamber was also equipped with a houselight (25 W), a tone generator (Sonalert Time Generator, 2900 Hz, Mallory, Indianapolis, IN), and a speaker for white noise (75 dB). Heroin reinforcement was controlled by an electronic circuit that operated a syringe pump (Model A, Razel Scientific Instruments, Stamford, CT). Events in the chamber and collection of the data were controlled on-line with a Pentium computer that used programs written in the Medstate notation language (MED Associates).

**Habituation and water-deprivation** – Over nine days prior to the start of training, all rats were water deprived and placed in the self-administration chamber for a 20 min period each day. During the first 5 min of the 20 min session, the rats were given access to water in the chamber via the right spout. An additional 1 h access to water was provided in the home cage each afternoon. This water-deprivation regimen (1 h access to water each afternoon) was continued throughout the training and extinction portions of the study as described previously (Grigson and Twining 2002).
Self-administration – All rats were given 14 daily, 3 h drug self-administration sessions. Sixteen rats self-administered heroin using licking on an empty spout as the operant behavior and were trained on a fixed ratio 10 (FR10) lick schedule of reinforcement. During acquisition, the rats were placed in individual operant chambers in which a house light was initially illuminated and white noise was broadcast. At the start of each session, two spouts extended into the chamber and a stimulus light was illuminated above the rightmost (active) spout. Every 10 licks on the active spout resulted in an iv infusion of heroin (0.06 mg/0.2 ml infusion), extinguishment of the cue light located above the active spout, illumination of the house light, and retraction of the spouts for a 20 sec timeout period indicated by onset of a 20 sec tone. Licks on the left (inactive) spout had no consequence, but were recorded. With the exception of spout licking, these general parameters are similar to those used in previous studies of heroin self-administration (Martin et al. 1998; Zhou et al. 2005; Chen et al. 2006).

Forced abstinence – Following the 2-week self-administration period, half of the rats (Group 1 Day: n=8) were returned to their home cage for a 24 h period of forced home cage abstinence, while the other half underwent a 14 day period of enforced home cage abstinence (n=8). During this time, rats were handled and weighed daily, food was available ad libitum, and water was available for 1 h each afternoon.
Extinction session - Following either 1 or 14 days of abstinence, all rats were subjected to a 90-minute extinction session during which completion of the 10 lick contingency on the active spout resulted in all of the same consequences (e.g. light extinguishment, tone) as during the 2-week training period, but saline, rather than heroin, was infused. Catheters in all but one of the rats were patent during the extinction session.

Behavioral Measures – Rats were weighed daily and licks on both spouts were recorded during acquisition and extinction. The latency to make the initial lick on each spout also was recorded, as was the total number of infusions (or infusion attempts) administered.

Drugs - Heroin HCl was provided by the National Institute on Drug Abuse (Research Triangle Institute, Research Triangle Par, N.C., USA). Drug was dissolved in sterile physiological saline at a concentration of 0.3 mg/ml.

Statistical Analyses – The number of responses (licks) and the log 10 latency to initiate responding were averaged for the final two days of self-administration and then analyzed using 2 x 14 repeated measures ANOVAs varying spout (active, inactive) and training day (1-14). Post hoc tests were conducted, where appropriate, using Newman-Keuls tests with alpha set at 0.05. Additionally, the number of responses and the log 10 latency to initiate responding were averaged across the 90 min extinction session. The extinction lick and latency data were
analyzed using 2 x 2 mixed factorial ANOVAs varying spout (active, inactive) and abstinence period (1 or 14 d). Post hoc tests were conducted, where appropriate, using Newman-Keuls tests with the alpha level set at $p < 0.05$.

*Goal-directed Behavior* – Goal-directed behavior is defined as the number of responses made on the active spout minus those made on the inactive spout (Fuchs et al. 1998; Lu et al. 2004a). This transformation also controls for differences in overall activity. Goal-directed behavior exhibited during extinction testing was compared following either 1 or 14 days of home cage abstinence using an unpaired Students $t$-test.
Results

**Number of responses.** Rats clearly distinguished between the active and inactive spouts during the acquisition sessions (see Figure 1, panel A). A significant Spout x Training day interaction, $F(13, 390) = 2.12, p < 0.01$, was detected for spout licks and post hoc analysis demonstrated that the number of responses made on the active spout was significantly greater than the number of responses made on the inactive spout beginning with Trial day 5 ($ps < 0.05$).

**Log 10 Latency to initiate responding.** Rats also exhibited clear drug-taking behavior by initiating licking more quickly on the active than on the inactive spout (see Figure 1, panel B). Support for this conclusion was provided by a significant main effect of spout, $F(1,28) = 143.58, p < 0.001$. The Spout x Training day interaction, however, was not statistically significant, $F(13,364) = 1.12, p = 0.34$. Thus, rats initiated licking on the active spout more quickly than on the inactive spout and this difference persisted pretty much unchanged throughout training.

Extinction.

**Number of responses.** During the 90-min extinction session, rats responded more on the active spout than on the inactive spout after 14 days of abstinence compared to 1 day of abstinence (Figure 2, panel A). Post hoc tests of a significant Spout x Abstinence Period interaction, $F(1,28) = 5.14, p < 0.03$, verified that responses on the active spout were significantly
Figure 1 – Behavioral data during self-administration. Left panel. The mean (+/− SEM) number of licks emitted on the active and the inactive spout across 14 days of acquisition training. Right panel. Mean (± SEM) log latency (seconds) to make initial contact with the active and the inactive spout across 14 days of acquisition training. * denotes statistical significance.
Figure 2. Behavioral data during extinction. Left panel. Mean (+/− SEM) number of licks emitted on the active vs. the inactive spout during the 90 min extinction test conducted after either 1 or 14 days of home cage abstinence. Middle panel. Mean (+/− SEM) log 10 latency (seconds) to make initial contact with either the active or the inactive spout during the 90 min extinction test conducted after either 1 or 14 days of home cage abstinence. Right panel. Mean (+/− SEM) goal-directed behavior (number of licks made on the active spout minus the number of licks made on the inactive spout) during the 90 min extinction test conducted after either 1 or 14 days of home cage abstinence. *denotes statistical significance.
higher after 14 days of abstinence than they were after 1 day of abstinence \( (p < 0.05) \). Additionally, responses were significantly higher on the active than the inactive spout during the extinction session after 14 days of abstinence \( (p < 0.05) \). The number of responses emitted on the active vs. the inactive spout did not differ when tested after only 1 day of abstinence \( (p > 0.05) \). Finally, after 1 day of abstinence, most saline infusions were earned during the first 45 minutes of the extinction session. After 14 days of abstinence, saline infusions were spread relatively evenly throughout the 90-min extinction session.

**Log 10 Latency to initiate responding.** Post-abstinence latency scores were analyzed using a 2 x 2 ANOVA varying spout and abstinence period (Figure 2, panel B). Unlike the lick data, the latency measure was not sensitive to incubation. Thus, the latency to initiate responding on the active vs. the inactive spout was very short, whether the extinction test occurred following 1 or 14 days of abstinence. This may represent a floor effect (i.e., the latency to lick cannot get much shorter). This conclusion was supported by a significant main effect of spout, \( F(1,28) = 50.9, p < 0.0001 \), and a non-significant Spout x Abstinence Period interaction, \( F(1,28) = 0.61, p = 0.44 \)

**Goal-Directed Behavior.** Goal-directed behavior (i.e., the number of responses emitted on the active spout minus those on the inactive spout) was significantly higher when tested following 14 days, as compared to 1 day, of abstinence \( (t(14)=-3.54, p < 0.003, \text{Figure 2, panel C}) \).
Discussion

During training, rats exhibited clear heroin self-administration behavior using spout-licking as the operant behavior. Licking on the active spout was initiated more quickly than on the inactive spout, and goal-directed behavior was evident with rats making more responses on the active than on the inactive spout. The water-deprivation regimen (i.e., having a history of water intake on the right most “active” spout) may have contributed to responding on the active spout during training, but cannot, alone, account for the behavior. This is because (a) water was never available in the chambers during either acquisition or testing and (b) saline-administering control rats, that were maintained on the same water-deprivation regimen, did not exhibit goal-directed behavior for the right-most “active” spout (see companion manuscript by Kuntz et al. and Grigson & Twining (2002)). Responding, then, was driven by the opportunity to self-administer the highly addictive agent, heroin.

During extinction testing, rats with a history of heroin self-administration were as likely to lick on the active as the inactive spout when tested after just 1 day of home cage abstinence. When tested after 14 days of abstinence, however, rats with a history of heroin self-administration made 3 times as many licks on the active than on the inactive spout. The longer period of abstinence, then, resulted in the development of directed heroin-seeking behavior. Indeed, the unexpected loss of reward (i.e., extinction) shifted the behavior of these rats from an adaptive “search-like” strategy across the two spouts (Day 1) to persistent drug-seeking on the previously active operandum (Day 14).
The incubation behavior described in this manuscript extends reports where the study of incubation is limited to an evaluation of active responses (Sorge and Stewart 2005). Difference scores can provide a measure of seeking-behavior and have been reported in studies assessing cocaine-seeking behavior (Tran-Nguyen et al. 1998). Lu and co-workers (Lu, et al., 2004b) reported on both active and inactive responding for cocaine. In that case, the rats clearly responded on the active lever more than the inactive lever when tested even after only 1 d of home cage abstinence. In the present heroin study, rats showed a preference for the active lever, but only following 14 d of abstinence. Possible explanations for the difference between the previous data (Lu, et al., 2004b) and those reported here include differences in drug, FR schedule, and operant behavior. The difference in drug seems an unlikely explanation as incubation has been reported with both cocaine and heroin (Shalev et al. 2001; Freeman et al. 2007). Regarding the schedule of reinforcement, rats in the present study were trained on a FR10 schedule of reinforcement, while the rats in the Lu et al. study were trained on an FR1 schedule of reinforcement. Relative to continuous reinforcement, partial reinforcement (i.e., an FR greater than 1) during training can elicit greater persistence in responding when tested during extinction (Valles et al. 2006). This difference, however, also seems an unlikely explanation because spout licking was both less and more goal-directed in the present study, depending only upon the length of the abstinence period. Finally, in reference to the operant response, lever pressing has a potential advantage over spout-licking because it offers a unique behavior that becomes exclusively associated
with drug taking. Even so, spout licking readily supports operant responding for sweets (Sclafani and Ackroff 2003; Hajnal et al. 2007) and for drugs (Liu and Grigson, 2005; Jones et al., 2002). Like lever pressing, spout licking for drug is orderly (Grigson & Twining, 2002) and, apparently, also is sensitive to incubation. We believe, then, that spout licking is a useful operant that may be even more sensitive to small changes in the strength of the goal-directed behavior than lever pressing. Further studies are required to test the accuracy of this conclusion and the merits of the resulting hypothesis that incubation involves a shift from an adaptive search strategy to a less plastic, tightly honed drug-seeking strategy.

Incubation occurred in rats in the present experiment despite the lower total daily intake of heroin and shorter daily exposure. As such, this study also extends the initial report by showing incubation of heroin-seeking behavior following increasing periods of abstinence (Shalev et al. 2001). Importantly, rats self-administered using an FR1 schedule of reinforcement in Shalev’s study and therefore his study utilized not only a different operant behavior, but also a different reinforcement schedule. Incubation has traditionally been associated with an increased response with time following prior exposure to some aversive stimulus (McAllister and McAllister 1967; Eysenck 1968; Houston et al. 1999). Recent reports suggest that the incubation phenomenon also occurs with rewarding stimuli. Cue-induced sucrose-seeking (Grimm et al. 2005), cocaine-seeking (Lu et al. 2005), and heroin-seeking (Shalev et al. 2001) increase when tested over increasing periods of abstinence. Incubation, then, is an adaptive phenomenon that, when engaged by drugs of abuse, could contribute to drug
relapse following abstinence. Increased craving with the abstinence period has been reported in humans (Gawin and Kleber 1986). This may help explain the increased likelihood of relapse, not only to the explicit cues and contexts that were previously associated with the drug, but also to cues and contexts that are remotely similar (Thiele et al. 1996; Houston et al. 1999). Incubation, in rats, also occurs with drug priming (Tran-Nguyen et al., 1998, but see Lu et al., 2004b) or following exposure to a stressor (Sorge and Stewart 2005b).

Studies focusing on the phenomenon of incubation are crucial to understanding this relapse model (Shepard et al. 2004). We can now conclude that the phenomenon of incubation, which develops after even relatively modest heroin self-administration, serves to narrow behavior to focus upon the goal. Further investigation of increased goal-directed behavior with abstinence will be needed to distinguish between learning and memory effects vs. physical craving. Additionally, characterization of the molecular underpinnings of this phenomenon is addressed in the companion paper, and ultimately may contribute to effective treatments for relapse prevention.

**Acknowledgements**

We thank Drake Morgan and David Roberts for their helpful comments on a draft of this manuscript and Chris Freet for programming. This work was supported by DA09815 and DA12473 to PSG, DA 13770 to KEV and DA021450 to KLK.
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Chapter 3

CNS Gene Expression Following Withdrawal and Cue-induced Drug-seeking Behavior

Kara L. Kuntz, Kruti M. Patel, Patricia S. Grigson, Willard M. Freeman, Kent E. Vrana

Published in Pharmacology, Biochemistry, and Behavior 90: 349-356 (2008). K.L.K., K.E.V., and W.M.F. developed the central theme of this article. K.L.K performed all of the writing and constructed the figures. P.S.G., W.M.F. and K.E.V. provided intellectual and editorial input into the manuscript. K.M.P. provided procedural guidance and synthesized the cDNA samples.
Abstract

In Chapter 2, we described incubation of heroin-seeking behavior in rats following 1 or 14 d 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 of 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 d of abstinence, but at 14 d 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, d 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.

Keywords: heroin self-administration, incubation, genes, contingency
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; O'Brien 2005; Krupitsky et al. 2006), 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 (see Chapter 2). Tissues from the rats from that report (supplemented with tissues from response-independent, investigator-administered heroin 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 observed in the NAc between animals self-administering drug (heroin or cocaine) versus those receiving infusions administered by an experimenter (Hemby et al. 1995; Hemby et al. 1997). Distinctive patterns of brain activity resulting from passive- versus self-administration of μ-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; Jacobs et al. 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; Jacobs et al., 2005; Koya et al. 2006), morphine (Ammon et al. 2003; Ammon-Treiber and Holtt 2005) or cocaine administration (Freeman et al. 2001; Freeman et al. 2002a; Freeman et al. 2002b). 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; Szumlinski et al. 2006; McClung and Nestler 2008; Szumlinski et al. 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.
Methods

Heroin self-administration and non-contingent drug delivery.

Subjects – Male, Sprague-Dawley rats (Charles River Laboratories, Raleigh, NC) weighing 250-300g at the start of the experiment, were individually housed in standard wire mesh cages and 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).

Surgery – Surgeries were performed one 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 one week.

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. (Chapter 2) for a more complete description of the apparatus.

*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 nine days. Water deprivation continued through the remainder of the study, with 1 h of water access in the home cage each afternoon.

*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 Chapter 2). 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 20s, and onset of a 20s tone. During this 20s period, responses on the “active” spout had no consequence. There were 14 such daily acquisition trials.

Forced Abstinence and Extinction session – Following 14 days of heroin (or saline) administration, half of the rats in each group (8 animals) received 1 d of abstinence, while the other half (8 per group) received an 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 one hour 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.

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.
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 0.3mg/ml. Each IV-injection was a 0.06mg/0.2ml infusion dose delivered over 6s.

Dissection and RNA isolation

Sacrifice and tissue dissection– Immediately following the 90-minute extinction session, all rats were sedated using Propofol (10mg/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 2.2mm 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 0.2mm was cut 0.5mm 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°C.

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).

**QRT-PCR analysis of gene expression.**

cDNA synthesis was performed on total RNA using Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA). 1µg RNA, 500ng Oligo (dT), and 10mM each dNTP, were incubated for 5 minutes at 65°C and then chilled on ice for 2 minutes. 5X First Strand Buffer (250mM Tris-HCL (pH8.3), 375mM KCL, and 15mM MgCl₂), 5mM DTT (final concentration), 40 U RNaseOut, and 200 U Superscript III RT were then added. The 20µl reaction was incubated for 60 minutes at 50°C followed by a final incubation at 70°C for 15 minutes 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.
Table 1 – Gene Expression Arrays. TaqMan Gene Expression Assays were used to quantify gene expression. The gene abbreviation, full name, and assay ID for each gene are listed in this table.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Name</th>
<th>Assay Number</th>
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<tbody>
<tr>
<td>Arc</td>
<td>Activity-regulated cytoskeletal-associated protein</td>
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</tr>
<tr>
<td>Beta-actin</td>
<td>Beta-actin</td>
<td>Rn00667869_m1</td>
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<tr>
<td>cdk5</td>
<td>Cyclin-dependent kinase 5</td>
<td>Rn00590045_m1</td>
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<td>Crystalline, alpha B</td>
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</tr>
<tr>
<td>EGR2</td>
<td>Early growth response 2, a.k.a. krox 20</td>
<td>Rn00586224_m1</td>
</tr>
<tr>
<td>Fos</td>
<td>c-fos</td>
<td>Rn02396759_m1</td>
</tr>
<tr>
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<tr>
<td>Homer 1 pan</td>
<td>Homer homolog 1, a.k.a ania-3, HOMER1F, Vesl-1</td>
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</tr>
<tr>
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<tr>
<td>Per2</td>
<td>Period homolog 2</td>
<td>Rn00581577_m1</td>
</tr>
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</table>

Statistical Analysis

Behavioral data were analyzed using 3 x 2 x 2 mixed factorial ANOVAs, varying group (self-administering, yoked heroin, yoked saline), spout (active, inactive), and abstinence period (1 or 14 d). Goal-directed behavior was determined by subtracting inactive spout responses from active spout responses. Gene expression values were evaluated using 3 x 2 mixed factorial ANOVAs varying
group (self-administering, yoked heroin, yoked saline) and abstinence period (1 or 14 d). 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: 1d and 14d 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.
Results

**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 +/- 9.7g on the first day of self-administration to 416.0 +/- 10.4g on the final day of self-administration. Weight increases for rats yoked to heroin or saline were 380.6 +/- 6.4g to 429.5 +/- 7.5g and 372.3 +/- 7.2g to 421.3 +/- 9.7g, 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 Chapter 2.

*Acquisition: Heroin self-administration and non-contingent administration*

The 16 self-administering rats increased their heroin intake from an average of 7.3 +/- 0.37 infusions on day 1 to 12.6 +/- 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 Chapter 2).

Self-administration behavior was also demonstrated by executive rats initiating licking more quickly on the active spout than on the inactive spout (10.7 +/- 3.6s vs. 3043.0 +/- 953.2s, 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 Chapter 2).

*Extinction: Incubation of drug-seeking behavior*

*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 spout than did rats that experienced only 1 day of abstinence (see Figure 2 in Chapter 2). 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 d of abstinence, responding on the active spout was nearly 3 times that made on the inactive spout when the extinction test followed 14 d 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 +/- 1.9s vs. 8.2 +/- 2.6s). Finally, active vs. inactive spout responding did not differ for either the yoked heroin or the yoked saline controls, whether tested following either 1 d or 14 d of abstinence, ps > 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 (Figure 1).

**Figure 1 – Goal-directed behavior.** 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.

**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). (Figure 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 1d ($F_{2,19} = 10.76$, $p < 0.001$) and 14d ($F_{2,19} = 17.65$, $p < 0.001$) of abstinence. Observed increases above yoked saline existed for self-administration following 1d abstinence ($p < 0.001$) and 14d abstinence ($p < 0.001$). Elevations above saline also existed for yoked heroin rats following 1d ($p < 0.05$) and 14d ($p < 0.005$) 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 ($p < 0.03$) (Figure 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 received yoked infusions of heroin displaying a dramatic increase in EGR2 gene expression (300%; $F_{2,16} = 5.37$, $p < 0.02$) (Figure 2, panel b).
Figure 2 – Early growth factor gene expression. (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. \(^{\text{^p}=0.001; 
\#p<0.01}\)

(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. \(^{\text{^p}<0.001; 
\#p<0.01; \; \#p<0.05}\)
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 ($F_{2,19} = 6.00, p < 0.02$) (Figure 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.

**Figure 3 – Fos gene expression.** 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. $^{#} p < 0.01; ^{\&} p < 0.05$. 
Changes Induced by Heroin that Do Not Persistent 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 (Figure 4). No significant changes were detected in the NAc.

![Graph showing Arc gene expression in mPFC and NAc](image)

**Figure 4 – Arc gene expression.** 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.
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 (Figure 5). Homer1 b/c was not significantly changed in the NAc regardless of duration of abstinence (although a non-significant trend is evident).

Figure 5 – Homer1 b/c gene expression. mPFC Homer1 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. $\alpha p < 0.05$. 
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 (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. Similarly, levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were also confirmed to be unchanged (data not shown).
### Table 2 – Non-significant changes in gene expression.

Average expression of genes that were not significantly changed (+/- S.E.M.) are depicted within this table.

#### A. mPFC

<table>
<thead>
<tr>
<th>Gene</th>
<th>1 day abstinence</th>
<th>14 days abstinence</th>
<th>1 day abstinence</th>
<th>14 days abstinence</th>
</tr>
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<tbody>
<tr>
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<td>Yoked Saline</td>
<td>Yoked Heroin</td>
<td>Self-Administering</td>
<td>Yoked Saline</td>
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<td>Per 2</td>
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<td>1.37 +/- 0.32</td>
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#### B. NAc

<table>
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<th>Gene</th>
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<th>14 days abstinence</th>
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<tr>
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<td>Yoked Saline</td>
<td>Yoked Heroin</td>
<td>Self-Administering</td>
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<td>alpha B-crystallin</td>
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<td>Homer1 a</td>
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**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 14d 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.
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

<table>
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<tr>
<th>Gene</th>
<th>Behavior</th>
<th>Session</th>
<th>Abs. Pd.</th>
<th>Correlation Coefficient</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crystallin ab</td>
<td>active</td>
<td>extinction</td>
<td>1 d</td>
<td>0.76</td>
<td>0.03</td>
</tr>
<tr>
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<td>acquisition</td>
<td>14 d</td>
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<td>0.03</td>
</tr>
<tr>
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<td>acquisition</td>
<td>14 d</td>
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Table 3 – Behavior – gene expression correlations. Significant correlations between behavior and gene expression were detected. Most correlations occurred between behavior and mPFC gene expression. Italic indicates the correlation was detected between behavior and NAc gene expression.
were observed when compared with rats experiencing only 1 day of abstinence (Figure 1 and Chapter 2).

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 antisense 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; Chavrier et al. 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 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
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; Uslaner et al. 2001b). 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. Homer 1 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 one 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 homer1a, 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. Homer1a 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 Homer1a also may have been elevated in our rats following exposure to the test chamber, but this elevation could not be verified 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. 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 14d 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 active spout) whether tested following either 1d or 14d 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 d 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 d of abstinence. Homer 1 b/c was decreased in the mPFC, but only when examined after 14 d of abstinence, and only in the yoked, noncontingently administered rats. The mean expression of Homer 1 pan was not altered in the mPFC, but the level of expression of Homer 1 b/c and Homer 1 pan varied as a function of the magnitude of the drug-seeking behavior when tested after 14 d 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 d 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.
Reference List


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Chapter 4

Gene Expression Changes in a Heroin Behavioral Incubation Model

Kara L. Kuntz-Melcavage, Robert M. Brucklacher, Patricia S. Grigson,
Willard M. Freeman, Kent E. Vrana

Submitted to *BMC Neuroscience*. The text (and formatting) conform to the requirements of the journal. K.L.K., K.E.V. and W.M.F. developed the central theme of this article. K.L.K performed all of the writing and constructed the figures. R.M.B. performed the technical microarray procedures and provided guidance for the data analysis. P.S.G., W.M.F. and K.E.V. provided intellectual and editorial input into the manuscript.
Abstract

Background: Several studies have investigated gene expression changes induced by drug exposure, but few reports describe changes that persist following relapse. In this study, genome-wide analysis of gene expression was conducted in rats that expressed behavioral incubation of heroin-seeking and goal-directed behavior. As an important modulator of goal-directed behavior, the medial prefrontal cortex (mPFC) was the target of genomic analysis. Rats were trained to self-administer heroin (0.06 mg/0.2 ml infusion) during 3h daily sessions for 14d. Following the self-administration period, rats were reintroduced to the self-administration chambers for a 90-minute extinction session. The extinction session occurred either 1d or 14d following the final self-administration session.

Results: Behavioral data demonstrated incubation (increased expression) of heroin-seeking and goal-directed behavior after the 14d abstinent period. Whole genome gene expression analysis was performed and results were confirmed by quantitative real-time PCR (RT-qPCR). Microarrays identified 66 genes whose expression was identified as changed by at least 1.4 fold (p<0.02) following 14d of abstinence and the 90-minute extinction session compared to the saline treated controls. Orthogonal confirmation by RT-qPCR demonstrated significant alterations in BDNF, Calb1, Dusp5, Dusp6, EGR1, NPY, RGS2.

Conclusions: Ontological analysis indicates that several of the genes confirmed to be changed are important for neuroplasticity, and through that role may impact learning and behavior. The importance of drug-seeking behavior and memory of
previous drug-taking sessions suggest that such genes may be important for relapse. The global gene expression analysis adds to the knowledge of heroin-induced changes and further highlights similarities between heroin and other drugs of abuse.

Keywords: relapse, heroin, microarray, gene expression
Background

The challenge for drug abuse treatment is maintaining abstinence despite a high propensity for patients to relapse to drug use. Although heroin has been abused for centuries, effective long-term preventions for heroin-relapse are still needed. Physiological and gene expression changes that may increase an individual's likelihood to relapse are known to exist well into a period of abstinence (Nestler 2000; Self 2004; Mohn et al. 2004; Hemby 2006; Freeman et al. 2008). Therefore, relapse to drug use is currently being investigated on both the molecular and behavioral levels (Lee et al. 2005; Lee et al. 2006; Kuntz-Melcavage et al. 2008; Stewart 2008; Kuntz et al. 2008a; Marie-Claire et al. 2008b). These studies have been aided by advances in systems biology tools, including large-scale discovery techniques such as microarrays and discovery proteomics. These approaches are useful for discovering novel targets affected by drug abuse and examining hypotheses concerning categories of genes that are affected by drug use (Freeman and Vrana 2005; Nielsen et al. 2008). As data on gene expression following relapse accumulates, the existence of a single "relapse gene" is becoming increasingly unlikely. Therefore, macroscopic views of gene expression (pattern identification) will prove very useful for guiding research into behavioral phenomena.

Neurobiological, environmental, cue, and stress mechanisms have all been implicated in relapse to drug use. The intense craving and motivation to seek drug, reported by humans during withdrawal from drug use, is challenging
to model in animals, but the need for a relapse model continues to motivate the design of new behavioral procedures. Incubation is a behavioral phenomenon that is characterized by increased drug seeking following increasing periods of abstinence after the last self-administration session (Lu et al. 2004). Increased drug-seeking has been inferred to represent the craving that drives humans to relapse (Berridge and Robinson 1998; Self 2004; Wise 2004).

The prefrontal cortex is important for decision making and guiding behavior (Schoenbaum and Shaham 2008), and the medial prefrontal cortex (mPFC) is known to be especially important for goal-directed behaviors (Hitchcott et al. 2007; George et al. 2008). Because of its role in guiding goal-directed, drug-seeking behaviors, understanding the gene expression changes in this region following a period of abstinence from drug self-administration will be useful for understanding the neurobiological basis to relapse following a period of drug self-administration. Additionally, the ventral mPFC is believed to play an essential role for expression of incubation of cocaine-seeking (Koya et al. 2008).

In this study, we performed a whole genome analysis of gene expression in the medial prefrontal cortex of rats that displayed incubation of goal-directed behavior following 2 weeks of heroin self-administration and 2 weeks of home-cage enforced abstinence. After the abstinence period, rats were reintroduced to the testing chambers for a 90-minute extinction session during which behavioral responses were recorded. Following this experience of re-exposure to drug-
associated context and cues, RNA was isolated from the mPFC for whole
genome microarray and qPCR analyses. Ontological analyses revealed that
many of the genes identified to be changed have the potential to be key
components to neuroadaptations that exist at the time of relapse.
Methods

Heroin self-administration

The behavioral procedures have been previously described (Kuntz et al. 2008a; Kuntz et al. 2008b). Briefly, rats that self-administered heroin displayed incubation of heroin-seeking when tested in a 90-minute extinction session that occurred after 2 weeks of enforced abstinence (see Figure 1). As described in previous work, rats were subjected to two weeks of heroin self-administration followed by a 90-minute extinction session that occurred either 1 day or 14 days after the final self-administration session. During the extinction session, responses on active and inactive spouts were recorded. Self-administering rats were yoked to rats that received infusions of saline. Immediately after the extinction sessions, the rats were sacrificed and brain regions were harvested.

Dissection and RNA isolation

Sacrifice and tissue dissection – Immediately following the 90-minute extinction session, all rats were sedated using Propofol (10mg/kg) and decapitated. Brains were rapidly removed from skulls, 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 2.2mm was cut along the forceps minor and the cortex medial to this cut was collected (medial prefrontal cortex, mPFC). This includes the cingulate, prelimbic cortex, and medial orbital cortex. Following dissection, the tissue was placed in prechilled tubes, immediately frozen on dry ice, and then stored at -80°C.
**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).

**Microarray analysis**

Microarray analyses were performed on samples from rats that experienced 14d of abstinence prior to an extinction session. Studies were performed by the Penn State College of Medicine Functional Genomics Core Facility on 12 arrays (n = 6 per treatment group: 14d abstinent self-administering and yoked saline) according to standard procedures (Brucklacher et al. 2008). Following the manufacturer’s protocol of the Low Input Fluorescent Linear Amplification Kit (Agilent, Santa Clara, CA), 500ng RNA with the addition of One-Color Spike Mix was denatured and incubated with T7 Promoter primer. Synthesis of cDNA followed with the addition of First-Strand buffer, DTT, dNTP mix, MMLV-RT and RNase Out and incubation at 40°C for 2 hours. Transcription of the product incorporated the Cyanine 3-CTP in the Master Mix which includes Transcription buffer, DTT, NTPs, PEG, RNase Out, pyrophosphatases and T7 RNA Polymerase, with incubation for 2 hours at 40°C. The resulting cRNA was purified using RNEasy columns (Qiagen) followed by assessment of purity,
concentration and quality using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE 19810) through calculated yield and Specific Activity. 1.65 µg from each sample was fragmented, denatured, and then hybridized to Agilent 4x44 rat whole genome microarray slides for 17 hours at 65°C. Slides were then washed according to protocol.

Microarrays were scanned with an Axon 4000B scanner with GenePix4 v4.0 software at a 5 µm resolution and 635 nm with laser power at 100%, PMT voltage at 600 V, focus position 0 µm, and lines to average = 1. Images were then imported into Agilent Feature Extraction Software. Initial quality control (positive and negative controls), exclusion of manufacturing defects (MSR spots), background subtraction was then performed and the results exported to GeneSpring GX 7.3 (Agilent Technologies).

**Microarray Data Analysis**

Microarray data were normalized following import into GeneSpring GX 7.3 (Agilent Technologies) by transforming signal values less than 5.0 to an intensity of 5.0. Normalization was done per chip to the 50th percentile, and per gene to the median. Values were then normalized on a per gene basis to the control group. Potential differential expression was determined with a one-way ANOVA (variances not assumed to be equal), p < 0.02 and filtered for 1.4 fold and greater differences in expression in accordance with standards for microarray analysis (Allison et al. 2006). The use of a combination of statistical and fold-change cutoffs as opposed to traditional multiple testing corrections (e.g., Bonferroni)
produce gene lists with the lowest rate of type I and type II errors (Osier et al. 2004). 1.4-fold was chosen as the fold-change cutoff, as this magnitude change is at the lower range of changes we find to be confirmable by RT-qPCR.

**RT-qPCR analysis of gene expression**

Complimentary DNA synthesis was performed on total RNA (n = 8 per treatment group: 14d abstinent self-administering and yoked saline) using Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA). One µg RNA, 500ng Oligo (dT), and 10mM each dNTP, were incubated for 5 minutes at 65°C and then chilled on ice for 2 minutes. 5X First Strand Buffer (250mM Tris-HCL (pH8.3), 375mM KCL, and 15mM MgCl₂), 5mM DTT (final concentration), 40 U RNaseOut, and 200 U Superscript III RT were then added. The 20µl reaction was incubated for 60 minutes at 50°C followed by a final incubation at 70°C for 15 minutes for termination. The resulting cDNA product was quantified and 20ng of product was used in each subsequent qPCR reaction.

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 (Brucklacher et al. 2008; Bowyer et al. 2008). Primer/probe sets used are listed in Table 1. SDS 2.2.2 software and the $2^{\Delta\Delta Ct}$ analysis method (Livak and Schmittgen 2001) were used to quantitate relative amounts using β-actin as an endogenous control.
Ontological, pathway, and network analysis

Ontological analysis used Gene Ontology (GO) categories to determine processes or functional categories that were differentially expressed, as described previously (Beissbarth and Speed 2004) using GeneSpring GX software. This analysis determined the number of genes in a category present on the array and the number of expression changes that would be part of that category by random chance given the number of differentially expressed genes.

<table>
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**Table 1 – Genes examined.** List of genes examined in this and previous studies.
Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, CA) was used to create a network from RT-qPCR confirmed gene expression results from the rats described in this study.

**Statistical Analysis**

Behavioral data were analyzed by t-tests at each time point of abstinence (1d and 14d). Goal-directed behavior was determined by subtracting inactive spout responses from active spout responses. RT-qPCR gene expression values were evaluated using t-tests between self-administering and yoked saline rats at each time point of abstinence (1 or 14 d). For both behavioral and gene expression data, levels of significance were determined with \( \alpha \) set at 0.05. Correlational analyses were performed to determine whether a correlation existed between the goal-directed behavior or active spout responses during the extinction session and gene expression.
Results

Behavior

Rats were allowed to self-administer heroin during 14 days of daily drug access. When rats were reintroduced to the self-administration chambers following 1d or 14d of abstinence, incubation of active spout responses was observed (defined as a significant increase in responses on the active spout with the progression of time; Kuntz et al. 2008a). Goal-directed behavior also incubated in self-administering rats and increased from an average of 80.6 +/- 42.5 responses following 1 day of abstinence to an average of 302.6 +/- 45.9 responses following 14 days of abstinence (Figure 1). Goal-directed behavior after 14 days of abstinence was significantly higher in self-administering vs. yoked saline rats (p < 0.001) and self-administering vs. 1 day abstinent self-administering rats (p < 0.01). Additionally, goal-directed responding on the active spout compared to the inactive spout was significantly increased only after 14 days of abstinence (p < 0.001).
Microarray analysis

Signals from 23,670 probes (of the approximately 41,000 total probes) were detected as being present on all of the arrays. Filtering the detected genes produced a list of 66 genes that were identified as being changed by at least 1.4 fold (at the p < 0.02 level of significance) relative to saline controls after 14 days of abstinence and a 90-minute extinction session.

RT-qPCR Confirmation and Validation

RT-qPCR was performed to confirm gene expression levels of genes for which significant differences in expression were detected by microarrays. Genes were chosen for confirmation analyses based on their ontological classifications and probable involvement in drug use. Many genes examined belonged to ontological categories of nervous system development or behavior. For each
gene examined, samples from self-administering and yoked saline rats from each of the abstinent period treatment groups (1 day and 14 days) were examined. Table 1 depicts genes on which RT-qPCR was performed either for this study, or in a previous study that used these same cDNA samples (Kuntz et al., 2008b). Additionally, Nr4a3 was detected by our arrays to be significantly increased and has been previously reported to be increased by another laboratory (Koya et al., 2006).

Among the genes that were detected by the arrays to be significantly changed, and confirmed by RT-qPCR, were genes that are important for intracellular signaling (Figure 2). Dual specificity phosphatase 5 (Dusp5) and dual specificity phosphatase 6 (Dusp6) expression levels were both increased by 20-40% after 14 days of abstinence, although only Dusp6 expression levels were increased after only 1 day of abstinence. Regulator of G-protein signaling 2 expression was increased by 20% following 14 days of abstinence.

![Figure 2 – Genes involved in regulation of intracellular signaling.](image)

RT-qPCR confirmed gene expression changes in self-administering compared to yoked saline rats after 1 and 14 days of abstinence. Data represent means ± SEM. * p < 0.01, # p < 0.05
Genes for additional intracellular molecules that can be linked to physiological changes following drug use were also confirmed to be significantly changed (Figure 3). Brain-derived neurotrophic factor (BDNF) and calbindin 1 (Calb1) both displayed increased expression levels, by 30% and 20% relative to saline controls. Neuropeptide Y gene expression was decreased by approximately 20% at 1 day and 14 days of abstinence.

![Figure 3 – Genes involved in neuronal adaptations to behavior](image)

Ontological, pathway, and network analysis

Analysis of gene ontology revealed that 24 genes that were identified as being different from saline controls by at least 1.4 fold ($p < 0.02$) following 14d of abstinence were involved in development. Of these genes, 4 were confirmed to be changed by RT-qPCR: BDNF, Calb1, Dusp6, Egr1 and one (Nr4a3) has been previously reported by another group of researchers (Koya et al. 2006). A
second ontological category of interest was behavior, and 17 genes from our list were included in this category. Among these genes were EGR1 and Crybb1.

The Ingenuity network analysis revealed that many genes validated in this study, together with genes previously reported from these samples to be significantly changed, interact in a network that is involved with behavior, nervous system development and function, and cellular development (Figure 4). All of the genes in this pathway were determined to be upregulated.

**Figure 4 – Ingenuity pathway analysis.** Gene expression changes that have been confirmed function together in a network that is important for nervous system development. Direct interactions are indicated by solid lines while indirect interactions are indicated by dashed lines.
**Correlational Analyses**

Behavioral data collected during extinction (goal-directed responses and active spout responses) was used in correlational analyses with gene expression data. Both Pearson’s and Spearman’s correlations were performed because of the small sample sizes that were not always normally distributed. When separate correlations were performed for data from each time point, Pearson’s analyses identified a positive correlation between the number of active spout responses and Dusp5 gene expression in rats that experienced 1 day of abstinence (p = 0.05; r = 0.70, data not shown). A significant negative correlation between Dusp5 gene expression and inactive spout responses (p < 0.03; r = -0.78) was detected for rats that experienced 14 days of abstinence by a Spearman’s analysis. Combining the data from both abstinent periods yielded 8 more significant correlations (Table 2).

<table>
<thead>
<tr>
<th></th>
<th>BDNF</th>
<th>Calb1</th>
<th>Dusp5</th>
<th>NPY</th>
<th>BDNF</th>
<th>Calb1</th>
<th>Dusp5</th>
<th>NPY</th>
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<tr>
<td><strong>Pearson</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Active Spout Responses</td>
<td>0.59</td>
<td>0.10</td>
<td>0.37</td>
<td>-0.20</td>
<td>0.59</td>
<td>0.09</td>
<td>0.42</td>
<td>-0.10</td>
</tr>
<tr>
<td>Goal-Directed Behavior</td>
<td>0.64</td>
<td>0.48</td>
<td>0.61</td>
<td>-0.55</td>
<td>0.65</td>
<td>0.53</td>
<td>0.48</td>
<td>-0.58</td>
</tr>
<tr>
<td><strong>Spearman</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Active Spout Responses</td>
<td>0.02*</td>
<td>0.73</td>
<td>0.18</td>
<td>0.49</td>
<td>0.02*</td>
<td>0.78</td>
<td>0.11</td>
<td>0.52</td>
</tr>
<tr>
<td>Goal-Directed Behavior</td>
<td>0.01*</td>
<td>0.07</td>
<td>0.02*</td>
<td>0.03*</td>
<td>0.09*</td>
<td>0.04*</td>
<td>0.07</td>
<td>0.02*</td>
</tr>
</tbody>
</table>

**Table 2 – Correlational analysis results.** Goal-directed response and active spout response data from extinction were correlated with RT-qPCR data. Eight significant correlations were detected (4 detected by Pearson’s analysis and 4 detected by Spearman’s analysis). The upper number in each block of the table is the correlation coefficient and the lower number in each block of the table is the P value.
Discussion

This study has provided the first report of whole genome analysis of CNS gene expression in rats that have expressed behavioral incubation. Given the relevance of incubation of drug-seeking behavior to studying relapse, the data produced by this research expands our knowledge of gene expression changes that exist after abstinence and during the time of relapse. The approach of using a preliminary screen of the entire genome, followed by rigorous RT-qPCR confirmation of expression changes for genes likely to be involved in behavior or neuronal changes has yielded information on genes, such as Dusp5 and Dusp6, whose role in addiction is only now beginning to emerge.

The majority of genes that were significantly changed using the criteria reported in this paper (52 of 66 genes) had increased expression levels in heroin self-administering rats when compared to yoked saline following 14 days of enforced abstinence. Increases in gene expression are well-documented following opiate use. An increase in gene expression has been found for opiate receptors in humans that died from an opiate overdose (Becker et al. 2004) and increases in gene expression are known to exist for molecules within the cyclic AMP signaling system following opiate use (Guitart and Nestler 1993). The reasons for a disproportionate number of down-regulated genes following abstinence from heroin self-administration remain to be determined. However, when gene expression is near the level of detection of the microarray platform, it is becomes more difficult to detect downregulations.
Phosphatase genes, such as Dusp5 and Dusp6 (MKP-3; MAP kinase phosphatase-3), encode proteins that have a direct impact on intracellular signaling. Dusp5 preferentially dephosphorylates ERK (Kwak and Dixon 1995; Jeong et al. 2007) and is intranuclear, while Dusp6 is cytoplasmic (Owens and Keyse 2007). The present findings suggest that heroin affects expression of these genes, but other drugs have also been reported to impact dual specificity phosphatase gene expression. MDMA (3,4-methylenedioxymethamphetamine) has been shown to increase expression of Dusp 1, Dusp 5, and Dusp 14 genes (Marie-Claire et al. 2008a), while methamphetamine treatment increases Dusp6 gene expression in multiple brain regions (Takaki et al. 2001). Both Dusp5 (Kovanen et al. 2007) and Dusp6 (Reffas and Schlegel 2000) regulate mitogen-activated protein kinase (MAPK). Numerous studies have suggested MAPK involvement in neuroadaptations that occur following drug use (Schulz et al. 2004; Ferrer-Alcon et al. 2004; Mattson et al. 2005). Beyond being affected by mere drug exposure, MAPK molecules have also been shown to play a role in morphine tolerance (Chen et al. 2008) and to be activated following opiate withdrawal (Schulz and Hollt 1998). Interestingly, the MAPK pathway plays a central role in the relationships between genes that were reported as changed in this study and our previous study of gene expression (Figure 4; Chapter 2). It is possible that many of the changes we have reported may be downstream effects of MAPK alterations that initially occur upon the initiation of drug use.
Opioid receptors are coupled to G-proteins, and molecules that regulate G-protein coupled receptor (GPCR) signaling have been shown to be essential for reinstatement of heroin-seeking behavior (Yao et al. 2005). The observed increase in RGS2 gene expression following 14 days of abstinence and contextual re-exposure may represent an intracellular signaling change that affects communication between receptors and transcription factors, ultimately affecting cellular and organismal physiology. A GPCR-regulating molecule (AGS3; Activator of G-protein signaling 3) was shown, through a gene knock-down approach, to be essential to activating protein kinase A (PKA) signaling and observing reinstatement of heroin-seeking (Yao et al. 2005). Although RGS2 functions to inactivate rather than activate intracellular signaling, the observed increase in RGS2 may be important for regulating additional signaling molecules that are essential for the expression of behavioral incubation of heroin-seeking.

An additional molecule whose role in incubation remains to be elucidated is neuropeptide Y (NPY). The microarray analysis detected an NPY receptor, NPY5R, to be significantly increased after 14 days of abstinence. While RT-qPCR for NPY5R failed to replicate this change, NPY, the ligand for this receptor, was confirmed to be changed. NPY gene expression differed from most other genes examined because it was decreased in rats that had self-administered heroin, both after 1 and 14 days of abstinence. Although NPY is a neurotransmitter most recognized for its role in regulating food intake (Meister
2007), it has been hypothesized that NPY may contribute to the negative motivational state of withdrawal (Koob 2008). Studies have shown that NPY has an affect on drug intake. Intracerebroventricular administration of NPY has been found to block increased ethanol intake in rats that have been rendered ethanol dependant by ethanol inhalation (Thorsell et al. 2005; Koob 2008), and overexpression of the NPY gene, using intra-amygdalar infusions of a viral expression vector, diminished alcohol intake that normally increased following longer periods of abstinence or repeated alcohol withdrawals (Thorsell et al. 2007). It remains to be established if similar infusions in rats that have self-administered heroin or cocaine result in a similar blunting of incubation of drug-seeking behavior.

Unlike NPY, whose expression was decreased after both 1 and 14 days of abstinence, the majority of genes on which RT-qPCR was performed displayed expression levels significantly different from saline controls only after 14 days of abstinence (Dusp5, RGS2, BDNF, Calb1). This suggests that these changes are attributable either solely to the extended drug abstinence or to the combination of extended abstinence and contextual re-exposure. Rats from both of the abstinence period treatment groups were re-exposed to the drug-paired context, yet many gene expression changes occurred only in the group that experienced extended abstinence (14 days). It is documented that exposure to environments previously paired with drug administration can affect gene expression (Ostrander et al. 2003; Hearing et al. 2008a; Hearing et al. 2008b), so future studies will be
required to discern whether the observed changes in gene expression after 14 days of abstinence resulted from contextual re-exposure, the pharmacological drug abstinence, or a combination.

Several genes whose expression was changed in this study have also displayed changed expression levels following several extinction sessions (Koya et al. 2006). In the Koya et al. study, rats self-administered heroin, using noke-poking as the operant behavior for 15 days, followed by 14 extinction sessions that spanned a 3-week period. In the mPFC, there was an increase in gene expression relative to controls for arc, homer1a, ania-3, MKP-1, c-fos, EGR1, EGR2, and Nr4a3. In contrast to the current study, RGS2 gene expression was reportedly not significantly increased. Homer1, EGR1, and Nr4a3 each were detected by the arrays to have increased expression values of at least 1.4-fold in the current study. The repeated extinction sessions in Koya’s study are a major difference from our study that may contribute to the differences in gene expression observed between studies. Genes that were changed in both Koya’s study, which included 14 extinction sessions, and the present study, which included 1 extinction session, are genes that apparently exhibit changed expression following a prolonged abstinence and maintain that change in expression regardless of the amount of environmental re-exposures. Therefore, such genes represent changes that persist into drug abstinence, preventing a former drug user from returning to a pre-drug use physiological state.
Twenty-one genes identified as significantly changed in the microarray experiment have been investigated in our laboratory using RT-qPCR: one was observed in our previous report of gene expression changes (Kuntz et al. 2008b) and twenty in the present study. Of those genes, 7 were validated by RT-qPCR to be changed at a significance level of at least $\alpha = 0.05$. Reasons for why all 21 genes were not detected as changed by RT-qPCR include differences in sample numbers ($n=6$ for arrays vs. $n=8$ for RT-qPCR), trends towards changed expression that did not reach significance, and low signal intensity for many of the genes that failed to replicate (enhancing the opportunity for detecting false positives). Correlational analyses detected that BDNF was significantly correlated with both goal-directed behavior and active spout responses. This observation supports the proposed importance on BDNF to drug-seeking behavior (Berglind, 2007).

The identities of the confirmed genes include not only transcription factors, but also genes for molecules involved in intracellular signaling and protein binding. The importance of Figure 4 is not merely the illustration that the MAP kinase pathway is a common link between many of the validated genes, but the variety of functions possessed by the molecules encoded by the genes. Some are transcription factors, which are rather expected because of the abundance of literature reporting transcription factor expression that is changed by drug use, but molecules that bind receptors and ions are also included, as are phosphatases. This range of functions provides a reminder that drug use elicits changes in entire intracellular networks. The importance of changes in gene
expression of certain proteins, such as Calb1 or Dusp6, to eliciting changed expression of genes encoding transcription factors, such as EGR1 or Fos, is an area for future investigation.

While this microarray analysis has detected genes whose expression changes have been confirmed by other laboratories, it has also identified novel targets for genomic research investigating incubation. This study has produced data that are relevant not only for future studies involving heroin, but also for understanding the molecular underpinnings of incubation of drug-seeking.
Conclusions

We have identified a group of genes whose expression is significantly changed following abstinence from heroin self-administration and incubation of heroin-seeking behavior. Confirmed genes are not limited to genes encoding transcription factors, but also encompass genes encoding molecules that are important for regulation of intracellular signaling. These regulatory molecules may be effective targets for drug interventions to prevent relapse.

Authors’ Contributions

KLK performed behavioral procedures, data analysis, and drafted the manuscript. RMB performed the microarray experimental procedures. PSG contributed to the behavioral design. WMF contributed to the design of the microarray experiment. KEV participated in the design and coordination of the study. All authors made contributions to drafts of the manuscript and approved the final manuscript.

Acknowledgements

We thank Georgina Bixler for her assistance with the RT-qPCR. This work was supported by DA021450 (KLK), DA13770 (KEV), and DA12473 (PSG).
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Chapter 5

DISCUSSION

Advantages of Combining Behavior and Functional Genomics
Specific genes that are potentially involved in relapse to heroin self-administration have been identified through this research. Establishing a behavioral model of heroin relapse provided the basis for molecular studies of gene expression. The completion of the research described in the preceding chapters has enhanced our understanding of the behavior and molecular neurobiology of heroin relapse. Continued studies of relapse are crucial to understanding the urge to resume drug use in the recovering addict. Regardless of how many drug users are forced from their habits (through incarceration and rehabilitation), the real threat to addicted individuals and the continuing societal threat exist in high rates of relapse. It is widely accepted that following drug abuse a person is invariably changed, both psychologically and physiologically.

Drug addiction is based in the interplay of physiology and psychology. Initial drug use elicits physical changes that partially underlie the psychological drive to engage in future use. Physical adaptations that occur in response to initial drug doses result in the development of tolerance, which requires progressively larger doses to yield the same euphoric experience that initially occurred in response to a smaller drug dose. In fact, the physical adaptations (including neuroadaptations) that occur in response to repeated drug use begin to demand that the drug be administered to prevent negative physical symptoms. It is at this point, when a drug of abuse is no longer used solely to achieve pleasurable feelings, but also to prevent unpleasant physical symptoms, that an individual has become dependent on the drug. Dependency is a hallmark
of addiction and is best characterized by the observation of withdrawal symptoms when the drug is not administered. In humans, symptoms of withdrawal from heroin are similar to a severe flu and include vomiting, diarrhea, chills, runny nose, and watery eyes. Rats exhibit teeth chattering, salivation, wet dog shakes and diarrhea as physical signs of opiate withdrawal, although studies have determined that physical withdrawal and motivational withdrawal (measured by food intake via an operant procedure) are separate measures. Indeed, motivational withdrawal can be observed in the absence of physical withdrawal symptoms (Higgins and Sellers 1994). Although physical signs of withdrawal were not observed in the research described in Chapter 2, the increased drug-seeking behavior observed after 14 days of abstinence can be inferred to indicate an elevation in drug craving, which may be associated with withdrawal. Despite the lack of physical withdrawal symptoms, relapse to drug seeking was successfully modeled in the animals studied, and molecular studies combined with the behavioral research to produce the work presented in this dissertation. Drug relapse is a problem for which a comprehensive approach is particularly well-suited.

Existing literature about drug abuse is dominated by a few drugs, namely alcohol, nicotine, and cocaine. Reasons for this include the demographics of heroin addicts, who often are less wealthy than users of more well-studied drugs, and the low prevalence of heroin use in the United States in comparison to other drugs of abuse (National Survey on Drug Use and Health, 2007). Specifically, 0.2 million persons aged 12 or older reported using heroin in the month
surveyed, compared to 2.1 million reports of cocaine use and 14.4 million reports of marijuana use. Although heroin is currently not the most abused drug in the United States, it is a widely abused drug on a global scale and is growing in popularity among in the United States, especially among youth (Armon 2008; Morera 2008) and prescription drug addicts who seek more affordable drugs to satisfy their cravings (Halpin 2008). The execution of studies about heroin abuse, coordinated with the findings on other drugs of abuse, will ultimately be helpful for understanding the overarching problem of addiction.

**Heroin Incubation**

If relapse to drug use is to be studied in a variety of drugs, a behavioral model that is applicable across a variety of drugs is desirable. Incubation of cocaine-seeking behavior has been reported in several studies (Grimm et al. 2001; Lu et al. 2004; Koya et al. 2008), but is not well-documented for other drugs of abuse. In Chapter 2 of this dissertation, the extension of incubation of drug-seeking behavior to a model of heroin self-administration is demonstrated. Also in this chapter, incubation is documented to occur for both drug-seeking behavior and goal-directed behavior. The discovery that incubation involves an increased focus on a goal occurring alongside increased physical seeking of a drug illustrates how this model is homologous to human drug relapse. Because human relapse is characterized by an increased focus on the goal of obtaining drug, this added knowledge about incubation strengthens the support for this animal model of relapse. In future studies into pharmacological and gene
therapy interventions for drug addiction, using the incubation behavioral model can assess both drug-seeking and goal-directed behavior in rats to determine whether a treatment is merely decreasing overall locomotion and responding, or specifically targeting goal-directed seeking.

**Individual Responsiveness**

Before reaching adulthood, most humans are aware that individual differences exist within their species. Some humans are “big eaters” while others seem to pick at their food. Some enjoy exercise, while others would rather read a book or watch television. It is often surprising for people who are unfamiliar with behavioral research to learn that rats, too, exhibit inter-individual variation. All rats do not express uniform behavior: some have higher intakes than others, both for sweets and drugs. In this study, rats demonstrated considerable individual differences in behavior and drug-intake. Inter-individual variation was a concern that was considered when designing the behavioral experiments, resulting in rats being assigned to triads with the intent of distributing rats that were high spout responders evenly across the treatment groups. Dividing the highest responding rats equally between the treatment groups minimized the confounding variable of inherent high responders. Although differences in gene expression profiles between high responding and low responding rats remain to be elucidated, a reasonable concern is that inherent differences in gene expression exist between the groups. Therefore, ensuring an even behavioral
distribution of rats was essential to minimizing the impact of inherent gene differences on the expression data.

Despite attempts to ensure that all treatment groups contained rats with similar response profiles, individual responses to drug were still highly variable. Pharmacogenomics is a branch of pharmacology that investigates how gene expression levels or genomic sequences impact the potency and efficacy of a drug. Individual differences in gene expression were observed between rats within the same treatment groups, and pharmacogenomics provides a link between variability in activity (such as the large differences in spout-responding in the yoked heroin rats) and variability in gene expression.

**Addiction Neuroanatomy**

Having established a behavioral model of relapse to heroin-seeking, the question of what molecular changes exist within the brain at the time of relapse was addressed. Studies focused on the medial prefrontal cortex (mPFC), a relevant area not only because of its involvement in the mesolimbic dopaminergic system, but also because of its importance in mediating goal-directed behavior. Neurons in the mesolimbic dopaminergic system originate in the ventral tegmental area (VTA), located in the midbrain region of the brainstem, and send axonal projections (gathered in the medial forebrain bundle) towards the forebrain. In addition to the mPFC, dopaminergic projections travel from the VTA to the nucleus accumbens (NAc), amygdala and, to a lesser extent, the dorsal striatum (Weiner et al. 2003). The PFC does not merely receive dopaminergic
projections, but also sends projections to other brain regions, including the NAc and VTA. Anterograde transport allows proteins that are transcribed in one neuronal area to travel within an axon and ultimately reside in a different area of the brain (Smith et al. 1997; Ng et al. 2007). Therefore, gene expression changes occurring in the mPFC have the potential to affect protein expression levels in brain regions to which mPFC neurons project.

Role of Contingency

The contingency with which a drug is administered can produce different physiological responses. When a subject receives drug after expressing a behavior that indicates their desire for the drug, contingent drug-administration occurs. However, drug administration is non-contingent if the behavior of a subject has no effect on when or how often drug is administered. EEG patterns recorded from heroin abusers are larger, in magnitude, when fentanyl (a synthetic opiate prescribed for pain management) is self-administered than when fentanyl is passively administered (Greenwald and Roehrs 2005). Heroin self-administration induces a more significant increase in temperature of both brain and body in rats when compared with rats who receive passively administered heroin (Kiyatkin and Wise 2002). The importance of contingency in determining responses to drugs is further highlighted by behavioral responses and differences in dendritic spine density depending on the contingency with which a drug has been administered in rodents, as well as by imaging studies in humans. Historically, studies of drugs of abuse using animal models have often involved
procedures in which an investigator administers drug to a subject. In this situation, drug receipt is non-contingent and has the potential to elicit intracellular effects that are different from what occurs when drug is self-administered. The gene expression results presented in Chapter 3 illustrate the idea that contingency can determine the gene expression response to a drug of abuse. Each of the genes that were found to have changed expression exhibited contingency-specific changes. While Arc changed only at 1 day and only in self-administering rats, EGR1 gene expression was significantly increased after both 1 and 14 days of abstinence, again only in self-administering rats. These studies have highlighted the importance of contingency when making observations and provide reason to consider the role drug-administration contingency may have played in previously published research. Studies of gene expression changes induced by non-contingently administered drug injections may have identified expression changes that are not applicable to real-life situations when drugs are self-administered. For example, accurate gene expression profiles following drug abuse may have been obscured by false positives. Alternatively, some genes may have expression that is significantly changed by drug self-administration, but unchanged by passively administered drug. The genes would not be detected in non-contingent studies. In both situations, the true gene expression profile following drug use has become obscured.
Regional Gene Expression Comparisons

Examining gene expression levels in three different neuroanatomical locations (mPFC, NAc, and dorsal striatum) revealed that, for the genes examined, more changes in expression occurred in the mPFC in response to the behavioral incubation model. The rats examined in this work had exhibited a high degree of goal-directed behavior just moments prior to their sacrifice, so the finding of a greater quantity of significant changes in gene expression in an area important for behavior compared with neuronal regions that are less involved in behavior is to be expected. Only 1 gene (EGR2) displayed a statistically significant change in expression in the NAc, and no statistically significant expression changes were detected in the dorsal striatum. Interestingly, the patterns of gene expression were similar in the NAc and dorsal striatum for EGR1, EGR2, and Arc, although the EGR2 increase in the dorsal striatum did not reach significance (Appendix B). These two regions both receive dopaminergic afferent projections and exhibit similar increases in dopamine upon cocaine self-administration (D’Souza and Duvachelle, 2006). The absence of widespread gene expression changes detected in the dorsal striatum and NAc in the studies reported within this dissertation does not suggest that the genomic profiles were unchanged in these regions. Rather, it is probable that following drug exposure and environmental re-exposures, several genes in these areas were changed. The genes for which RT-qPCR analyses occurred represent a small portion of the genome, and global analyses on neuronal areas in addition to the mPFC would be the most efficient approach to discover gene expression changes in
those areas. In fact, a microarray approach has been reported to examine gene expression changes within the dorsal striatum following 1 week of cocaine self-administration (Lynch et al. 2008).

**Gene Therapy to Treat Addiction**

Gene therapy has evolved from a fantastical notion to a fledgling treatment choice for disorders in humans (Cideciyan et al. 2008). Negative critics of gene therapy have proclaimed that, while the approach is effective in treating a disorder whose physical effects are localized, problems as complex as psychiatric disorders (including addiction) involve too many genomic changes and neuronal areas to be effective. Just as with more traditional pharmacological approaches, additional studies will be required to determine whether approaches that are effective in animals can translate to human treatments. A gene whose role in addiction has been implicated in both human and rodent studies encodes dopamine D2 receptors (DRD2; Chapter 1). In rats, an upregulation in dopamine D2 receptor gene expression, via intracranial infusions of an adenovirus carrying the D2R gene, in the nucleus accumbens of rats that have been trained to self-administer cocaine attenuates drug-seeking (Thanos et al. 2008). In humans, a mutated dopamine D2 receptor allele is associated with human compulsive behavior, including drug and alcohol addiction (David et al. 2003; Li et al. 2006). While differences in gene sequence are admittedly not the same as differences in gene expression, the expression of a flawed gene sequence ultimately decreases expression of the functional gene. Therefore, replacing the addiction-
associated alleles in humans with wild-type D2 receptor genes may ultimately increase the amount of wild-type receptors and prevent the physiological drive to relapse. For genes that are widely expressed in the body, site-specific manipulation will be crucial. Also, overcoming the psychological relapse component would likely continue to involve therapy.

Fortunately, a behavioral treatment that effectively compliments physical treatments already exists. Contingency management is an approach to addiction treatment in which subjects are provided positive reinforcers for drug abstinence (Carroll and Rounsaville 2007). Although it has some shortcomings, such as the expense of positive reinforcements, and relatively short duration of effectiveness, supplementing pharmacotherapies with contingency management is effective in prolonging drug abstinence. One can envision how an approach in which incentives are offered could motivate former users to overcome unpleasant aspects of gene therapy.

Current gene therapy trials in humans focus on replacing faulty genes. Through the development of these procedures, the challenges involved in gene therapy have become clearer. The blood-brain barrier's isolation of brain cells is a major obstacle to developing gene therapy treatments for the brain. A combination of craniotomy and local viral vector delivery is the approach most often used in animal models. Current neurosurgery techniques for humans render this a possible approach, but the limited location of drug delivery provided through intracranial infusions may negatively affect the efficacy of this approach for treating disorders that involve multiple brain sites. Additionally, viral vectors
can introduce toxicity issues that deem treatments undesirable. Liposomes present a non-toxic method of trans-blood brain barrier small molecule delivery (Schlachetzki et al. 2004). Using liposomal delivery, siRNAs can be administered intravenously and delivered throughout the brain. In this approach, however, liposomes are delivered throughout the body. Therefore, site-specific promoters must be incorporated into the small-molecule delivered so that transcription of the delivered gene will only occur in appropriate areas within the body. If gene therapy still seems an improbable treatment for addiction, it should be considered alongside the role of memory in addiction.

**Role of Learning and Memory in Addiction**

Often, drug addiction research is considered to have relevance primarily confined to drug addiction studies. However, learning and memory play a crucial role in addiction, especially during relapse to drug use. Therefore, the behavioral model of incubation is useful in studying not merely relapse, but also memory. The incubation of goal-directed behavior reported in Chapter 2 is an example of this broad relevance. The finding that goal-directed behavior incubates with the passage of time suggests that the memory for obtaining drug becomes more pronounced as time passes since the last contextual, drug-associated, exposure. Neuroscience research has identified a physical process that describes memories as something beyond abstract thoughts in one’s mind. Through rodent studies, the necessity of protein synthesis for long-term memory formation has been well-documented (Schafe and LeDoux 2000; Nader et al. 2000). Memory
storage is not a one-way process: after a memory has moved from short-term memory to long-term memory through a process termed “consolidation”, future recall of that memory retrieves it from long-term memory storage. Following memory retrieval, a second process, termed “reconsolidation,” stores the memory once again (Figure 1). In addition to protein synthesis, synthesis of mRNA is required for both consolidation and reconsolidation (Duvarci et al. 2008). Notably, two of the genes identified to have changed expression in

![Figure 1 – Schematic of memory storage process.](image)

Chapter 3 and 4 are important in the memory storage process. BDNF is essential for consolidation, but not reconsolidation. On the contrary, EGR1 is essential for reconsolidation, but not consolidation (Lee et al. 2004; Monfils et al. 2007; Ou and Gean 2007). In the present work, both of these genes displayed
heightened expression upon contextual re-exposure and drug-seeking, with BDNF gene expression being significantly increased compared to saline controls only after 14 days of abstinence, while EGR1 gene expression was significantly increased above saline controls after both 1 and 14 days of abstinence (Chapters 3 and 4). BDNF’s differential expression between time points may provide a clue about its role in relapse. Following extinction at both 1 and 14 days, the contextual memories and the behavioral drug-seeking memory undergo reconsolidation, so observing increases in EGR1 gene expression at both time points corroborates its role in reconsolidation. However, the importance of BDNF to consolidation, rather than reconsolidation, suggests that following 14 days of abstinence, the extinction session may be accompanied by the formation of a new memory. Because goal-directed behavior is more defined after 14 days of abstinence than 1 day of abstinence, different values of learning may be assigned to the extinction session depending on whether it occurs after 1 or 14 days of abstinence. After 1 day of abstinence, the drug-seeking memory has only been stored for a short time and extinction behavior merely modifies that memory. After 14 days of abstinence, the memory has incubated and become very pronounced. The inability to obtain drug in the context of the self-administration chamber may be a distinct experience from the initial drug-seeking event and result in the formation of an entirely new memory.

Memories are important in the lives of all humans, regardless of their history of drug use. Those who suffer from post traumatic stress disorder (PTSD) are plagued by memories of terrifying events. Memories can also
contribute to depressive and anxiety disorders. Currently, methods based on extinction are used to help subjects overcome these memories. Such techniques, especially the virtual reality treatments that currently exist for PTSD treatment, provide situations in which memories are retrieved. If gene interventions were applied at the time of memory retrieval, the negative memory would fail to undergo reconsolidation and essentially be lost.

Rodent gene manipulation studies have demonstrated that gene interference can, in fact, inhibit reconsolidation. Gene manipulation studies suggesting that EGR1 is an important gene for addiction are discussed in Chapter 1. In those studies, reinstatement of drug-seeking behavior is blocked by antisense RNA. Gene manipulation and behavioral studies have also combined to examine the importance of another gene, beta-catenin, in memory formation (Maguschak and Ressler 2008). Site-specific and time-specific deletion of Ctnnb1, the gene encoding beta-catenin, in the amygdala of mice that have experienced fear-conditioning suggests that mice with beta-catenin gene deletions are impaired in their ability to form long-term memories. Ctnnb1 was identified by the microarray results reported in Chapter 4 to be significantly changed, although RT-qPCR did not validate this change.

**Correlational Analyses**

Considerable inter-individual variations existed not only in reference to behavior, but also for gene expression. Correlational analyses between the genes examined in Chapter 3 (Arc, cdk5, Cryab, EGR1, EGR2, Fos, Homer 1
b/c, Homer 1 pan, Nr4a1, and Per2), Chapter 4 (Dusp5, Dusp6, RGS2, BDNF, Calb1, and NPY) and behavior were performed (Appendix A). Although the small group sizes limited the statistical power of the analyses, a few statistically significant correlations were detected. Specifically, significant correlations were detected between various behavioral measures and gene expression of EGR1, Fos, Nr4a1, Homer 1 b/c, Homer 1 pan, Cryab, BDNF, Dusp5, and NPY (Chapters 3 and 4).

Figures 2, 3, 4 and 5 provide comprehensive views of gene expression results associated with correlational results for each of the genes for which a change in expression was detected. In each table, an arrow indicates the direction of change in gene expression when compared with yoked saline controls. Large boxes depict Pearson’s correlation values and inset boxes depict p-values of significance. For each behavior-gene expression comparison, the data in the top box results from a correlation involving behavioral data from the final 2 days of training and the bottom box contains results from a correlation involving behavioral data from the extinction session.
Figure 2 - Chapter 3 Gene Correlations After 1 Day of Abstinence. Correlation values (large boxes) and significance values (inset boxes) for comparisons between behavioral measures and gene expression in the mPFC for self-administering rats after 1 day of abstinence from heroin self-administration. Arrows to the left of the boxes indicate the direction of gene expression change compared with samples from yoked saline rats, as indicated by qRT-PCR. NC indicates no change in gene expression, and colored boxes with asterisks denote comparisons with p-values < 0.05. For each behavior-gene expression comparison, the data in the top box results from a correlation involving behavioral data from the final 2 days of training and the bottom box contains results from a correlation involving behavioral data from the extinction session.
Figure 3 - Chapter 4 Gene Correlations After 1 Day of Absinence. Correlation values (large boxes) and significance values (inset boxes) for comparisons between behavioral measures and gene expression in the mPFC for self-administering rats after 1 day of abstinence from heroin self-administration. Arrows to the left of the boxes indicate the direction of gene expression change compared with samples from yoked saline rats, as indicated by qRT-PCR. NC indicates no change in gene expression, and colored boxes with asterisks denote comparisons with p-values < 0.05. For each behavior-gene expression comparison, the data in the top box results from a correlation involving behavioral data from the final 2 days of training and the bottom box contains results from a correlation involving behavioral data from the extinction session.
Figure 4 - Chapter 3 Gene Correlations After 14 Days of Abstinence. Correlation values (large boxes) and significance values (inset boxes) for comparisons between behavioral measures and gene expression in the mPFC for self-administering rats after 14 days of abstinence from heroin self-administration. Arrows to the left of the boxes indicate the direction of gene expression change compared with samples from yoked saline rats, as indicated by qRT-PCR. NC indicates no change in gene expression, and colored boxes with asterisks denote comparisons with p-values < 0.05. For each behavior-gene expression comparison, the data in the top box results from a correlation involving behavioral data from the final 2 days of training and the bottom box contains results from a correlation involving behavioral data from the extinction session.
**Figure 5 - Chapter 4 Gene Correlations After 14 Days of Abstinence.** Correlation values (large boxes) and significance values (inset boxes) for comparisons between behavioral measures and gene expression in the mPFC for self-administering rats after 14 days of abstinence from heroin self-administration. Arrows to the left of the boxes indicate the direction of gene expression change compared with samples from yoked saline rats, as indicated by qRT-PCR. NC indicates no change in gene expression, and colored boxes with asterisks denote comparisons with p-values < 0.05. For each behavior-gene expression comparison, the data in the top box results from a correlation involving behavioral data from the final 2 days of training and the bottom box contains results from a correlation involving behavioral data from the extinction session.
After 14 days of abstinence, Fos is correlated with each of the three behavioral measures. These correlations do not exist for rats that experienced only 1 day of abstinence prior to the extinction session. Therefore, Fos gene expression may change during the time in which drug-seeking incubates to provide an indication of past drug-seeking behavior, or it may undergo a rapid change in expression upon re-exposure to cues. It is important to note that because group sizes for correlational analyses were small (n= 7 or 8), individual data points that appeared to be outliers strongly influenced the data and, in some cases, resulted in significance for correlations.

**Future of Genomics Research**

Epigenetics is an emerging field that examines how changes in the structure of DNA and chromatin within the nucleus elicit changes in gene transcription (Colvis et al. 2005). Gene expression within an organism is essentially regulated by epigenetics. As depicted in Figure 6, the methylation state of portions of DNA affects how tightly chromatin is wound around histones. Methylation promotes tight winding around histones, and therefore prevents transcription. Because transcription is only possible for chromatin segments that are not tightly wound around histones, epigenetic changes are precursors to changes in gene expression for molecules who function throughout the cell. Epigenetic modulation provides a link between environmental influences and gene expression (Jirtle 2008) and therefore is undoubtedly affected by drug use. Improving the understanding of epigenetic modulation will establish a mechanism
that produces drug-induced changes in gene expression, such as the ones described in the present work. The usefulness of such knowledge to the development of pharmacological treatments is probable, although the intermediate steps remain to be elucidated.

**Study Improvements**

As with most scientific studies, in hindsight there are procedural changes that would have eliminated some confounding variables and answered questions that have arisen from this study. Although many precautions were taken to minimize group differences (such as balancing rats’ body weight and water intake), the effect of conducting self-administration training during different months was not considered. The behavioral study for rats that experienced 1
day of abstinence prior to extinction occurred 2 months before the start of the behavioral study for rats that experienced 14 days of abstinence. Although the amount of training chambers necessitated the study being divided into 2 parts, if half of the rats from each abstinence period treatment group were included in each of the 2 behavioral trials, questions about whether gene changes were the result of abstinence period or some other factor (such as seasonal physiology changes) would have been eliminated. Sacrificing some rats without them first experiencing extinction would have provided insight about which of the gene expression changes we observed were attributable to the contextual re-exposure. This is especially likely because the majority of genes inspected were immediate early genes (IEG) and the 90-minute period was more than sufficient for changes in gene expression to occur. The impact of the 90-minute extinction session on gene expression could be assessed by replicating the behavioral procedures and introducing additional groups for each time point that are sacrificed without experiencing an extinction session. Adding the additional groups seems like a logical approach, and one might ask why the experiment was not initially designed without the groups, or why the extinction session was added? The extinction session was initially included because an attempt was made to record ultrasonic vocalizations (USV) from rats. It is documented that rats emit USVs in response to both distress and pleasure. Vocalizations around 20 kHz are elicited by aversive stimuli (startling stimuli, intruders) while vocalizations around 55 kHz are emitted in pleasurable situations (feeding, sexual behavior). A difference in USV intensity between self-administering and
yoked heroin rats was expected. Furthermore, the extinction session was expected to evoke aversive USVs in self-administering rats that had previously emitted pleasurable USVs when receiving drug infusions. Unfortunately, a technical problem prevented the USV data from being recorded. The approach of correlating behavior with gene expression changes has great potential, and larger groups of rats in each treatment group would have likely increased the correlation coefficients that were detected and yielded higher levels of significance.

Considering these suggestions for improvements naturally gives rise to several ideas for future studies. Performing studies involving gene interference, and examining the feasibility of these studies in humans is a lucrative area for possibilities. Interference studies of EGR1 have already been performed, and suggested to effectively prevent memory reconsolidation, but the locations of these studies have been limited. The amygdala has been the area of focus for EGR1 gene intervention studies, so it will be important to know whether EGR1 inhibition in other neuronal areas has similar effects. The mPFC may be an area in which memory disruption is also possible because of its role in memory formation. Inhibition of additional genes, such as BDNF, may also be effective in disrupting reconsolidation. Further development of pharmacological agents to deliver treatments site-specifically will be beneficial for many diseases and disorders. The approach of combining pharmacotherapies with behavioral therapies holds a lot of promise for relapse prevention.
Importance of Gene Expression Research

Advances in genomic research during the past decade have been accompanied by other advances in scientific research, such as proteomics and epigenetics. One might ask why researchers continue to examine gene expression when it is problems exist on organismal levels and can be seen in protein expression. Such a question is similar to asking why one studies cells when diseases are visible in entire organisms. Examining the starting point of physiological disorders holds the promise of combating problems at their source and efficiently eliminating the disorder in its entirety. Without examining the most basic level at which disorder-induced changes occur, treatments at more complex levels may be futile.

The importance of gene expression is well-known to proponents of personalized medicine, a fledgling concept that takes an individual’s genetic composition into consideration when prescribing treatments. Gene expression underlies protein expression, and knowledge of gene expression profiles that exist in disease states provides information about the causes of macroscopic changes that are visible on the level of protein expression or even behavior. When designing treatments, it may be preferable to intervene at the source of an affliction than at a superficial level. Lasting changes are accomplished by intervening at the source, and relapse to drug use is an excellent example of where treatments focusing primarily on behavior or physical conditions fail to truly rectify the problem.
Scientific Contribution of This Dissertation

The contributions of this research include the addition of information concerning the genomic underpinnings of heroin relapse, as well as distinguishing the purely pharmacological effects on gene expression from changes in gene expression that occur in voluntary drug-taking situations. Specific findings of gene expression changes are summarized in Figure 7.

<table>
<thead>
<tr>
<th></th>
<th>Arc</th>
<th>EGR1</th>
<th>EGR2</th>
<th>Fos</th>
<th>Homer 1 b/c</th>
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<th>Calb1</th>
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<td>+</td>
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<td>nc</td>
<td>nc</td>
<td>+</td>
<td>-</td>
<td>nc</td>
<td></td>
</tr>
<tr>
<td>Self-administration Incubation only (14 day)</td>
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<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

Figure 7 - Summary of gene expression changes reported in this dissertation.

As discussed in earlier chapters, the gene profile that exists upon expression of incubation of drug-seeking highlights specific genes that may be optimal targets for relapse prevention. Knowledge of the drug relapse model of incubation has been expanded to include heroin-seeking and goal-directed behavior. The gene expression research performed following the behavioral work has demonstrated how behavioral and molecular work can be combined to maximize the information yielded from an experiment. Combining behavioral and gene expression data has added relevance to the changes in gene expression, beyond merely reporting gene expression levels that exist after 14 days of abstinence. The correlational analyses suggest that gene expression profiles may be affected by drug-seeking behavior, and the global analysis of gene expression determined that several genes that function in nervous system development are changed in
the mPFC following extended abstinence and re-exposure to a drug-associated environment. Figure 8 presents a visual summary of the research included in this dissertation. Both behavioral and molecular data are included to emphasize that macroscopic views are essential for tackling a problem as complex as drug addiction.
Figure 8 – Summary of dissertation data.
Reference List


Morera,N. Heroin use rising dramatically among area youth. The Buffalo News.
8-29-2008.


Appendix A: Correlational Studies

Correlational analyses, included in this appendix, were only partially represented in Chapters 3 and 4. Therefore, a more complete description of correlation data are presented within this appendix.
Rationale: The large amount of data collected by this dissertation research provides opportunity for higher-level analyses. Individual differences existed in both behavioral data and gene expression data, so correlational analyses were performed to determine whether any of the genes on which quantitative real-time PCR were performed were significantly correlated with behavior.

Approach: Data from the behavioral and gene expression experiments were entered into a spreadsheet and analyzed using Sigma Stat. Pearson's correlational analyses were performed on all data. In addition to the Pearson analysis, Spearman’s correlational analyses were performed on gene expression data reported in Chapter 4. Both of these analyses assess the strength and direction of a linear relationship between two variables. Pearson's correlational analysis is a parametric measure that is most appropriate for normally distributed data while Spearman’s correlational analysis is a non-parametric test that can be used when data are not normally distributed.

Data:
Table 1 depicts behavioral variables within this research. Correlational analyses were performed to investigate relationships between different behavioral variables and relationships between behavior and gene expression.
Analyses that compared goal-directed behavior and active spout responses during either the final 2 days of acquisition or the extinction session demonstrates that the 2 behavioral measures are positively correlated (Figure 1). That is, the more responses that were made on the active spout, the more goal-directed was the behavior. Data from the final 2 days of acquisition are remarkably highly correlated (when an aberrant high responding rat is removed from the analysis), while the correlation from the extinction session, while still significant, is not as strong.

Responding of a rat during acquisition does not appear to predict responding levels during the extinction session (Figure 2). Rats who were the highest responders during acquisition, either for active spout responses or goal-directed behavior, were not necessarily the highest responders during extinction.
Figure 1 – Behavioral correlations. Graphs depicting correlations between the 2 behavioral measures (active spout responses and goal-directed behavior) during the final 2 days of acquisition (top panel) and the extinction sessions (bottom panel).
Figure 2 – Acquisition vs. extinction correlations. Graphs depicting correlations between acquisition and extinction responding on the active spout (top panel) and goal-directed behavior (bottom panel).
Behavior Median Splits

Median splits were performed to discern whether subgroups of rats influenced the behavioral data. The two self-administering groups (1 day abstinent and 14 day abstinent) each contained 8 rats, so each of these groups were divided into smaller groups containing 4 rats, and the groups were termed “high responders” and “low responders.” Median splits were generated using 4 different approaches to create the divisions: the average responding during the final 2 days of acquisition and extinction, average responding during the extinction session, average goal-directed behavior during the final 2 days of acquisition, and average goal-directed behavior during the extinction session.

Interestingly, after 1 day of abstinence only half (n=2) of the rats who expressed the highest active spout responses during the final 2 days of acquisition were included in the group of rats who expressed the highest active spout responses during the extinction session. After 14 days of abstinence, only 1 rat who was a high responder during the final 2 days of acquisition was also a high responder during the extinction session. A median separation of rats, with the split based on average responding during the final 2 days of acquisition, showed statistically significant differences in self-administration behavior (licks on the active spout) when extinction occurred after 14 days of abstinence (p=0.04). When the median split was generated based on average responding on the active spout during extinction, a significant difference in responses on the active spout exists between high and low responders after 1 day (p=0.012) and 14 days (p=0.016) of abstinence. When the median split is based on goal-directed
behavior during the extinction session, significant difference between groups only at 1 day (p=0.045). Although the extinction session was the endpoint of the behavioral study, examining data from the self-administration period, termed “acquisition”, provides insight into whether statistically significant behavioral phenotypes existed during self-administration. A median split based on average active spout responding during the final 2 days of acquisition yields a significant difference between high responders and low responders that went on to experience 1 day of abstinence (p=0.037). An extremely high-responding rat in the group of rats that went on to experience 14 days of abstinence results in the data failing to pass a normality test and requiring a Mann-Whitney rank sum test to be performed. This test indicates that a significant difference exists between the 2 groups of responders (9=0.029). A median split based on goal-directed behavior during the final days of acquisition detects a significant difference after 1 day (p=0.037) and 14 days (p=0.029).

**Analyses of Gene Expression Data Using Behavioral Median Splits**

Data from median splits (highest 4 rats vs. lowest 4 rats) was analyzed for both behavior and gene expression. Unfortunately, difficulties in obtaining cDNA that was optimal for RT-qPCR from all 8 samples at each time point prevented gene expression data from being obtained from all rats. No significance was detected between gene expression in high responding vs. low responding rats, which is unsurprising because of the small and uneven group sizes used for these analyses.
Correlational Analyses Between Behavior and Gene Expression

Correlation coefficients and levels of significance were determined by a Pearson’s correlation analyses for comparisons between the gene expression data in Chapter 3 and behavioral measures during the final 2 days of acquisition (Figure 3) and extinction (Figure 4). The analysis revealed relatively few significant correlations. As was mentioned in Chapter 3, a single outlier appeared to be responsible for the correlations that were detected as significant. The outlier in the top panel of Figure 2 provides an example of the strong influence one data point can have on a correlation. In that case, removing a single data point results in a correlation coefficient shifting from a negative to a positive value.

Validated genes reported in Chapter 4 were correlated with active spout responding and goal-directed behavior during extinction (Figure 5).
Brain Region: medial prefrontal cortex  
Behavioral Session: Average of Final 2 Self-Administration Days  
Treatment: Self-Administering

**P-value (Correlation Coefficient)**

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<th>Arc</th>
<th>cdk5</th>
<th>cry ab</th>
<th>EGR1</th>
<th>EGR2</th>
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<th>Homer1 pan</th>
<th>Nr4a1</th>
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<tr>
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<td>0.95 (-0.03)</td>
<td>0.48 (0.30)</td>
<td>0.62 (-0.10)</td>
<td>0.42 (0.34)</td>
<td>0.73 (0.15)</td>
<td>0.84 (0.08)</td>
<td>0.59 (0.23)</td>
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<tr>
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<td>0.07 (0.67)</td>
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<td>0.94 (-0.03)</td>
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<td>0.72 (0.15)</td>
<td>0.83 (0.09)</td>
<td>0.43 (0.32)</td>
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<th>EGR2</th>
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<td>0.19 (-0.56)</td>
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<td>0.53 (0.29)</td>
<td>0.37 (0.41)</td>
<td>0.27 (-0.48)</td>
<td>0.46 (0.34)</td>
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<td><strong>Active Spout</strong></td>
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<td>0.68 (0.12)</td>
<td>0.17 (-0.37)</td>
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**Figure 3 – Chapter 3 acquisition correlations.** Correlations between behavioral measures and genes examined in Chapter 3.
Brain Region: medial prefrontal cortex
Behavioral Session: Extinction
Treatment: Self-Administering

## Abstinent Period: 1 day

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<td>0.18</td>
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## Abstinent Period: 14 days

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<td>0.61</td>
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<tr>
<td>Goal-Directed</td>
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## Combined Data From Both Abstinent Periods

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**Figure 4 – Chapter 3 extinction correlations.** Correlations between behavioral measures and genes examined in Chapter 3.
Figure 5 – Chapter 4 extinction correlations. Correlations between behavioral measures and genes in Chapter 4 that were confirmed to be changed.
**Discussion:** The analyses between behavioral measures have added depth to the understanding of incubation of heroin-seeking. Behavioral correlations show that the rats with highest active spout responding also had highest amount of goal-directed behavior. This importantly shows that high-responding rats were not indiscriminately responding on spouts. The more robustly a rat responded on the active spout, the more it discriminated between spouts. Active spout responding and goal-directed behavior is very highly correlated during the final acquisition sessions and, although still significant, less highly correlated during the extinction sessions. This drop in correlational strength may indicate that individual rats differ in their abilities to retain either memory for the active spout or motivation to seek drug.

The large number of genes examined in Chapters 3 and 4, in combination with the multiple behavioral measures that were collected, provides ample opportunity for correlational analyses. Correlations between behavior and gene expression that were found to be significant are reported in Chapters 3 and 4.

Because of the inter-individual variability that exists between rats (Chapter 5), correlational analyses were conducted to compare the behavior and gene expression of high responding versus low responding rats. Dividing data into these 2 groups is referred to as a median split analysis. Analyses conducted solely on behavioral data had the advantage of always having 4 data points in each responding group. In analyses performed using gene expression data, fewer data points were available because one sample failed to yield cDNA that
was useful in RT-qPCR. Therefore, many of the median split analyses contained 4 samples in one group and 3 samples in the other group.

An analysis of gene expression between high responding rats compared with low responding rats revealed that, in some cases, elevated gene expression levels detected in the overall treatment group are attributable to a subset of rats. For example, EGR2 gene expression was significantly higher in the heroin treated rats that were yoked to low responders than in the rats yoked to high responders (p<0.05). Per2 and cdk5 gene expression were higher following one day of enforced abstinence in high responding self-administering rats and their yoked heroin counterparts (p<0.02; p<0.001), although the difference in gene expression between treatment groups did not reach statistical significance when an overall analysis was performed.

A major challenge to drug design research is the decision about which gene(s) on which to focus study. The approach of correlating gene expression with behavior presents the opportunity of locating a gene whose expression is indicative of behavior. High expression of a gene that predisposes one to compulsive behavior may allow that individual to take extra precautions. In cases in which gene expression provides a record of past behavior, examining gene expression may be a more definitive approach to determine the past behavior of a person who is prone to lying (as, unfortunately, is often the case for drug addicts). In both situations, gene expression analysis provides a possibility for enhancing known information about an individual.
Appendix B: Dorsal Striatum Gene Expression
Rationale: The gene analyses presented in Chapter 3 were limited to two neuronal regions: the medial prefrontal cortex (mPFC) and the nucleus accumbens (NAc). In an effort to obtain a more global picture of gene expression within the brain, quantitative real-time PCR was performed to examine expression of genes in the dorsal striatum. The specific genes examined were EGR1, EGR2, and Arc.

Approach: RNA was isolated from dorsal striatum samples that were harvested at the same time and from the same rats as the mPFC and NAc. The isolation procedure is described in Chapters 3 and 4. Immediately following the 90-minute extinction session, all rats were sedated using Propofol (10mg/kg) and decapitated. Brains were rapidly removed from skulls, 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 2.2mm was cut along the forceps minor and the cortex medial to this cut was collected (medial prefrontal cortex, mPFC). This includes the cingulate, prelimbic cortex, and medial orbital cortex. Following dissection, the tissue was placed in prechilled tubes, immediately frozen on dry ice, and then stored at -80°C.

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).
cDNA synthesis was performed on total RNA using Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA). 1µg RNA, 500ng Oligo (dT), and 10mM each dNTP, were incubated for 5 minutes at 65° C and then chilled on ice for 2 minutes. 5X First Strand Buffer (250mM Tris-HCL (pH8.3), 375mM KCL, and 15mM MgCl₂), 5mM DTT (final concentration), 40 U RNaseOut, and 200 U Superscript III RT were then added. The 20µl reaction was incubated for 60 minutes at 50° C followed by a final incubation at 70° C for 15 minutes 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.
Figure 1 - EGR1 Gene Expression. In dorsal striatum, t-test of YS vs. SA at 1 day: $p < 0.04$
Figure 2 – EGR2 Gene Expression. Significant differences were detected by an ANOVA of YS vs. YH in NAc (p < 0.03), ANOVA of YSW vs. YHW in NAc (p < 0.01), and t-test of YSW vs. SAW in NAc (p < 0.01).
Figure 3 – Arc gene expression. No significant differences were detected for Arc gene expression in either the NAc or dorsal striatum.
Discussion: Expanding gene expression analyses to another brain region has provided additional information about a few of the genes that were reported in Chapter 3 to be significantly changed in rats treated with heroin compared to saline controls. The dorsal striatal region was a particularly interesting area to investigate because it has been reported that inhibition of the dorsal striatum via a combination of baclofen and muscimol attenuates cocaine-seeking that occurs following 2 weeks of abstinence (See et al. 2007). Although no studies investigating the effect of inhibition of the dorsal striatum on heroin-seeking, the generalization of incubation behavior between cocaine-seeking and heroin-seeking that was established Chapter 2 suggests that inhibition of the dorsal striatum may produce a similar attenuation for heroin-seeking behavior. Furthermore, a role of the dorsal striatum in addiction has been emerging with studies highlighting the involvement of this brain region, traditionally thought of as having a primary role in motor function, to the learning of habitual tasks associated with addiction (Knowlton et al. 1996; Packard and Knowlton 2002).

One facet of common knowledge in drug addiction research is that most drugs of abuse enhance dopamine levels in the mesolimbic dopaminergic pathway. A study designed to compare dopamine levels in rat nucleus accumbens and dorsal striatum immediately following a self-administered cocaine infusion determined that dopamine is higher in the NAc than dorsal striatum under normal conditions, although the magnitude of increase in dopamine elicited by cocaine self-administration is comparable between the two regions (D’Souza and Duvauchelle, 2006). The NAc and dorsal striatum possess
similarities in neurotransmitter content and cytoarchitecture, with over 90% of cells in each region being GABAergic medium spiny neurons (Gerdeman et al. 2003). These two regions both receive dopaminergic projections, although the VTA projects almost exclusively to the NAc rather than the dorsal striatum (Joel and Weiner 2000). Dopaminergic input to the dorsal striatum arises largely from the substantia nigra.

Gene expression results from the RT-qPCR analyses of EGR1, Arc, and EGR2 are remarkably similar in both the nucleus accumbens and dorsal striatum. One-way ANOVAs were performed at each time point to compare gene expression in the three treatment groups (yoked saline, yoked heroin, self-administering), and the only case in which significance was detected in both the dorsal striatum and nucleus accumbens was for EGR2 after 14 days of abstinence and an extinction session. Gene expression in rats that received yoked infusions of heroin was significantly higher than in rats that received yoked infusions of saline (p<0.02). Although significance was not detected in any of the other comparisons, the pattern, after 14 days of abstinence, of heightened gene expression in yoked heroin rats compared to the two other treatment groups is evident in both brain regions for all 3 genes. This may indicate that rats who receive heroin non-contingently experience neuroadaptations during withdrawal that are distinct from rats that self-administer drug. Such a finding may be important for understanding narcotics dependencies that develop following recreational drug use compared with medical prescription of narcotics. Perhaps the increased likelihood of developing a drug dependency following recreational
drug use is partly attributable to differences in drug-induced changes in gene expression.

Reference List


CIRRICULUM VITAE

EDUCATION:
Pennsylvania State University College of Medicine, Hershey, Pennsylvania
August 2003: Huck Institute for Life Sciences, Neuroscience Ph.D. Program

East Tennessee State University, Johnson City, Tennessee
Bachelor of Science degree in Biology, minor in Humanities, University Honors Scholar, Magna cum Laude (May 2002)

PUBLICATIONS:


AWARDS AND HONORS:
National Institutes of Health Predoctoral Fellow (August 2006 – present)
Intercollege Graduate Student Outreach Achievement Award (2008)
NIDA Director’s Travel Award (2007)
Committee on Women in Neuroscience Travel Award (2006)

TEACHING EXPERIENCE:
Adjunct Professor, Elizabethtown College, Psychology Department (2007-2008)
Student Lecturer, Penn State Pharmacology Department (2005, 2007 – 2008)
Neuroanatomy Laboratory Teaching Assistant (Spring 2006)