

NOVEL ROLES FOR CYCLIN-DEPENDENT KINASE INHIBITOR 1a IN
COCAINE-INDUCED RESPONSE AND MOTIVATED BEHAVIORS

by

NATALIE ELIZABETH SCHOLPA

(Under the Direction of Brian S. Cummings)

ABSTRACT

Although the neurobiological mechanisms of cocaine are well studied, the extent of cocaine's influence on the body has not been fully characterized. Similarly, the mechanisms by which cocaine induces these effects are not completely understood. This series of studies addressed this deficit by testing the hypothesis that cocaine induces alterations in hippocampal p21 expression via epigenetic mechanisms, and these alterations mediate cocaine-induced response. Both experimenter-administered, non-contingent and self-administered, contingent cocaine exposure increased p21 expression in the brain of rodents exclusively in the ventral sector of the hippocampus. Further, cocaine increased histone 3 acetylation of the p21 promoter region specifically in the ventral hippocampus, correlating to the increased expression observed. Suggestive to its wide breadth of physiological effects, cocaine also significantly altered the blood lipidome of mice, particularly affecting sphingolipids.

We utilized a knockout mouse model to determine the role of p21 on drug-induced response. Genetic ablation of p21 resulted in altered cocaine-induced

behavior in two separate assays. *p21*^{-/-} mice portrayed a decreased locomotor response to an initial cocaine exposure, as well as increased cocaine-induced conditioned place preference and κ -opioid-induced conditioned place aversion. The effects of p21 also extended to the blood lipidome, as the abundance of several lipid species involved in various neurological processes significantly differed between the two mouse models. *p21*^{-/-} mice also expressed higher levels of NeuN and DCX, two markers of neurogenesis, in the dorsal hippocampus compared to wild-type mice. While the behavioral differences observed between the two models could be attributed to this increased neurogenesis and, subsequently, enhanced cognitive ability, these inherent differences between *p21*^{-/-} and wild-type mice could have implications reaching beyond addiction.

The data presented here demonstrate the novel findings that cocaine increases p21 expression in the brain region-specifically, correlating to alterations in the epigenetic status of p21. We observed that mice lacking functional p21 respond differently to cocaine exposure. Moreover, basal differences in neurogenesis and lipid profile between *p21*^{-/-} and wild-type mice suggest a more comprehensive role for this cyclin-dependent kinase inhibitor in neuronal functions.

INDEX WORDS: p21, cocaine, ventral hippocampus, locomotor sensitization, conditioned place preference

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ABBREVIATIONS

5-HT = serotonin

5-mC = 5-methylcytosine

ANOVA = analysis of variance

ASK1 = apoptosis signal-regulating kinase 1

CA = cornu ammonis

CAK = Cdk activating kinase

Cdk = Cyclin-dependent kinase

Cdkn1a = Cyclin-dependent kinase inhibitor 1a

CEBI = Chemical Entities of Biological Interest

ChIP = chromatin immunoprecipitation

Cip = Cdk-interacting protein

CPP = conditioned place preference

CS = conditioning stimulus

DCX = doublecortin

DNMT = DNA methyltransferase

ESI-MS = electrospray ionization-mass spectrometry

EZH2 = enhancer of zeste homologue 2

FR-1 = fixed ratio schedule-1

HAT = histone acetyltransferase

HDAC = histone deacetylase

HMDB = Human Metabolome Database

IDP = intrinsically disordered protein

Ink = inhibitor of Cdk

iPLA2 = calcium-independent phospholipase A2

JNK = c-Jun N-terminal kinase

KEGG = Kyoto Encyclopedia of Genes and Genomes

Kip = kinase inhibitor protein

LMS = locomotor sensitization

MOZ = monocytic leukemia zinc finger

MT = mutant

NAc = nucleus accumbens

NSC = neural stem cells

NSDUH = National Survey on Drug Use and Health

p53-RE = p53-responsive element

PCNA = proliferating cell nuclear antigen

PLS-DA = partial least squares-discriminant analysis

Prc2 = polycomb-repressive complex 2

PUFA = polyunsaturated fatty acid

qPCR = quantitative polymerase chain reaction

Rb = retinoblastoma

S1P = sphingosine-1-phosphate

WT = wild-type

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Cyclin-dependent kinase inhibitor 1a (p21)

p21 was identified as the first Cyclin-dependent kinase (Cdk) inhibitor in 1993, leading to one of its many designations: cyclin-dependent kinase inhibitor 1a (Cdkn1a) (Cmielova and Rezacova 2011). It was originally discovered as part of a quaternary complex in human fibroblasts consisting of cyclinD1, a Cdk, proliferating cell nuclear antigen (PCNA) and p21 (Yoon et al. 2012). With a molecular weight of 20 kDa, p21 is a polypeptide consisting of 164 amino acids. The *Cdkn1a* gene is located on chromosome 6 (6p21.2) in humans, though this location varies between species (**Table 1.1**). Courtesy of alternative promoters and splicing, this gene codes not only for p21, but also for a smaller protein of 132 amino acids, p21B. p21 inhibits several different Cdks including Cdk1, Cdk2, Cdk3, Cdk4 and Cdk6, all of which are directly involved in cell cycle phase transitions. There are two families of Cdk inhibitors: the “inhibitor of Cdks (Ink)” family, which specifically bind Cdk4 and Cdk6, and the “kinase inhibitor protein (Kip)” or “Cdk-interacting protein (Cip)” family, which bind and inhibit a wider range of Cdks. p21 is a member of the latter family and is, therefore, also known as Kip1/Cip1 (Cmielova and Rezacova 2011; Jung et al. 2010; Lu and Hunter 2010). In addition to its inhibitory properties, p21 is also a direct target of the

tumor suppressor p53 (Jung et al. 2010). After the discovery of its relationship with p53, p21 was also termed “wild-type p53 activated factor,” or Waf1 (Cmielova and Rezacova 2011). p21 was initially considered a negative regulator of cell cycle and a tumor suppressor. However, p21 is now implicated in many different biological functions, including cell cycle regulation, cell death, DNA repair, senescence and aging (Romanov et al. 2012).

Role of p21 in Cell Cycle

Activation of Cdk/cyclin complexes drives cell cycle progression. The Cip family of proteins, including p21, inhibits Cdk activity at multiple cell cycle checkpoints (Yoon et al. 2012), without altering Cdk protein levels (Lu and Hunter 2010). The inhibitory pathways of nuclear p21 are outlined in **Figure 1.1**. The G1/S phase transition is critical to DNA replication, and the checkpoint between these two phases is responsible for preventing the replication of damaged DNA (Cmielova and Rezacova 2011). Prior to the G1/S phase checkpoint, retinoblastoma (Rb) protein is bound to and inactivating E2F transcription factors (Jung et al. 2010). Cdk4,6/cyclinD complexes phosphorylate Rb protein in early G1 phase, and during the end of this phase, the Cdk2/cyclinE complex completes this phosphorylation. Fully phosphorylated Rb protein then frees the bound E2F transcription factors, allowing the transcription of genes necessary for the phase transition and S phase, thereby leading cells through the G1/S checkpoint. p21 associates with Cdk/cyclin complexes when DNA damage takes place (Jung et al. 2010), causing an accumulation of hypophosphorylated Rb protein, which remains bound to the E2F transcription factors, blocking the

transition and leading to cell cycle arrest (Cmielova and Rezacova 2011). p21 can also associate directly with and inactivate E2F, again causing cell cycle arrest (Jung et al. 2010). Normal cells undergo DNA replication during the S phase of the cell cycle; however, after DNA damage, cell cycle is halted to allow time for DNA repair to take place before initiating the next phase. If p21 is not available to induce cell cycle arrest, DNA synthesis can continue, increasing the chance for abnormal or mutated DNA, such as that observed in cancer cells (Cmielova and Rezacova 2011).

p21 is also involved in the transition of cells from the G2 phase into mitosis. Prior to this transition, the Cdk1/cyclinB complex is inactive due to phosphorylation of Cdk1 at Tyr15 and Thr14. Cdc24 phosphatase eliminates these phosphate groups, activating Cdk1 as the cell enters mitosis. Cell cycle arrest at this point mainly occurs via regulation of Cdc24 phosphatase activity, causing the Cdk1/cyclinB complex to remain inactive. However, p21 can also regulate the active form of this complex. Although its affinity for p21 is low compared to other Cdk/cyclin complexes, p21 can inhibit Cdk1 in multiple ways. First, p21, if available at high enough concentrations, can bind directly to and inhibit the complex. Second, p21, while not interfering with the dephosphorylation of the two inhibitory phosphorylation sites, can interfere with the activating phosphorylation of Thr161 of Cdk1 by Cdk activating kinase (CAK) (Cmielova and Rezacova 2011; Smits et al. 2000). Interestingly, cytoplasmic p21, in contrast to the ability of nuclear p21 to inhibit Cdk/cyclin complexes, can actually mediate the assembly and translocation of Cdk4,6/cyclinD complexes into the

nucleus (Yoon et al. 2012). Additionally, p21 can also act as a bridge facilitating the binding of Cdk4,6 to cyclinD prior to this translocation (Coqueret 2003), thereby acting as a positive regulator of cell cycle (**Figure 1.2**).

Role of p21 in Cell Death

In response to stress, a cell can either undergo cell cycle arrest or cell death; cell type, strength of insult and p53 target genes, including p21, are hypothesized to help control the outcome (Jung et al. 2010). p21 has conflicting roles in apoptosis: it can both promote and inhibit this form of cell death (Yoon et al. 2012). The role of p21 in response to DNA damage depends, in part, on the extent of the damage itself. When the damage is low, the protein is stabilized and the level of nuclear p21 increases to induce cell cycle arrest as discussed above. After massive amounts of DNA damage, however, p21 levels are reduced and apoptosis takes place (Cmielova and Rezacova 2011).

p21 can bind to a number of pro-apoptotic proteins in the cytoplasm causing their inactivation (Cmielova and Rezacova 2011; Romanov et al. 2012). For example, p21 can bind to procaspase-3 and prevent its activation into caspase-3, thereby blocking a main pathway of apoptosis. This binding inhibits both receptor-mediated extrinsic pathways of apoptosis, as well as mitochondrial intrinsic pathways (Cmielova and Rezacova 2011). p21 can also bind to and inhibit pro-apoptotic kinases, furthering its anti-apoptotic role in the cytoplasm. One such kinase is apoptosis signal-regulating kinase 1 (ASK1), which is an important element in stress-activated and cytokine-induced apoptotic

mechanisms. ASK1 is involved in the activation of stress activated protein kinases and the p38 family of MAP kinases, and increased levels of ASK1 result in the induction of apoptosis. The complex formed by p21 binding to ASK1 inhibits this mechanism (Cmielova and Rezacova 2011). Cytoplasmic p21 can also inhibit JNK (c-Jun N-terminal kinase) family MAP kinases. Activation of these kinases is also an important part of the stress-induced apoptosis pathway (Romanov et al. 2012). This anti-apoptotic activity is a mechanism by which p21, while known to act as a tumor suppressor, can also act as an oncogene. The role of cytoplasmic p21 is outlined in **Figure 1.2**.

p21 Induction and Regulation

Several classes of chemicals have been identified to induce p21 expression and alter cellular processes involving p21. Environmental exposure to arsenic and heavy metals, such as cadmium and lead, has been found to induce cell cycle arrest or apoptotic pathways in parallel with alterations in p21 expression (Chatterjee et al. 2014; Choi et al. 2011; Dutto et al. 2015; Yih and Lee 2000). Mercury, a well-known neurotoxin, increases p21 expression and cell cycle alterations both *in vitro* and *in vivo* (Faustman et al. 2002; Ou et al. 1999). Even aluminum, a common element of household tools and cosmetics, causes DNA damage and increased p21 expression (Sappino et al. 2012). Studies have also reported increased cytoplasmic p21 expression in correlation with inhibition of apoptosis in the large airway epithelium of cigarette smokers (Chiappara et al. 2013). The increase in p21 expression induced by cigarette smoke correlates to oxidative stress, growth arrest and cellular senescence (Marwick et al. 2002;

Tsuji et al. 2004). Organic pesticides and phytochemicals also alter p21 expression, leading to subsequent alterations in cell cycle and cell proliferation (Dutto et al. 2015). The ability of these diverse stimuli to alter p21 expression suggests the importance of p21 in mediating a variety of cellular processes. Further, these studies also show the various possible regulatory mechanisms for p21 expression.

p21 expression and activity is regulated via multiple mechanisms (Jung et al. 2010; Romanov et al. 2012). p53-dependent regulation is the most common to consider when discussing p21. In normal cells, the majority of p53 is found in the cytoplasm. Following DNA damage, p53 accumulates in the nucleus, where it acts as a transcription factor regulating the expression of various proteins, including p21 (Cmielova and Rezacova 2011). p53 directly binds to one of two p53-responsive elements (p53-RE) within the p21 gene. Two p53 homologues can also bind to the p53-REs and transactivate p21 (Jung et al. 2010). Other factors can recruit or assist p53 in the regulation of p21 expression, acting as p53-coactivators. These include BRCA1 and monocytic leukemia zinc finger (MOZ), which acetylate and stabilize p53. Pin1 is a prolyl isomerase that manipulates the structure of p53 in response to DNA damage to facilitate the protein's phosphorylation. This phosphorylation results in p53 activation and, eventually, activation of p21.

p21 is also regulated independently of p53. This can be mediated by additional binding elements also present within the p21 promoter. One of these is an Sp1/Sp3 binding site that regulates p21 expression in response to various

stress signals and, in doing so, plays a role in DNA repair, cell growth, differentiation and apoptosis. In some cell types, p21 can be induced via these sites by the Rb protein. In other cell types, however, Sp1 represses p21 transcription, indicating that regulation of p21 by means of these sites is cell-dependent. The transcription factor AP-1 can also increase the expression of p21 through direct binding (Du and Wu 2012), as well as indirectly as it is a complex of c-Fos and c-jun. c-jun can bind to and block the Sp1 binding site of the p21 gene, preventing its transcription and expression (Stepniak et al. 2006). The formation of AP-1 decreases the amount of free c-jun, thereby resulting in disinhibition of p21 expression. These are not the only transcription factors that target the p21 promoter region. In fact, a large quantity of transcription factors can regulate p21 expression in response to different signals. These are discussed in detail in a review by Jung et al. (2010).

Several studies have shown that p21 can be regulated epigenetically (Duan et al. 2005; Fan et al. 2011; Geyer 2010; Li et al. 2013; Majid et al. 2008; Scholpa et al. 2014; Vijayaraghavalu et al. 2013). The study of epigenetics, or heritable changes in gene expression not associated with alterations in DNA sequence, is an emerging scientific field with the potential to revolutionize our understanding of toxicological mechanisms of action. Epigenetic events, summarized in **Figure 1.3**, include histone modifications, such as acetylation and methylation, DNA methylation and nonprotein coding RNA-mediated effects. Evidence of the importance of epigenetic alterations in various cellular processes is increasing rapidly.

Role of Epigenetic Mechanisms in Gene Expression and p21

DNA methylation is an epigenetic event that affects cell function by altering gene expression (Phillips 2008). Essentially, it is the covalent addition of a methyl group to the 5-carbon position of a cytosine residue forming 5-methylcytosine (5-mC) (Jaenisch and Bird 2003). This methyl group projects into the major groove of DNA, impeding the binding of transcription proteins, decreasing transcription and expression. The formation of 5-mC occurs almost exclusively at CpG sites, where a cytosine nucleotide is located next to a guanine nucleotide. The majority of CpG dinucleotide sites are heavily methylated in coding regions of genomic DNA. CpG clusters, known as CpG islands, in promoter regions, on the other hand, are largely un-methylated to allow gene expression to occur (Lister et al. 2009). Expression is repressed if a CpG island within a promoter region becomes methylated. DNA methylation is carried out by DNA methyltransferases (DNMTs), which use S-adenosyl methionine as the methyl group donor.

Multiple studies suggest roles for DNA methylation in p21 expression. Treatment of drug-resistant breast cancer cells with a DNA demethylating agent increased p21 expression and subsequently enhanced cell cycle arrest at the G2/M phase transition (Vijayaraghavalu et al. 2013). Our own studies showed that treatment of kidney cells with the DNA methyltransferase inhibitor 5-azacytidine increased p21 protein expression. This same study showed that sub-chronic low-dose bromate treatment biphasically altered p21 expression, correlating to alterations in the DNA methylation status of the p21 coding region

((Scholpa et al. 2014), Appendix), further indicating a role for DNA methylation in regulation of p21 expression.

DNA is tightly wrapped around histones, the main proteins of chromatin, to allow it to be condensed within the nucleus. For a gene to be transcribed, the chromatin structure must temporarily change to enable the binding of transcription proteins to the relevant DNA. Alterations in promoter-specific histone modifications can result in improper silencing or transcription, which could lead to cellular transformation, carcinogenesis or cancer progression (Fraga et al. 2005; Shiio and Eisenman 2003). Such structural changes can occur via acetylation, methylation, phosphorylation or ubiquitination of histone tails, which can either allow or prevent access to the DNA. Different histone modifications have varying effects on structure and eventually gene expression.

Histone acetylation generally induces gene activation through post-transcriptional acetylation of lysine residues at the N-terminal tail of each of four histones: H2A, H2B, H3 and H4 (Luger et al. 1997; Vaquero et al. 2003). Histone acetyltransferase (HAT) catalyzes the transfer of an acetyl group from acetyl-coenzyme A to the histone. The addition of this acetyl group decreases the interaction of the histone with the negatively charged phosphate groups of DNA. This results in less condensed chromatin, referred to as euchromatin, which is more readily and easily transcribed. Histone deacetylases (HDAC) remove these acetyl groups, condensing the chromatin and forming heterochromatin. Altering histone acetylation has been found to contribute to p21 expression (Duan et al. 2005; Fan et al. 2011; Geyer 2010; Li et al. 2013; Majid et al. 2008). For

example, HDAC recruitment to the p21 promoter decreases its expression in myeloid cells (Duan et al. 2005). Additionally, our own studies with the HDAC inhibitor trichostatin A showed that this compound increases p21 protein expression in kidney cells ((Scholpa et al. 2014), Appendix).

Histone methylation can also affect p21 expression. Methylation can only occur on lysine and arginine residues, and results in active or inactive regions of transcription depending on which residues are methylated (Wang and Jia 2009). For example, trimethylation of the lysine 4 residue of histone 3 induces transcriptional activation, while dimethylation of histone 3 at lysine 9 causes silencing (Gupta et al. 2010). Enhancer of zeste homologue 2 (EZH2) is a catalytic subunit of Polycomb-repressive complex 2 (Prc2) whose primary function is global gene transcription repression through histone methylation. p21 expression is enhanced upon EZH2 knockdown, and depletion of EZH2 also removes HDAC1 from the p21 promoter region, increasing histone 3 acetylation, leading to activation and transcription (Fan et al. 2011).

In addition to direct alteration of histone structure, histone modification has also been linked with alterations in DNA methylation. Some studies suggest that methylation of certain histone residues is required for DNA methylation to take place, in that DNA methyltransferases may only act on chromatin that is methylated (Kouzarides 2002; Zhao et al. 2009). Additionally, DNA methylation is thought to be a signal to recruit histone deacetylase and histone methylase activity so as to maintain a repressed state (Kouzarides 2002).

Several diseases have been linked to epigenetic mechanisms, including Alzheimer's disease, autoimmune disorders and cancer (Barrow and Michels 2014; Daniel and Tollefsbol 2015; Egger et al. 2004; Farh et al. 2015; Lakshmaiah et al. 2014; Portela and Esteller 2010; Sezgin and Dincer 2014). In many cancers, the balance of epigenetic modifiers is altered compared to normal cells, and as such, several chemotherapeutics target epigenetic regulation to increase the expression of tumor suppressors, as well as decrease the expression of known oncogenes (Barrow and Michels 2014; Christman 2002; Dobosy et al. 2007; Fraga et al. 2005; Lakshmaiah et al. 2014). Environmental exposures, such as to the known oxidant bromate, can also alter the epigenetic status of genes such as p21 (Cortessis et al. 2012; Marsit 2015; Scholpa et al. 2014). Everyday life-style choices such as diet and exercise have been associated with long-lasting epigenetic modifications that may contribute to the onset of diseases (Daniel and Tollefsbol 2015; Hardy and Tollefsbol 2011; Horsburgh et al. 2015; Ling and Ronn 2014; McKay and Mathers 2011; Ntanasis-Stathopoulos et al. 2013). Epigenetic alterations can also occur following exposure to drugs of abuse, such as cocaine (LaPlant and Nestler 2011; Nielsen et al. 2012; Robison and Nestler 2011; Wong et al. 2011).

Neurological Activity of Cocaine

Cocaine is a powerfully addictive psychoactive substance, and abuse of the drug has been a persistent problem for over 100 years. Cocaine is currently classified as a Schedule II drug, meaning it has a high potential for abuse, but can still be administered by a doctor for legitimate medical purposes. Cocaine

blocks Na⁺ channels, resulting in anesthetic effects, making it useful for certain eye, ear and throat surgeries (2010). The National Survey on Drug Use and Health (NSDUH) estimates that in 2013 there were 1.5 million current cocaine users of age 12 or older in the United States alone and over 850,000 of these individuals met the Diagnostic and Statistical Manual of Mental Disorders, 4th edition criteria for dependence or abuse of cocaine (2014). The cocaine hydrochloride salt can be snorted directly and absorbed into the blood through the nasal tissue, or dissolved in water and injected directly into the blood stream. Heated rock crystal, or “crack” cocaine, produces vapors that can be smoked. Through inhalation, the absorption into the blood stream is comparable to that with injection. The intensity and duration of the “high” produced by cocaine depends on the route of exposure, with faster absorption resulting in a faster, shorter high (2010; Nestler 2005).

Upon exposure, cocaine enters the blood stream and penetrates the brain where it alters the dopamine pathway, a neural circuit involved in reward-driven learning and considered fundamentally important to survival (Nestler 2005). Cocaine binds to dopamine transporters preventing dopamine reuptake and resulting in accumulation within the synaptic cleft, where dopamine continues to bind to and activate dopamine receptors. This occurs throughout the brain, but the high experienced with the drug is due primarily to its effects within the mesolimbic reward pathway (Hyman and Malenka 2001; Kalivas and McFarland 2003; Koob et al. 1998; Nestler 2001). The limbic system links emotional responses with memories and includes several brain structures such as the

nucleus accumbens (NAc), the hippocampus and the frontal cortex (Amunts et al. 2005; Nestler 2005). NAc cells produce feelings of pleasure when stimulated by dopamine and the majority of research regarding cocaine addiction has focused on alterations in gene expression induced therein (LaPlant and Nestler 2011; Larson et al. 2010; Meyer et al. 2009; Nestler 2012; Renthal et al. 2009; Robison and Nestler 2011). Although there have been many studies on the neurochemical and neurobiological effects of various drugs of abuse, there is little information on what makes one individual more likely to become addicted than another, and once clean, what makes one more likely to relapse. Additionally, there is currently neither an effective pharmacological treatment for addiction nor prevention for relapse.

There is limited information regarding the relationship between cocaine and p21. A study by Hu et al. (2006) exposed human fetal brain-derived neural precursor cells to cocaine and observed an increase in p21 expression, correlating to inhibited proliferation. Cocaine has been found to alter the expression of several proteins involved in regulation of p21 including p38, c-Fos and ERK, among others (Larson et al. 2010; Nestler 2012; Robison and Nestler 2011; Yang et al. 2010). The effects of these alterations on p21 expression, however, have not been examined.

There has recently been a rise in interest in the link between drugs of abuse and epigenetics. Cocaine generally induces a more permissive transcription state, leading to increased activation. This occurs mainly through alterations in histone acetylation; however changes have also been observed

with DNA methylation and histone methylation (LaPlant and Nestler 2011; Nielsen et al. 2012; Renthal et al. 2009; Robison and Nestler 2011; Schmidt et al. 2013; Wong et al. 2011; Zhou et al. 2011). Interestingly, these alterations have been shown to be gene- and region-specific as opposed to global changes. Additionally, treatment with epigenetic inhibitors in conjunction with cocaine exposure has been shown to alter drug-seeking and drug-taking behaviors (LaPlant and Nestler 2011; Nielsen et al. 2012; Renthal et al. 2007; Wong et al. 2011). The ability of cocaine to induce epigenetic alterations and the potential for p21 to be epigenetically regulated beg the question if cocaine can alter p21 expression via epigenetic mechanisms. In a large-scale genome-wide analysis on the effect of cocaine on histone acetylation using chromatin immunoprecipitation, Renthal et al. (2009) found that cocaine altered the epigenetic status of p21 chromatin in mouse NAc, but the effect on transcription and expression of p21 was not discussed.

While the NAc is a common target when investigating drugs of abuse, addiction also involves other key brain regions. The hippocampus is a structure of the limbic system involved in the formation of long-term memories (Larson et al. 2010; Nestler 2012; Robison and Nestler 2011; Scoville and Milner 1957), a process integral to addictive behaviors. The hippocampus is structurally and functionally divided into two distinct compartments: the dorsal hippocampus and the ventral hippocampus (**Figure 1.4**). The dorsal hippocampus, which corresponds to the posterior hippocampus in primates, is primarily involved in performing cognitive functions and spatial learning and memory (Moser et al.

1995). The ventral, or anterior, hippocampus, on the other hand, is related to stress response and emotional behaviors (Henke 1990) and has more connections with structures that have been implicated in cocaine-seeking behaviors (Rogers and See 2007), specifically in cocaine-primed reinstatement models of relapse (Lasseter et al. 2010). The hippocampus in its entirety consists of two major regions separated by the hippocampal sulcus (**Figure 1.5**): the dentate gyrus, made up of granule cells whose axons form mossy fiber bundles, and the hippocampus proper, also known as the cornu ammonis (CA), which is made up of pyramidal cells that receive contact from the mossy fibers of the dentate gyrus (O'Keefe and Nadel 1978). The dentate gyrus of the hippocampus is a primary site for adult neurogenesis, or the ability to give rise to new neurons (Abrous et al. 2005). Interestingly, levels of adult hippocampal neurogenesis are negatively correlated with drug-taking and drug-seeking, suggesting an important role for adult hippocampal neurogenesis in drug addiction (Noonan et al. 2010).

Adult Hippocampal Neurogenesis

The first observations of adult neurogenesis took place in the 1960s by Altman (1962). Although originally met with controversy, the notion of adult-born neurons gained footing in the 1980s courtesy of several studies by Kaplan (Kaplan 1981; Kaplan 1985; Kaplan and Bell 1983; Kaplan et al. 1985). Since this time, the sequence of events for the process has been fairly well established, particularly in rodents. Neurons are generated predominantly in two regions of the brain, the subgranular zone of the dentate gyrus and the subventricular zone lining the lateral ventricles, also referred to as the subependymal zone, which

generates olfactory neurons (Kazanis 2009; Marqués-Torrejón et al. 2013). In the subgranular zone, stem cells, morphologically resembling radial glial cells give rise to amplifying neural progenitor cells, which then differentiate into new immature neurons (**Figure 1.6**). While many newborn cells die shortly after formation, some become integrated into surrounding brain tissue (Dayer et al. 2003; Schouten et al. 2012). Granule cells of the dentate gyrus are the primary neuron type added during neurogenesis (Kohler et al. 2011). After being formed, new granule cells migrate from the subgranular layer into the granule cell layer. They then will extend axons and dendrites and make appropriate connections within the hippocampal circuit, becoming functionally integrated (Kohler et al. 2011; Markakis and Gage 1999). Maturation of new granule cells is accompanied by changes in neuron-specific protein expression. The two most widely assessed proteins with regard to neurogenesis are doublecortin (DCX), which is present in immature neurons for only approximately two weeks, and NeuN, which increases in mature neurons as DCX decreases (Brown et al. 2003).

In mammalian cells, proliferation, which is integral to neurogenesis, is tightly regulated at the G1 phase of the cell cycle (Nevins 2001). As discussed, p21 induces cell cycle arrest at the G1 phase through inhibition of Cdk/Cyclin complexes, thereby restraining cell proliferation (Cmielova and Rezacova 2011; Yoon et al. 2012). To date, few studies have examined the role of p21 in the hippocampus and on neuronal and neural stem cell (NSC) proliferation within the dentate gyrus. p21, however, has been found to inhibit NSC proliferation and hippocampal neurogenesis (Pechnick et al. 2008; Zonis et al. 2013). Additionally,

neuronal progenitor cells derived from mice lacking p21 show higher proliferation than those derived from wild-type mice (Pechnick et al. 2011). Similarly, suppression of p21 using shRNA in these wild-type cells increases proliferation (Pechnick et al. 2011). These data support the finding that mice that lack p21 have higher baseline neurogenesis than wild-type mice (Pechnick et al. 2011). A limitation of these studies, however, is the lack of discrimination between the dorsal and ventral sectors of the hippocampus during the analyses. Mice lacking p21 also have more NSCs and increased NSC proliferation in the forebrain and the subependymal zone than wild-type mice (Kippin et al. 2005; Marqués-Torrejón et al. 2013).

Other Cocaine-Induced Effects

In addition to the effects within the limbic system, recreational doses of cocaine can affect other biological systems as well. Cocaine's activity as a Na⁺ channel blocker, while giving it anesthetic properties, also causes cardiovascular consequences, decreasing left ventricular function and inducing arrhythmia (Schwartz et al. 2010). As a stimulant, cocaine increases sympathetic output, body temperature, heart rate and blood pressure, all of which increase myocardial oxygen demand. Myocardial oxygen supply, however, is decreased through vasoconstriction, meaning demand may exceed supply resulting in ischemia or myocardial infarction (Schwartz et al. 2010). In general, cocaine-related deaths are due to cardiac arrest or seizure followed by respiratory arrest (2010). Side effects induced by cocaine depend upon the route of exposure. Snorting cocaine can result in a loss of sense of smell, nosebleeds and voice

hoarseness. Injecting cocaine can cause allergic reaction as well as increased risk of blood-borne diseases, and ingestion can result in bowel gangrene from reduced blood flow (2010). Some studies also suggest that cocaine exposure may alter neurogenesis (Andersen et al. 2007; Garcia-Fuster et al. 2010; Sudai et al. 2011; Yamaguchi et al. 2004).

Recent studies suggest that the biological effect of drugs of abuse such as cocaine may also extend to the lipidome. Over the last few decades, increasing evidence has suggested a correlation between low plasma cholesterol levels and various psychiatric disorders, namely depression, aggressive tendencies, antisocial personality disorder and substance abuse (Buydens-Branchey and Branchey 2003; Gettler 1991; Glueck et al. 1994; Ho et al. 1977; Maccari et al. 1991; Morgan et al. 1993; Virkkunen 1979; Virkkunen 1983). Heroin addicts were found to have lower cholesterol levels than non-addicts in several studies spanning three decades (Gettler 1991; Ho et al. 1977; Maccari et al. 1991). Multiple studies have reported a relationship between cholesterol and cocaine, with Gettler (1991) observing decreased cholesterol in cocaine abusers and Buydens-Branchey and Branchey (2003) observing a correlation between low cholesterol and relapse rates in detoxified addicts. There is much less data available, however, regarding the relationship between drugs of abuse and phospholipids and their metabolites.

Studies suggest a connection between low levels of some polyunsaturated fatty acids (PUFAs), a type of phospholipid metabolite, and psychiatric disorders, again, including depression, aggression and addiction (Buydens-Branchey et al.

2003). Levels of PUFAs were altered in the blood of individuals addicted to heroin compared to normal participants (Wannasirindr et al. 2000). Buydens-Branchey et al. (2003) also found that low blood PUFA levels in humans were a better predictor of relapse to cocaine than drug use, sociodemographic or clinical parameters. Further, it has been shown that altering the fatty acid composition within the diet can alter neurotransmitter level, including dopamine, with rats fed a diet low in fatty acids having lower levels of endogenous dopamine (Delion et al. 1994; L'Hirondel et al. 1995). This effect could be due in part to arachidonic acid-induced inhibition of dopamine reuptake and stimulation of dopamine release (L'Hirondel et al. 1995; Zhang and Reith 1996). Therefore, it was hypothesized that in cocaine addicts with lower levels of fatty acids, particularly arachidonic acid, less dopamine is released than that in addicts with higher arachidonic acid content, leading to increased cocaine consumption necessary to increase synaptic levels of dopamine (Buydens-Branchey et al. 2003). While this hypothesis could explain altered cocaine intake in individuals with different fatty acid content in the diet, there is little data dissecting a direct relationship between cocaine and blood levels of various fatty acids.

The mechanisms by which cocaine-induced alterations in lipid profiles occur are not fully understood. Several studies have indicated that lipids can also alter the expression of p21. Sphingolipids contribute to membrane biology and to signaling events involved in eukaryotic cellular behavior and function. Sphingolipid metabolites such as ceramide and sphingosine-1-phosphate (S1P) have been shown to influence multiple cellular processes including proliferation,

migration, and survival (Fyrst and Saba 2010; Lee et al. 2011). A recent study by Hait et al. (2009) showed that S1P can directly act on nuclear targets and alter gene expression. Nuclear S1P can be assembled into corepressor complexes containing HDAC1 and HDAC2, which can inhibit deacetylation of histone tails, altering DNA binding and leading to increased p21 expression (Hait et al. 2009). Pharmacological inhibition of sphingolipid biosynthesis has been shown to have anti-cancer effects through p53-dependent increases in p21 expression, subsequently leading to cell cycle arrest and decreased proliferation both *in vitro* and *in vivo* (Lee et al. 2011; Lee et al. 2012). Ceramide also induces cell cycle arrest at the G2/M phase checkpoint via induction of p21, eventually leading to apoptosis (Phillips et al. 2007).

Sphingolipids are not the only class of lipids known to affect p21 expression. Some short-chain fatty acids have been shown to act as HDAC inhibitors (Chen et al. 2003). A study by Rocchi et al. (2005) found that p21 is a common target of increased transcription and expression by multiple short-chain fatty acid HDAC inhibitors including valproic acid, sodium butyrate and tributyrin *in vitro*. Additionally, inhibition of calcium-independent phospholipase A₂ (iPLA₂), which degrades phosphatidylcholine, a major component of phospholipids in mammalian cells, has been found to block cellular proliferation via G1 cell cycle arrest, correlating to up-regulation of p21 (Zhang et al. 2006). Despite the available information regarding the relationship between cocaine and the lipidome, and that of p21 and the lipidome, to our knowledge, there are no data or studies available discerning the connection between all three.

Summary and Conclusions:

Recreational drug abuse and addiction remain a prevalent problem not only within the United States, but worldwide. Although the biological activities of the majority of common drugs of abuse have been determined, there remains no approved pharmacological treatment for addiction. Sufficient evidence supports a connection between cocaine, p21 and epigenetic regulation, however the extension of this relationship into the hippocampus, as well as its behavioral significance remains unclear. Similarly, a role for hippocampal p21 expression in neurogenesis is suggested, however the sector-specificity, functional relevance and effect of stimuli exposure on this expression have not been fully characterized. Additionally, there is little to no information on the extent of the involvement of p21 in cocaine-induced effects, for example, extending into the lipidome. Filling these gaps in knowledge may identify epigenetic regulation of p21 as a potential therapeutic target for addictive behaviors.

Hypothesis and Specific Aims

Based on the above information this study tested the hypothesis that cocaine induces alterations in hippocampal p21 via epigenetic mechanisms and these alterations mediate cocaine-induced response. The specific aims proposed for investigation of this hypothesis are as follows:

Specific Aim 1: Test the hypothesis that cocaine alters p21 expression in the brain region-specifically. Aim 1 determines the effect of contingent and non-

contingent cocaine exposure on p21 mRNA and protein in limbic structures of rats and mice.

Specific Aim 2: Test the hypothesis that p21 affects cocaine-induced behavior.

Aim 2 studies the role of p21 in behavioral response to cocaine using a p21 knockout ($p21^{-/-}$) mouse model. The relationship between p21, cocaine and neurogenesis will also be determined, as well as the effects of cocaine and p21 on the blood lipidome and correlations between relative abundance of specific lipid species and cocaine-induced behaviors.

Specific Aim 3: Test the hypothesis that cocaine alters p21 via epigenetic mechanisms. Aim 3 investigates the mechanisms by which cocaine induces alterations in p21 expression, with a focus on DNA methylation and histone acetylation.

Successful completion of these studies will further characterize the relationship between cocaine and p21, as well as provide insight into the role for p21 in motivated behaviors and neurological processes such as neurogenesis. These studies could therefore be expanded to other drugs of abuse with alternate mechanisms of action, as well as additional neuropathologies. The knowledge gained from this work could provide a potential therapeutic avenue for the treatment or prevention of addiction involving exploitation and manipulation of p21 regulation.

Table 1.1 Location of the *Cdkn1a* Gene

Species	Common Name	Chromosome
<i>Homo sapiens</i>	Human	6
<i>Mus musculus</i>	Mouse	17
<i>Rattus norvegicus</i>	Rat	20
<i>Danio rerio</i>	Zebra fish	22
<i>Bos taurus</i>	Cattle	23
<i>Canis lupus familiaris</i>	Dog	12
<i>Gallus gallus</i>	Chicken	26
<i>Macaca mulatta</i>	Rhesus monkey	4
<i>Felis catus</i>	Cat	B2
<i>Sus scrofa</i>	Pig	7
<i>Meleagris gallopavo</i>	Turkey	28
<i>Pan troglodytes</i>	Chimpanzee	6
<i>Chlorocebus sabaeus</i>	Green monkey	17
<i>Capra hircus</i>	Goat	23
<i>Macaca fascicularis</i>	Crab-eaten macaque	4
<i>Microtus ochrogaster</i>	Prairie vole	LG2
<i>Chrysemys picta</i>	Painted turtle	4
<i>Ficedula albicollis</i>	Collared flycatcher	26
<i>Gorilla gorilla</i>	Western gorilla	6
<i>Papio anubis</i>	Olive baboon	4
<i>Callithrix jacchus</i>	White-tufted-ear marmoset	4
<i>Nomascus leucogenys</i>	Northern white-cheeked gibbon	17
<i>Anolis carolinensis</i>	Green anole	4
<i>Pongo abelii</i>	Sumatran orangutan	6
<i>Oryctolagus cuniculus</i>	Rabbit	12
<i>Ovis aries</i>	Sheep	20
<i>Equus caballus</i>	Horse	20
<i>Monodelphis domestica</i>	Opossum	2
Information obtained from National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/gene).		

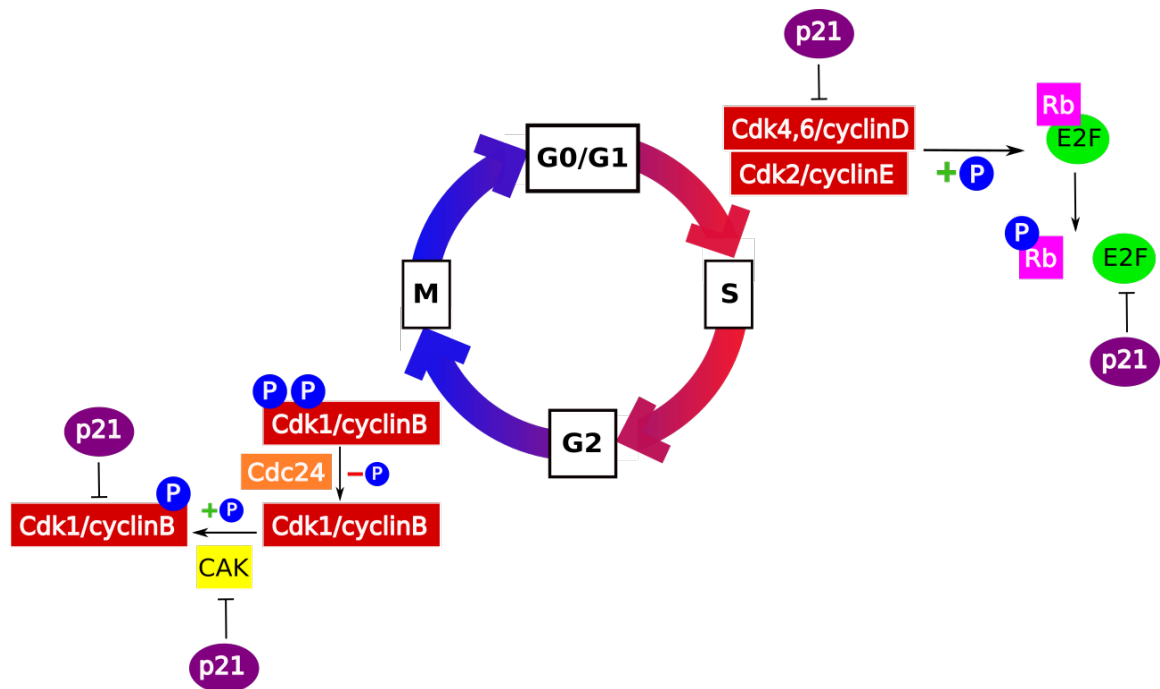


Figure 1.1. Role of Nuclear p21 on Cell Cycle Progression. Schematic derived using Inkscape (www.inkscape.org). During G1 phase, p21 can bind to and inhibit the activity of Cdk2, 4 and 6, causing hypophosphorylation of Rb. E2F is a transcription factor that allows the transcription of several genes necessary for the transition into S phase and for S phase itself. Hypophosphorylated Rb remains bound to E2F, preventing this activity and progression into S phase. p21 can also directly bind to and inhibit E2F. Progression from G2 to M phase requires dephosphorylation of the Cdk1/cyclinB complex by Cdc24, followed by activating phosphorylation by CAK. p21 can inhibit this activating phosphorylation or bind directly to and inhibit Cdk1, ceasing cell cycle progression.

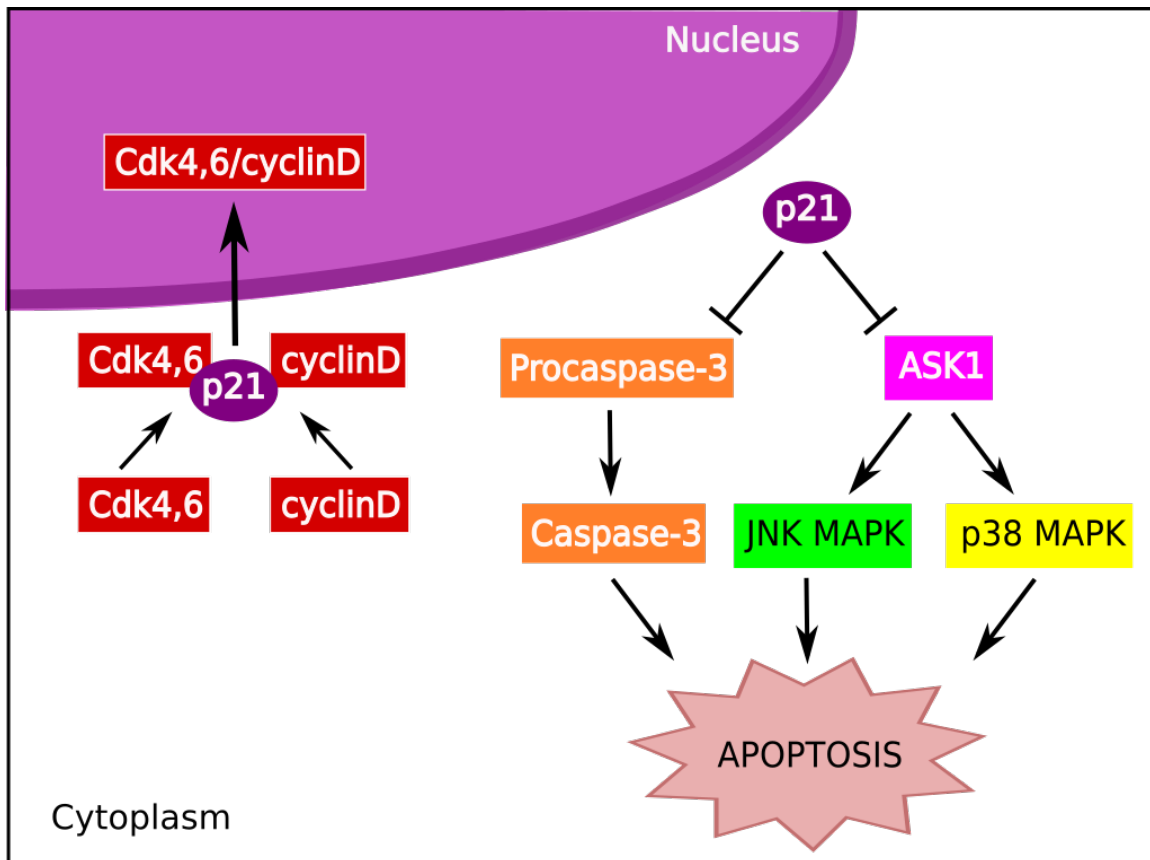


Figure 1.2. Role of Cytoplasmic p21. Schematic derived using Inkscape (www.inkscape.org). Cytoplasmic p21 can act as a bridge and facilitate the association of Cdk4 and 6 to cyclinD. Following binding, p21 can enable translocation of the complex into the nucleus, thereby acting as a cell cycle promoter. p21 can also inhibit the activity of several pro-apoptotic proteins within the cytoplasm, including procaspase-3 and ASK1. This prevents the activation of apoptotic pathways such as the caspase-3 pathway, JNK and p38 MAPK pathways, inhibiting apoptotic cell death.

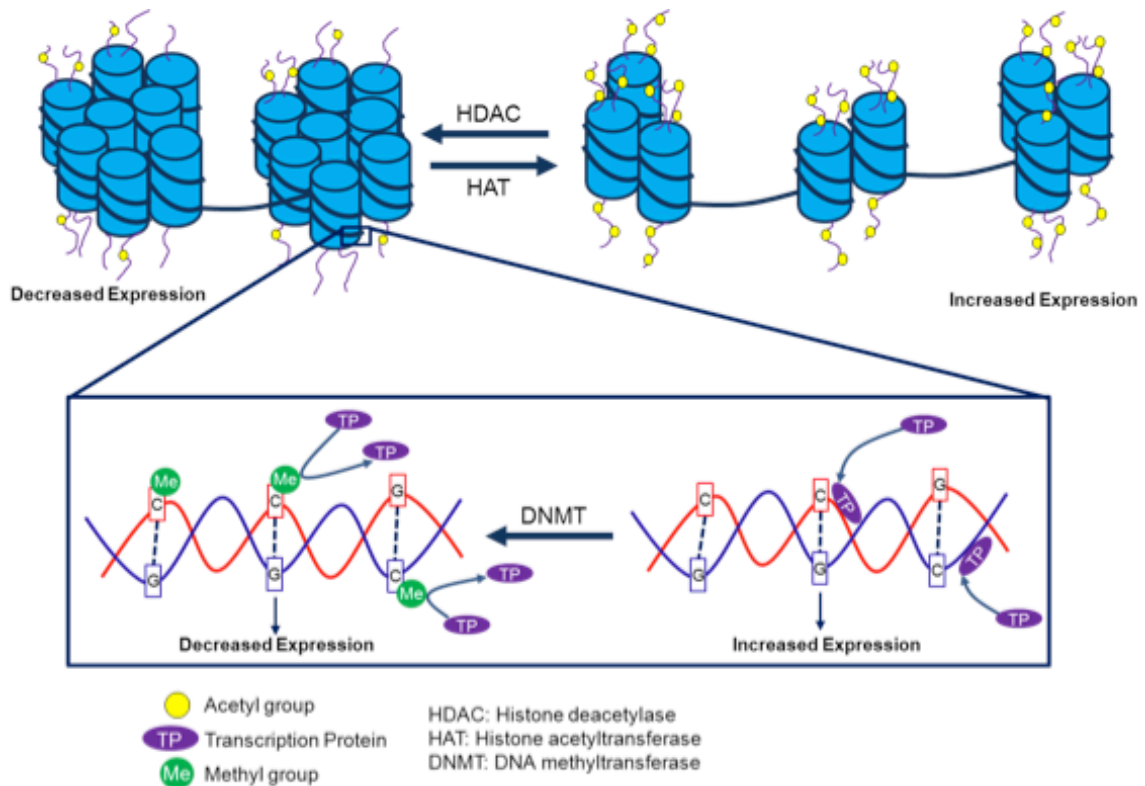


Figure 1.3. Epigenetic Mechanisms of Regulation. Schematic derived using Microsoft Power Point. DNA is tightly wrapped around histones. Histone acetylation (yellow circles) decreases the interaction between DNA and the histone, resulting in a looser conformation, known as euchromatin, and increased transcription and expression. Histone acetylation is catalyzed by histone acetyltransferases (HATs) and deacetylation is catalyzed by histone deacetylases (HDACs). The 5-carbon position of a cytosine residue can be methylated (green circle), catalyzed by DNA methyltransferases (DNMTs), forming 5-methylcytosine. This methyl group impedes the binding of transcription proteins (purple oval), decreasing transcription and expression.

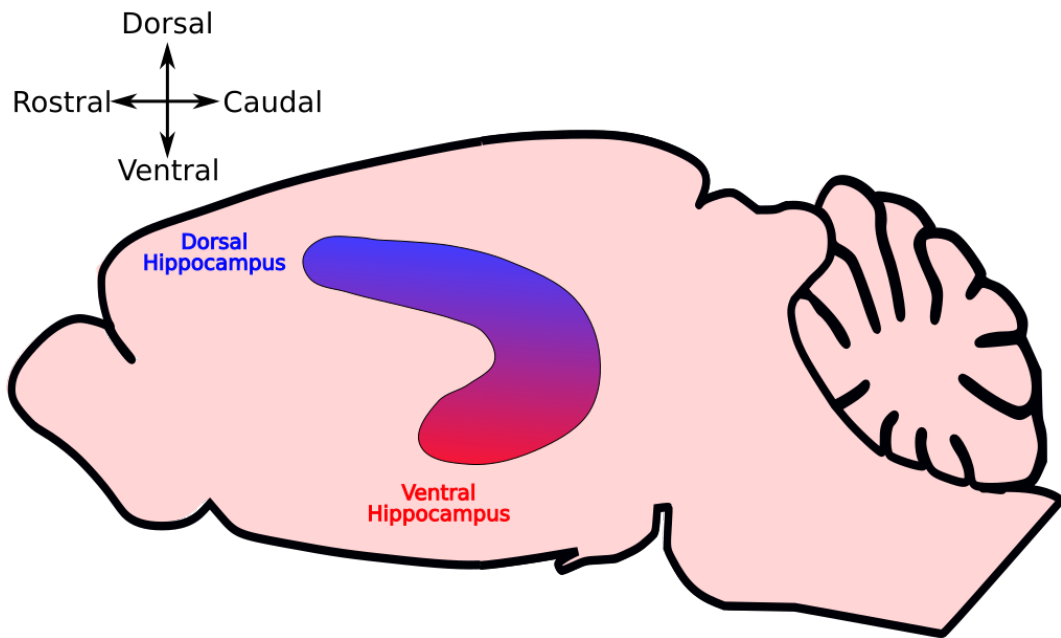


Figure 1.4. Longitudinal Axis of Rodent Hippocampus. Schematic adapted from O’Leary and Cryan (2014), and derived using Inkscape (www.inkscape.org). In mice and rats, the hippocampus extends both along the dorsoventral axis and the rostrocaudal axis. The dorsal hippocampus (blue) is both dorsal and rostral and the ventral hippocampus (red) is both ventral and caudal. Therefore, the most rostral coronal sections contain only dorsal hippocampus and more caudal sections contain both. Similarly, and not represented in the above image, more medial sagittal sections contain only the dorsal sector, while more lateral sections contain both.

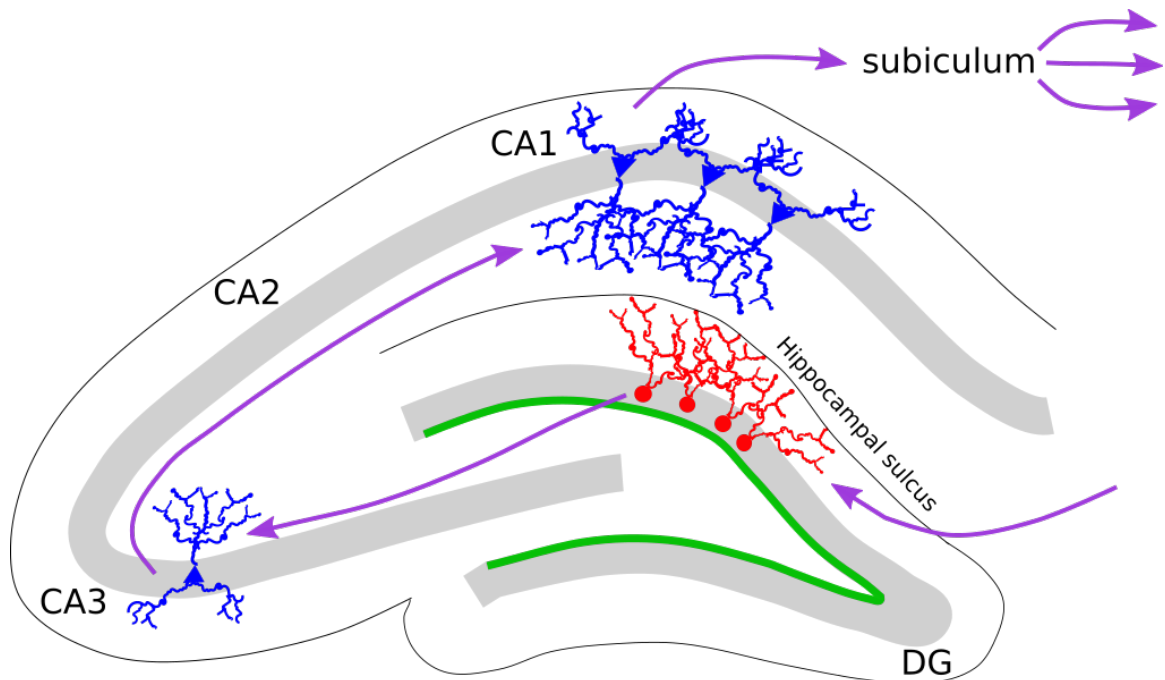


Figure 1.5. Hippocampal Anatomy and Circuitry. Schematic derived using Inkscape (www.inkscape.org). The hippocampus consists of the dentate gyrus (DG) and cornu ammonis (CA), separated by the hippocampal sulcus. The DG is the major input structure of the hippocampus and is made up of granule cells (red) whose axons form mossy fiber bundles that connect with the pyramidal cells (blue) of the CA3 region. From the CA3, communication carries through the CA2 to the CA1 and finally the subiculum, which serves as the major output structure. Neurogenesis takes place in the subgranular layer of the dentate gyrus, shown in green.

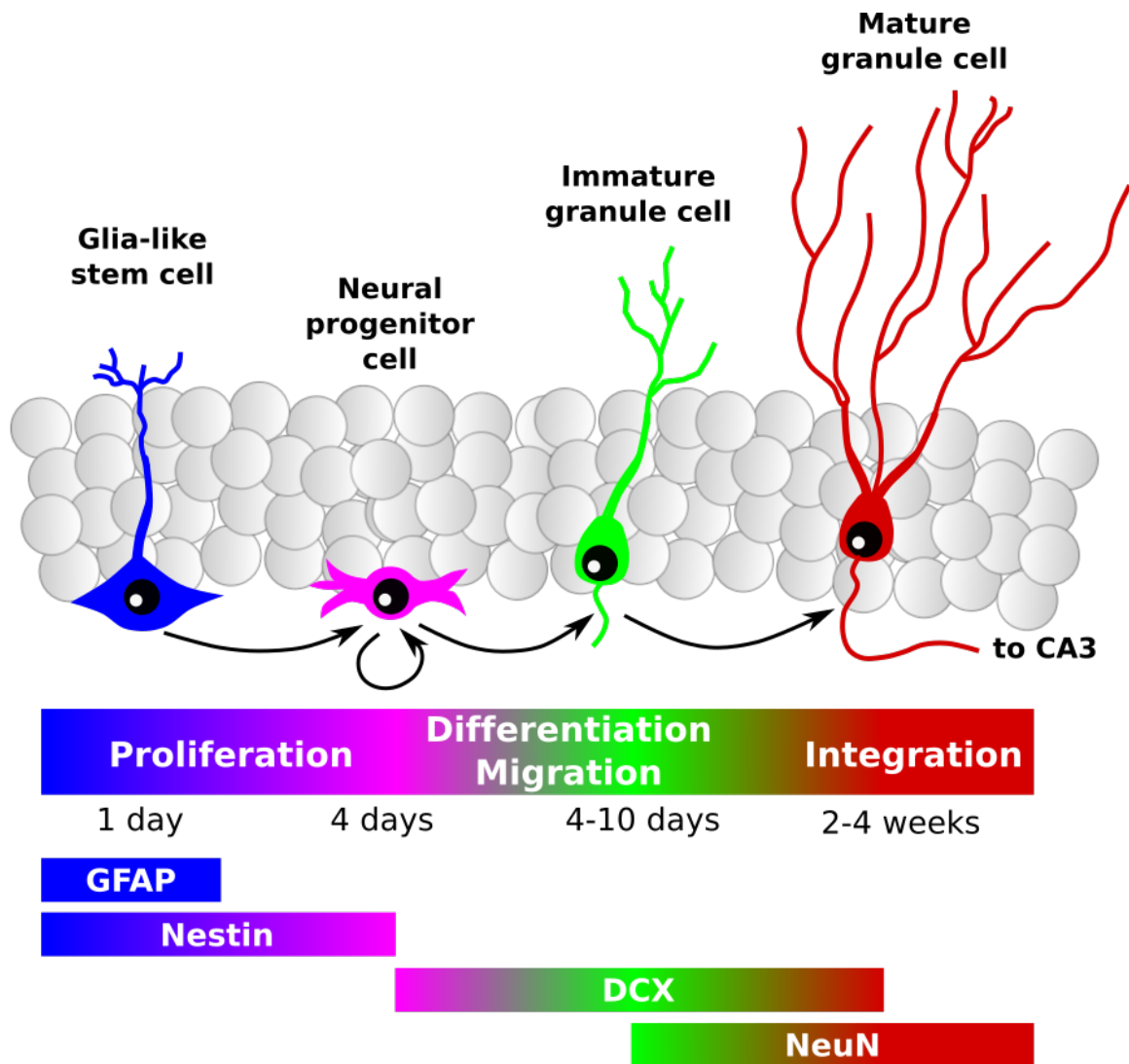


Figure 1.6. Subgranular Zone Neurogenesis. Schematic adapted from Lucassen et al. (2010) and Schouten et al. (2012), and derived using Inkscape (www.inkscape.org). Neuronal stem cells (blue) give rise to amplifying neural progenitor cells (pink), which then differentiate into new immature neurons (green). Progenitor cells can also be selectively eliminated via apoptosis prior to differentiation. New neurons then migrate into the granule cell layer of the dentate gyrus and mature into fully functioning granule cells (red). Protein expression varies throughout this process.

CHAPTER 2

MATERIALS AND METHODS

Animal Maintenance

Behavior assays were performed on Sprague–Dawley male rats (Harlan, Indianapolis, IN) and wild-type (B6;129SF2/J) and $p21^{-/-}$ (B6;129S2-*Cdkn1a*^{tm1Tyj}/J) mice purchased from the Jackson Laboratory (Bar Harbor, ME). Rats were housed in pairs and mice were kept in the groups in which they arrived (3-5 mice) in clear plastic cages. They were maintained on a 12 hr light/dark cycle (0700 hr/1900 hr) with food and water available ad libitum. Animals were allowed to acclimate to their home cages for at least one week and were habituated to handling (rats: 3 days, mice: 1 day) prior to testing. Sessions were conducted between 1000 hr and 1600 hr and these studies were approved by the University of Georgia Institutional Animal Care and Use Committee and conducted in accordance with the Guide for the Care and Use of Laboratory Animals.

Drugs

Cocaine hydrochloride was obtained from NIDA (RTI International, NC), (±)-U50,488 hydrochloride was obtained from Tocris (Bristol, UK) and

methiothepin maleate was obtained from Santa Cruz Biotechnology (Dallas, TX). Drugs were dissolved in 0.9% saline and filtered prior to use.

Apparatus

Behavioral testing was carried out in 43.2 x 43.2 cm chambers with clear plastic walls and a smooth solid floor (Med Associates Inc., St. Albans, VT). Each chamber was located in a sound-attenuating box equipped with two house lights (20 lx) and a ventilation fan. Two banks of 16 infrared photo beams detected horizontal activity and Activity Monitor software (Med Associates Inc., St. Albans, VT) was used to count beam breaks.

Conditioned place preference testing took place in a modified open field chamber using a two-compartment insert (Med Associates Inc., St. Albans, VT) specifically designed for use with mice. The insert divided the chamber into two compartments separated by a black partition with a guillotine door. When the door was removed, the mouse could access either compartment. When in place, the door confined the animal's activity to one compartment for conditioning purposes. One compartment contained flooring of rod-like steel bars and black plastic walls, which allowed for the darkening of that side of the chamber insert. The other compartment contained a wire mesh grid floor and transparent walls. These were the only ways that the two compartments differed and preliminary studies suggested that this arrangement yielded an equal preference between compartments.

Locomotor Sensitization and Conditioned Place Preference/Aversion

The experimental design for the behavior assays was strongly based on that described in previous studies (Seymour and Wagner 2008). Mice were first tested in a Pretest session where each was placed in the light/grid compartment of the chamber insert and the guillotine door was removed, allowing free access to both compartments for 15 min. The time spent in each compartment was measured to assure that no animals had a strong bias toward one side or another. The chamber insert was removed during the Activity Day (day 2) and the animal was placed in the center of the open field chamber for 30 min to determine baseline activity. An i.p. injection of either 0.9% saline, cocaine (15 mg/kg) or U50,488 (5 mg/kg) was given and the animal placed back into the open field chamber for an additional 60 min.

Conditioning sessions took place on experiment days 3-6. Mice were divided into six groups: wild-type saline ($n=14$), wild-type cocaine ($n=12$), wild-type U50,488 ($n=6$), $p21^{-/-}$ saline ($n=14$), $p21^{-/-}$ cocaine ($n=12$) and $p21^{-/-}$ U50,488 ($n=6$). Animals were given an i.p. injection of either saline, cocaine (20 mg/kg) or U50,488 (5 mg/kg) and placed in one of the two insert compartments for 15 min and then returned to their home cage. 4 hrs later, animals were injected with either saline or drug and confined to the opposite compartment for the second daily conditioning session. Following the four days of conditioning, the mice underwent a drug-free place preference Post-test on experiment day 7. One week after the final test, the open field Challenge test was conducted in the same manner as the Activity test in order to assess sensitization.

For the dose response study, animals underwent a locomotor sensitization assay as described above using additional concentrations of 7.5 and 25 mg/kg for the wild-type mice ($n=4$ /group), and 20 and 25 mg/kg for the $p21^{-/-}$ mice ($n=6$ /group) to determine the locomotor response to an initial dose of cocaine at various concentrations. These animals also underwent the remainder of the LMS/CPP assay described. However, on the four conditioning days (days 3-6), the cocaine-paired mice were pretreated with 50 μ g/kg i.p of the 5-HT receptor antagonist methiothepin maleate in the home cage 30 min prior to cocaine exposure to determine the role of serotonin on CPP in wild-type and $p21^{-/-}$ mice (Hnasko et al. 2007). The mice were exposed to saline in the home cage 30 min prior to saline conditioning sessions.

Self-Administration Assay

The experimental design for the self-administration assay is described in detail in Kelamangalath and Wagner (2010). Male Sprague-Dawley rats were sufficiently anesthetized and a silastic catheter (Dow Corning, Midland, MI) inserted into the right jugular vein and secured. Then, the rat was placed in a stereotaxic frame (Stoelting, Wood Dale, IL) and a right-angled cannula (Plastics One, Roanoke, VA) was mounted to the top of the skull. Animals were treated with gentamicin (5 mg/kg, i.v.) immediately after surgery and daily for 5 days. To maintain patency, the catheters were flushed with saline prior to each self-administration session and with heparin (10 USP/mL) after the session.

Operant chambers (Med Associates Inc., St. Albans, VT) were equipped with two levers: one “active” and one “inactive,” with lights positioned above each. The cannula atop the rat’s skull was connected to a liquid swivel, which was connected to a syringe pump containing either cocaine or saline. Each infusion delivered a volume of 0.125 mL/kg body weight, making the dose self-administered 0.5 mg/kg/infusion. MED-PC software (Med Associates Inc., St. Albans, VT) was used to record the number of active and inactive lever presses and the number of infusions.

Animals underwent self-administration training for 22 days with one session each day. For the first 15 days, the session lasted 90 min. For days 16-22, the sessions were 6 hr. Active lever presses not only triggered an infusion, but also the conditioning stimulus (CS) for 30 sec. Any lever presses during this 30 sec were counted, but had no effect. For the first 10 days, the rats were trained on a fixed ratio schedule-1 (FR-1). For the next 12 days of training, an FR-3 schedule was used, where three active lever presses were required to trigger an infusion and the CS. Each animal was placed in the same chamber throughout the experiment.

After the period of self-administration, rats underwent extinction training during which responses on the active lever had no effect. Following this training, rats were tested for cocaine-primed reinstatement. The animals were placed in their original chambers and active and inactive lever presses were measured for the first 60 min in order to allow any context-induced reinstatement to subside.

Rats then received a single non-contingent cocaine-prime dose (0.5 mg/kg) and lever pressing for the next 30 min was observed.

The sucrose self-administration assays were performed as described previously (Kelamangalath et al. 2009). Briefly, rats were allowed to self-administer sucrose pellets for one 90 min session each day under the same conditions described for cocaine, where active lever presses released a 45 mg sucrose pellet. After the self-administration days, the animals underwent an extinction protocol followed by sucrose-primed reinstatement.

Tissue Extraction

In order to identify persistent effects, tissue was extracted 7 days following final drug exposure. Animals were anesthetized with halothane prior to decapitation in compliance with protocols approved by the University of Georgia Animal Care and Use guidelines. The brain was removed from the cranium and cooled in ice-cold oxygenated (95% O₂/5% CO₂) dissection artificial cerebrospinal fluid containing 120 mM NaCl, 3 mM KCl, 4 mM MgCl₂, 1 mM NaH₂PO₄, 26 mM NaHCO₃ and 10 mM glucose. Regions of interest were extracted and flash frozen in liquid nitrogen. For $n \geq 3$ mice/group, the left hemisphere was placed midline down on a coverslip, coated in O.C.T. Embedding Compound (VWR, Radnor, PA), placed in -90°C isobutanol for 30 sec and kept at -80°C until cryosectioning. The tissue was cryosectioned into 10 µm sagittal slices at -20°C for immunohistochemistry.

Genotyping PCR

RNA was isolated region specifically from flash frozen tissue using TRIzol (Invitrogen, Carlsbad, CA) based on the manufacturer's protocol. Total RNA (100 ng) was used for cDNA synthesis using the iScript cDNA Synthesis Kit (Biorad, Hercules, CA). cDNA (100 ng) with a 260:280 >1.8 was used for genotyping PCR with HotStarTaq (Sigma-Aldrich, St. Louis, MI) and a protocol suggested by the Jackson Laboratory (Bar Harbor, ME). The primers used are as followed:

Common sense: 5'-AAGCCTTGATTCTGATGTGGGC-3'

Wild-type p21 anti-sense: 5'-TGACGAAGTCAAAGTTCCACC-3'

Mutant p21 anti-sense: 5'-GCTATCAGGACATAGCGTTGGC-3'

PCR conditions were 94°C for 3 min, followed by 35 cycles of 94°C for 30 sec, 64°C for 1 min and 1 min at 72°C, and finally 2 min at 72°C. For tissue positive for the wild-type p21, a product of 872 bp was produced. For mutated p21, the product was approximately 700 bp.

qPCR

RNA was isolated region specifically from flash frozen tissue using TRIzol (Invitrogen, Carlsbad, CA) based on the manufacturer's protocol. Total RNA (100 ng) was used for cDNA synthesis using the iScript cDNA Synthesis Kit (Biorad, Hercules, CA). cDNA (100 ng) with a 260:280 >1.8 was used for quantitative

PCR using iTaq Universal SYBR Green Supermix (Biorad, Hercules, CA) with primers specific for p21 and GAPDH. The primers used are as follows:

Mouse p21	sense: 5'-CTCTCCCAGTCTCCAAACTTAAA-3'
	anti-sense: 5'-TCATCCTAGCTGGCCTTAGA-3'
Mouse GAPDH	sense: 5'-TCAACAGCAACTCCCCTCTTCCA-3'
	anti-sense: 5'-ACCCTGTTGCTGTAGCCGTATTCA-3'
Rat p21	sense: 5'-ACGTGGCCTTGTCGCTGTCTT-3'
	anti-sense: 5'-TAAGGCAGAAGATGGGGAAGAG-3'
Rat GAPDH	sense: 5'-CACGGCAAGTTCAACGGCACAGTCA-3'
	anti-sense: 5'-GTGAAGACGCCAGTAGACTCCAGG AC-3'

PCR conditions were 95°C for 30 sec, followed by 45 cycles of 95°C for 5 sec and 60°C for 40 sec, then 1 min at 95°C, 1 min at 60°C, and finally 80 cycles of 60°C for 10 sec to generate the melt curve. qPCR results were analyzed via $2^{-\Delta\Delta C_t}$ to determine fold change between saline- and cocaine-treated animals normalized to the housekeeping gene GAPDH.

Immunoblot Analysis

Protein analysis was performed region-specifically using approximately 10-15 mg of flash frozen tissue. Tissue was briefly sonicated in the presence of 300 μ L of standard RIPA buffer. An additional 600 μ L of RIPA buffer was then

added and the samples maintained at constant agitation for 2 hr at 4°C. The samples were then centrifuged for 20 min at 16,000 x g at 4°C, the supernatant transferred to a new tube and the pellet discarded. Protein concentrations were determined using the BCA assay and samples (40 µg) were separated on 4%/9% stacked SDS-PAGE gels and transferred to nitrocellulose membranes. The membranes were blocked in 5% (w/v) milk powder in TBS-T for 2 hrs and exposed to antibodies overnight (p21 F-5 1:200, Santa Cruz Biotechnology Inc., Santa Cruz, CA; doublecortin 1:1000, Abcam, Cambridge, MA; NeuN 1:500, EMD Millipore, Billerica, MA). Membranes were then incubated with a peroxidase conjugated secondary antibody (1:2500 dilution, Promega, Madison, WI) for 4 hrs and bands were visualized using SuperSignal Chemiluminescent substrate (Thermo Scientific, Waltham, MA) and the intensities quantified using Alpha Innotech FluorChem™ HD2 system (ProteinSimple, Santa Clara, CA).

Immunohistochemistry

Immunohistochemistry was performed using Vectastain Universal (Anti-Mouse IgG/Rabbit IgG) Elite ABC Kit (Vector Laboratories, Burlingame, CA) based on the manufacturer's protocol for staining frozen sections. Briefly, sections were removed from -80°C and allowed to air dry at room temperature. They were then fixed for 10 min in pre-chilled acetone at -20°C before rinsing in PBS. The sections were then placed in 0.3% H₂O₂ in methanol for 30 min to quench endogenous peroxidase activity. After washing for 5 min in PBS, the sections were incubated in diluted blocking serum provided in the kit for 20 min. The sections were again washed in PBS and incubated overnight with primary

antibody against p21 (C-19, 1:100, Santa Cruz Biotechnology Inc., Santa Cruz, CA) diluted in 0.1% BSA in PBS at room temperature. The sections were washed in PBS and incubated for 30 min each with diluted biotinylated secondary antibody solution and the Vectastain Elite ABC Reagent. Sections were stained with DAB reagent (Vector Laboratories, Burlingame, CA) and counterstained using Gill's Hematoxylin before mounting with fluoromount. Visualization of staining was performed using a Nikon AZ100 microscope (Tokyo, Japan).

Immunofluorescence

Sections were removed from -80°C and allowed to air dry at room temperature for 20 min. They were then fixed in 1X paraformaldehyde fixing solution for 30 min followed by three 10 min washes with PBS. A permeabilization step was performed with 0.2% Triton X-100 for 2 hrs. The tissue was washed three times for 10 min with PBS containing 0.05% Triton X-100 and then blocked with 5% BSA for 2 hrs. The sections were exposed to primary antibody against NeuN (1:100, Millipore, Billerica, MA) or DCX (1:500, Abcam, Cambridge, MA) overnight and then washed three times in PBS-Triton wash buffer with 0.5% BSA for 30 min. After washing, slides were exposed to fluorescent-tagged secondary antibody for 2 hrs protected from light. The tissues were again washed with PBS-Triton wash buffer with 0.5% BSA three times for 30 min followed by three 5 min washes with PBS. All steps were performed at 4°C and on an orbital shaker at low speed. Fluoromount was used to mount the tissue and visualization performed using an Olympus IX71 microscope (Center Valley, PA).

Methylation-Specific PCR

DNA was isolated region-specifically from flash frozen tissue using a DNeasy tissue kit (Qiagen, Valencia, CA) based on the manufacturer's protocol. Bisulfite conversion was performed using the EZ DNA Methylation kit (Zymo Research Corporation, Irvine, CA) following the manufacturer's instructions. Converted DNA was amplified using PCR with forward and reverse primers covering no CpG sites. Amplification products were confirmed by electrophoresis on a 1% agarose gel. Primers used are as follows:

Methylated rat p21 sense: 5'-TGTATTAGTTATAGGTATTATGTTCTGA-3'

anti-sense: 5'-ACCCCTACAACAAAACCGAA-3'

Un-methylated rat p21 sense: 5'-TGTATTAGTTATAGGTATTATGTTTGA-3'

anti-sense: 5'-CTAACCCCTACAACAAAACCAAA-3'

Methylated mouse p21 sense: 5'-GTTTCGTGGATAGTGAGTAGTTGC-3'

anti-sense: 5'-ACCTTAAACAACCCTAAACTCCG-3'

Un-methylated mouse p21 sense: 5'-GGTTTTGTGGATAGTGAGTAGTTGT-3'

anti-sense: 5'-ACCTTAAACAACCCTAAACTCCA-3'

PCR conditions were 95°C for 5 min, followed by 40 cycles of 95°C for 30 sec and 52°C for 30 sec, then 45 sec at 72°C.

Acetyl-Histone 3 Chromatin Immunoprecipitation

Chromatin immunoprecipitation for acetylated histone 3 was performed using the EpiQuick Tissue Acetyl-Histone H3 ChIP Kit based on the manufacturer's protocol (Epigentek, Farmingdale, NY). Briefly, approximately 40 mg of flash frozen tissue was cross-linked using a 1% formaldehyde solution and 1.25 M glycine solution, and disaggregated. The samples were then centrifuged and the supernatant discarded. Cells were then lysed and the DNA sheared using sonication. The solution was again centrifuged and the supernatant separated and diluted using a ChIP dilution buffer. A portion of the diluted supernatant was saved as "input DNA". Diluted supernatant for each sample was added to Acetyl-Histone 3 antibody-bound wells and incubated on a rocking platform for 1 hr. After a series of washes, the bound DNA was released and the cross-linking reversed. Immunoprecipitated and input DNA was then bound to a spin column and precipitated with ethanol prior to elution. qPCR was then performed on input and immunoprecipitated DNA using primers for the p21 promoter region obtained from Yuan et al. (2013). The primers used are as follows:

Forward Primer: 5'-G TTCAGCCCTGGAACCGAAG-3'

Reverse Primer: 5'- GTACCAAACACCCTTCACCTGGTAC-3'

PCR conditions were 95°C for 5 min, followed by 50 cycles of 95°C for 30 sec, 59°C for 45 sec and 72°C for 45 sec, then 72°C for 10 min. Results were analyzed via $2^{-(\Delta\Delta C_t)}$ to determine the effect on p21 promoter region H3

acetylation between saline- and cocaine-treated animals normalized to the input DNA.

Bligh-Dyer Lipid Extraction

Phospholipids were extracted using chloroform and methanol according to the method of Bligh and Dyer (Bligh and Dyer 1959). Upon sacrifice, trunk blood was collected and suspended in 1 mL of methanol: water (2.0:0.8 v/v). The liquid was transferred to a glass test tube and 1.25 mL chloroform was added. Tubes were vortexed for 30 seconds and allowed to sit for 10 minutes on ice. Tubes were centrifuged at 213 x g for 1 minute and the bottom chloroform layer was transferred to a new test tube. The extraction steps were repeated a second time and the chloroform layers combined. The collected chloroform layers were dried under nitrogen, reconstituted with 50 μ L of methanol: chloroform (2:1 v/v), and stored at -20°C.

Lipid Phosphorus Assay

7 μ L of lipid samples and various concentrations of phosphate standard solutions were placed in glass test tubes and 400 μ L of 10 N sulfuric acid solution added. Samples were incubated for 1 hr in a heat block at 180-200°C. The samples were allowed to cool and 100 μ L of 30% H₂O₂ was added to each tube. The tubes were vortexed and placed back in the heat block for 1.5 hr at 180-200°C. The samples were removed from the heat block and allowed to cool. 4.6 mL of molybdate reagent was added to each tube and the tubes vortexed. 100 μ L of 15% ascorbic acid was added to each tube and the tubes vortexed.

Samples were then placed in 100°C water bath for 7-10 min, then removed from the water bath and allowed to cool. 150 µL of the samples and standards were added to a 96-well plate and the absorbance read at 830 nm. A standard curve was created using the phosphate standards and the concentration of phosphorous in each sample determined.

Electrospray Ionization-Mass Spectrometry (ESI-MS)

Lipid extract samples (500 pmol/µL) were reconstituted in chloroform: methanol (2:1, v/v). ESI-MS was performed as described previously (Kinsey et al. 2008; Peterson et al. 2008; Zhang et al. 2005) using a Trap XCT ion-trap mass spectrometer (Agilent Technologies, Santa Clara, CA) with a nitrogen drying gas flow-rate of 8 L/min at 350°C and a nebulizer pressure of 30 psi. The scanning range was from 100 to 2200 *m/z* on 5 µL of sample scanned in both positive and negative ion mode for 2.5 min with a mobile phase of acetonitrile: methanol: water (2:3:1) and 0.1% ammonium formate.

Multivariate Statistical Analysis of Blood Lipids

Multivariate techniques were executed using MetaboAnalyst 2.0 (<http://www.metaboanalyst.ca/MetaboAnalyst/faces/Home.jsp>) with default algorithms. Automatic peak detection and spectrum deconvolution was performed using a peak width set to 0.5. Data were normalized to the total ion count, and scaled to the z-value. No outliers were removed from the analysis.

Select lipids that were identified using a volcano plot analysis were further studied for correlations to cocaine-induced behaviors and further identified using SimLipid (Premier Biosoft), a comprehensive lipidome data analysis software that allows users to tentatively identify lipid species using m/z values from MS data based on a continuously updated database containing links from Kyoto Encyclopedia of Genes and Genomes (KEGG), Human Metabolome Database (HMDB), Chemical Entities of Biological Interest (CEBI), PubChem Substance database, LIPIDBANK and LIPIDAT. This analysis was confirmed by screening of m/z values in Lipid Maps. Following identification, the expression of each parent lipid was normalized to the total ion count, and the change in expression compared to control was determined. This method is standard for lipidomic analysis as reported in our previous studies (Cummings et al. 2015; Kinsey et al. 2008; Peterson et al. 2008).

Statistical Analysis

Tissue isolated from a single animal or a single animal's behavior data represented one experiment ($n=1$). Unless otherwise indicated, data are presented as mean $\pm$ SEM of at least 3 different animals ($n\geq 3$). For data sets with confirmed Gaussian distribution following normality testing, unpaired two-tailed Student's T-test was used to compare data across two groups. For those without Gaussian distribution, a nonparametric test such as the Mann-Whitney test was used. To compare across multiple groups, One-way ANOVA followed by Bonferonni post-hoc test was performed. In all cases, GraphPad Prism software (La Jolla, CA) was used and $p < 0.05$ was considered indicative of a statistically

significant difference between mean values. Statistical analysis of lipid data was performed using MetaboAnalyst 2.0 software with a t-test threshold of $p < 0.05$ and fold change of 1.5.

CHAPTER 3

REWARDING STIMULI ALTER HIPPOCAMPAL p21 EXPRESSION SECTOR- SPECIFICALLY¹

¹Scholpa N.E., Briggs S.B., Wagner J.J and Cummings B.S. To be submitted to *Journal of Pharmacology and Experimental Therapeutics*.

Abstract

This study tested the effect of cocaine on the expression of cyclin-dependent kinase inhibitor 1a (Cdkn1a or p21) in the rodent brain. Cocaine exposure increased p21 expression in the rat hippocampus, but not in the dorsal or ventral striatum, where the nucleus accumbens is located. The increase in p21 was specific to the ventral sector of the hippocampus and was observed following contingent and non-contingent drug administration, suggesting that the effect is not specific to protocol or route of exposure. This increased expression was accompanied by increased histone acetylation of the p21 promoter region. Sucrose self-administration also increased p21 expression in the ventral hippocampus, demonstrating that a natural rewarding stimulus could mimic the effects of cocaine. Similarly to rats, cocaine administration increased p21 expression exclusively in the ventral hippocampus of mice, indicating that the effect is also not species-specific. Analysis of basal p21 expression in control rats and mice demonstrated higher basal levels in the dorsal hippocampus, compared to the ventral hippocampus. This finding correlated with higher basal histone 3 acetylation of the p21 promoter region, with more acetylation being present within the dorsal sector of the hippocampus. These data indicate that basal p21 expression and reward-induced increases in p21 in the ventral hippocampus may be regulated via epigenetic mechanisms.

Introduction

While many data support a cognitive role for the hippocampus, contributing to the formation of declarative memories, there is also support for hippocampal involvement in emotional regulation and stress response. This dichotomy suggests the existence of dual “basic” functions of this divisive brain region. Increasing evidence indicates that this separation of hippocampal function exists along the dorsoventral axis, with the dorsal sector serving the cognitive function and the ventral sector serving the emotional function (**Figure 1.4**). Additionally, gene expression and synaptic projection patterns vary along this axis, suggesting that the hippocampus may not only be divided functionally, but also anatomically (Fanselow and Dong 2010).

Considerable behavioral testing has reinforced the idea of the aforementioned functional separation within the hippocampus, and has implicated the dorsal sector in spatial navigation and memory. The Morris water maze, during which a rodent must swim to a hidden platform using spatial cues placed outside of the pool, is a common assay for assessing spatial cognition. It has been found that lesioning as little as 25% of the dorsal hippocampus impairs successful completion of the water maze task, while damage to the ventral hippocampus has no effect (Moser et al. 1995). Similar results were observed when using the radial arm maze, another method of examining spatial memory (Pothuizen et al. 2004). In line with this behavioral data, compared to the ventral hippocampus, the dorsal hippocampus has been shown to have significantly more place cells, which are specific pyramidal neurons within the hippocampus

that become active upon entering a certain place in an animal's environment (Fenton et al. 2008; Jung et al. 1994). Together, these place cells are thought to act as a cognitive representation of a particular location, or "cognitive map" (O'Keefe and Nadel 1978). In contrast with findings from the dorsal hippocampus, lesioning of the ventral hippocampus has been found to reduce anxiety-like behaviors in rodents, such as freezing and fear response (Bannerman et al. 2003; Bannerman et al. 2004; Kjelstrup et al. 2002). Additionally, it has also been shown that increasing the activity of granule cells within the dentate gyrus of the ventral hippocampus, but not the dorsal hippocampus, attenuates these anxiety-related behaviors (Kheirbek et al. 2013).

Substantiating hippocampal dichotomy, the ventral and dorsal sectors also have distinct efferent and afferent connectivity (O'Leary and Cryan 2014; Strange et al. 2014). The dorsal subiculum sends projections to brain regions primarily involved in processing visual and spatial information and memories, including the anterior cingulate cortex, retrosplenial cortex and mammillary nuclei. Conversely, the ventral hippocampus has more connections to structures involved in behavioral responses to stress, anxiety, motivation and reward, including the medial prefrontal cortex, amygdala and nucleus accumbens (O'Leary and Cryan 2014). For example, ventral hippocampal lesions have been shown to lessen stress-induced increases in corticosterone in plasma, presumably through efferent circuitry to the hypothalamus via the ventral subiculum (Kjelstrup et al. 2002; O'Leary and Cryan 2014). Similar findings have been observed between

the posterior and anterior hippocampus of humans, analogous to the dorsal and ventral hippocampus of rodents, respectively (Poppenk et al. 2013).

Interestingly, both the dorsal and ventral hippocampus have circuitry connecting to the ventral tegmental area (VTA) (O'Leary and Cryan 2014). The VTA is comprised of dopaminergic neurons, the basis of the best-characterized reward circuit in the brain. Dopaminergic neurons from the VTA project to various other brain structures including the hippocampus, amygdala and, most notably regarding the reward pathway, the nucleus accumbens (NAc). Interestingly, in terms of the dopamine pathway, reference to 'the hippocampus' generally refers to the ventral hippocampus, as the majority of dopaminergic projections from the VTA innervate the ventral, not the dorsal, sector (Verney et al. 1985). These brain structures are inter-connected via complex circuitry outlined in **Figure 3.1**. The medium spiny neurons of the NAc have GABAergic projections to the VTA. Additionally, there are GABAergic projections from the NAc to the ventral pallidum, which also has GABAergic projections to the VTA; so while the NAc can directly inhibit the VTA, it can also result in disinhibition of this structure via the ventral pallidum. The NAc also receives glutamatergic innervation via the medial prefrontal cortex, hippocampus and amygdala, which also have glutamatergic connections with each other. Each of these regions also has GABAergic interneurons, which attenuate their functional output. Furthermore, many of these regions receive serotonin projections from the raphe nucleus and norepinephrine projections from the locus coeruleus (Russo and Nestler 2013). While the VTA-NAc pathway is crucial for recognition of rewarding stimuli, these

regions also respond to aversive stimuli (Koob and Le Moal 2008). For this reason, a more accurate description of this circuitry may be the 'dopamine motivation pathway.'

The dorsal and ventral sectors of the hippocampus also have varying responses to rewarding stimuli, including drugs of abuse. Inactivation of the dorsal hippocampus was found to impair contextual reinstatement to cocaine (Fuchs et al. 2005), which is not surprising considering the prominent role of the dorsal hippocampus in spatial learning and memory. Selective disruption of the ventral hippocampus, on the other hand, was found to diminish cocaine-seeking following either drug-paired cues or drug-priming (Rogers and See 2007). It is hypothesized that this effect is largely due to the connections between the ventral hippocampus and regions involved in drug-seeking, such as the amygdala, which is involved in cocaine-paired associative learning (See 2005), and the NAc shell, which is implicated in reinstatement of cocaine-seeking behavior (Kalivas and McFarland 2003). These data, among others, suggest that the ventral hippocampus may have an integral role in relapse and cocaine addiction. The extent of the separation in function between the dorsal and ventral sectors of the hippocampus with regard to reward and the dopamine pathway, however, has not yet been fully characterized.

Accumulation of available data suggests that neurogenesis may also be segregated along the dorsoventral axis of the hippocampus (O'Leary and Cryan 2014). New neurons have distinct features depending on the location of their synthesis within the hippocampus. For example, not only are more neurons

synthesized within the dorsal sector, but newly born neurons also mature faster in this sector than in the ventral hippocampus (Jinno 2011b; Piatti et al. 2011; Snyder et al. 2009). Although controversial, some studies have also shown that stress decreases neurogenesis in the ventral hippocampus, but not the dorsal hippocampus (Jayatissa et al. 2006; O'Leary and Cryan 2014; Tanti et al. 2012). Additionally, the natural decrease in neurogenesis observed with aging appears to occur at a faster rate within the ventral sector (Jinno 2011a). Given the role of the ventral hippocampus in emotion and reward, it is hypothesized that this phenomenon may contribute to the development of depression preceding dementia in older individuals (Jinno 2011a). An increasing number of studies have indicated that drugs known to alter hippocampal neurogenesis, such as antidepressants, may be doing so sector-specifically (O'Leary and Cryan 2014). Based on this information and the physiological variation between the dorsal and ventral hippocampus, it is plausible that the expression of proteins that control cell proliferation and neurogenesis, such as the cell cycle modulator p21, may not only have differential expression within hippocampal sectors, but may also be differentially regulated in response to stimuli.

Results

Effect of Cocaine on p21 mRNA Expression in Rats

Brain tissue from rats that had been sensitized to cocaine using an established protocol (Keralapurath et al. 2014; Seymour and Wagner 2008) underwent region-specific qPCR analysis for p21 mRNA. These studies were performed using the hippocampus, the dorsal striatum and ventral striatum, which is where the NAc is located (Scimeca and Badre 2012). No differences in p21 mRNA expression were observed between saline- and cocaine-treated rats in either the dorsal or ventral striatum; however, p21 mRNA expression increased 2-fold in the hippocampus of rats exposed to cocaine compared to that of rats exposed to saline (**Figure 3.2**).

Effect of Cocaine Self-Administration on p21 mRNA in Rats

We also performed the aforementioned studies on tissue from animals that had taken part in a cocaine self-administration assay, which is considered more relevant to human circumstances. Based on the above data, we confined our analyses to the hippocampus, further dividing the region into the dorsal hippocampus and the ventral hippocampus. Cocaine exposure did not alter the expression of p21 mRNA in the dorsal hippocampus, but did induce an approximate 3-fold increase in p21 mRNA expression in the ventral hippocampus, compared to saline treatment (**Figure 3.3A**).

Effect of Cocaine Self-Administration on p21 Protein Expression in Rats

Immunoblot analysis was performed to demonstrate if alterations in mRNA expression were reflected in protein. As expected, no significant difference in p21 protein expression was detected between cocaine- and saline-treated animals in the dorsal hippocampus. However, in accordance to what was observed with p21 mRNA expression, p21 protein expression was significantly increased in the ventral hippocampus of rats that had self-administered cocaine compared to those that had self-administered saline (**Figure 3.3B, C**).

Effect of Sucrose Self-Administration on p21 mRNA and Protein Expression in Rats

Similar to what was observed following cocaine exposure, treatment of rats with sucrose did not alter p21 mRNA expression in the dorsal hippocampus as compared to saline-treated rats. In contrast, sucrose self-administration increased p21 mRNA expression by approximately 3.5-fold in the ventral hippocampus of rats, as compared to those exposed to saline (**Figure 3.3A**). Not surprisingly, sucrose administration did not alter p21 protein expression in the dorsal hippocampus compared to saline, yet there was a significant increase in p21 protein expression in the ventral hippocampus of sucrose-exposed rats as compared to saline controls (**Figure 3.3B, C**).

Effect of Cocaine on p21 mRNA Expression in Mice

The dorsal and ventral sectors of the hippocampus were extracted from mice that had taken part in non-contingent experimenter-administrated cocaine behavior assays (timeline depicted in **Figure 3.4**), and region-specific p21 mRNA

expression was determined. Similar to the data reported in rats, cocaine-treatment increased p21 mRNA expression specifically in the ventral hippocampus (**Figure 3.4**). These data suggest that cocaine-induced increases in hippocampal p21 mRNA may be conserved across species.

Effect of Cocaine on p21 Protein Expression in Mice

Immunohistochemistry for p21 protein expression was performed to verify the expression data reported above. **Figure 3.5** shows representative staining for the dorsal and ventral hippocampus of wild-type control mice and those exposed to cocaine. Similar to data reported by Pechnick et al. (2011), positive p21 staining was present in the subgranular zone of the dentate gyrus. In addition, positive staining was also observed in the pyramidal cell bodies of the CA region of the dorsal and ventral hippocampus of both saline- (**Figure 3.5A, B**) and cocaine-treated (**Figure 3.5C, D**) mice. While there was no difference in staining intensity between saline- and cocaine-treated animals in the dorsal hippocampus (**Figure 3.5A, C**), there was an increase in staining observed in the ventral hippocampus of mice exposed to cocaine compared to those exposed to saline (**Figure 3.5B, D**). This increase in positive staining was particularly present in the CA1 region/ventral subiculum (**Figure 3.5**, white arrows) and the dentate gyrus (**Figure 3.5**, black arrows).

Effect of Cocaine Self-Administration on p-p53 (Ser15) Protein Expression in Rats

To begin to attempt to assess the mechanism of the cocaine-induced increase in p21 expression observed, immunoblot analysis was performed on tissue from rats that had self-administered cocaine to determine if the increase in p21 expression could be correlated to alterations in p53 phosphorylation. No significant difference in p-p53 (Ser15) protein expression was detected between cocaine- and saline-treated animals in either the ventral hippocampus (**Figure 3.6**) or the dorsal hippocampus (data not shown).

Effect of Cocaine on DNA Methylation of the p21 Coding Region in Rodents

To investigate the role of epigenetic regulation in cocaine-induced increases in hippocampal p21, we first performed bisulfite conversion and methylation-specific PCR to determine DNA methylation of the p21 coding region in the hippocampus of rodents exposed to cocaine compared to those exposed to saline. We observed an increase in un-methylated p21 in the hippocampus of cocaine-treated rats compared to saline-treated rats, with no change in methylated p21 (**Figure 3.7A**), while no difference was observed in the ventral or dorsal striatum (data not shown). An increase in un-methylated DNA, or decrease in methylated DNA, is associated with increased transcription and expression. These findings, therefore, correlate with the increase in p21 mRNA and protein expression observed in the hippocampus of rats exposed to cocaine, compared to those exposed to saline.

Even though there was also an increase in p21 mRNA expression in the ventral hippocampus of cocaine- compared to saline-exposed mice, there was no

difference in DNA methylation of the p21 coding region observed (**Figure 3.7B**). These data suggest that while changes in DNA methylation of the p21 coding region may contribute to the effects observed in cocaine-treated rats, this is not the sole mechanism of cocaine-induced increases in p21 mRNA and protein expression.

Effect of Cocaine on H3 Acetylation of the p21 Gene

qPCR analysis on acetylated histone 3 chromatin immunoprecipitated DNA demonstrated no difference in p21 promoter histone 3 acetylation in the dorsal hippocampus of cocaine self-administration rats compared to saline self-administration rats. In contrast, there was an approximate 5-fold increase in histone 3 acetylation of the p21 promoter region in the ventral hippocampus of rats that had self-administered cocaine compared to saline self-administration rats (**Figure 3.8**). Immunoprecipitated DNA was compared to input DNA for each sample.

Region-Specific Basal Hippocampal Expression of p21 in Rodents

Interestingly, qPCR analysis in saline self-administration rats indicated higher basal p21 expression in the dorsal hippocampus than in the ventral hippocampus of these control animals (**Figure 3.9A**). Additionally, there was a significant dorsal/ventral difference in the status of the saline control animals with regard to histone 3 acetylation of the p21 promoter, with the basal level being approximately 5-fold higher in the dorsal hippocampus than in the ventral hippocampus of the same animals (**Figure 3.9B**), correlating to the mRNA

expression pattern. The immunohistochemistry also indicated higher basal protein expression of p21 in the dorsal hippocampus compared to the ventral hippocampus of mice (**Figure 3.5A, B**) and again, this was observed with p21 mRNA via qPCR analysis (**Figure 3.9A**).

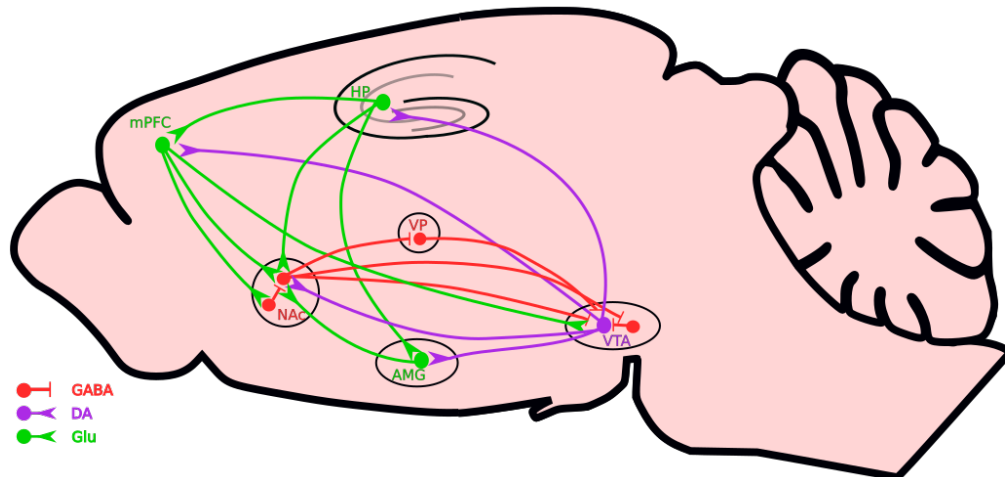


Figure 3.1. Dopamine Motivational Pathway Circuitry. Schematic derived using Inkscape (www.inkscape.org). Dopaminergic neurons (purple) from the VTA innervate several brain structures including the hippocampus (HP), medial prefrontal cortex (mPFC), nucleus accumbens (NAc) and amygdala (AMG). The mPFC, HP and AMG have glutamatergic projections (green) to various structures within the pathway. GABAergic neurons (red) from the NAc inhibit the VTA, as well as project to inhibitory interneurons within the VTA, causing disinhibition. Disinhibition can also occur via NAc GABAergic neurons projecting to the ventral pallidum (VP), inhibiting GABAergic projections to the VTA.

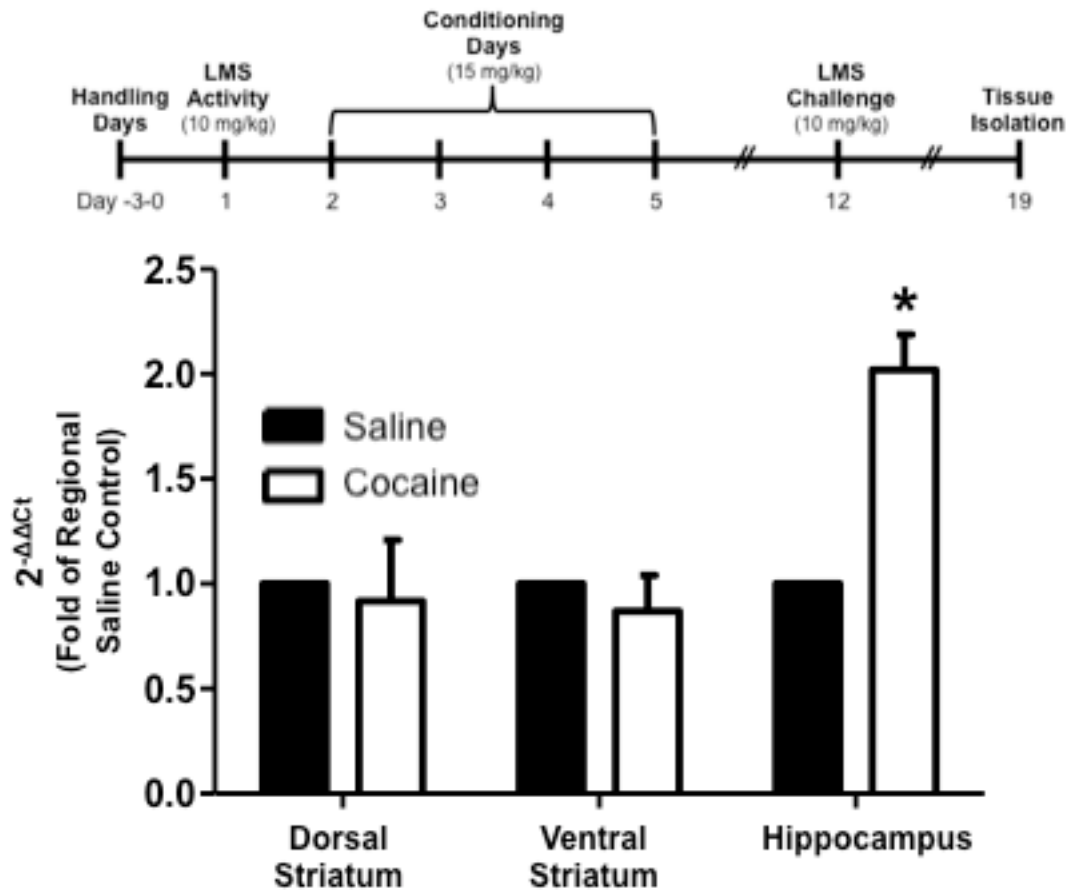


Figure 3.2. Effect of Cocaine on p21 mRNA Expression in Rat Brain Tissue. Rats were injected i.p with either cocaine or saline under conditions shown to induce sensitization to cocaine. Seven days after the final exposure, the indicated brain tissue was collected and assessed for p21 mRNA expression using qPCR. Data are indicative of at least 3 separate animals per group ($n \geq 3$) and are expressed as $2^{-(\Delta\Delta Ct)} \pm \text{SEM } \Delta Ct$ for each group ($*p < 0.05$ compared to saline-treated control).

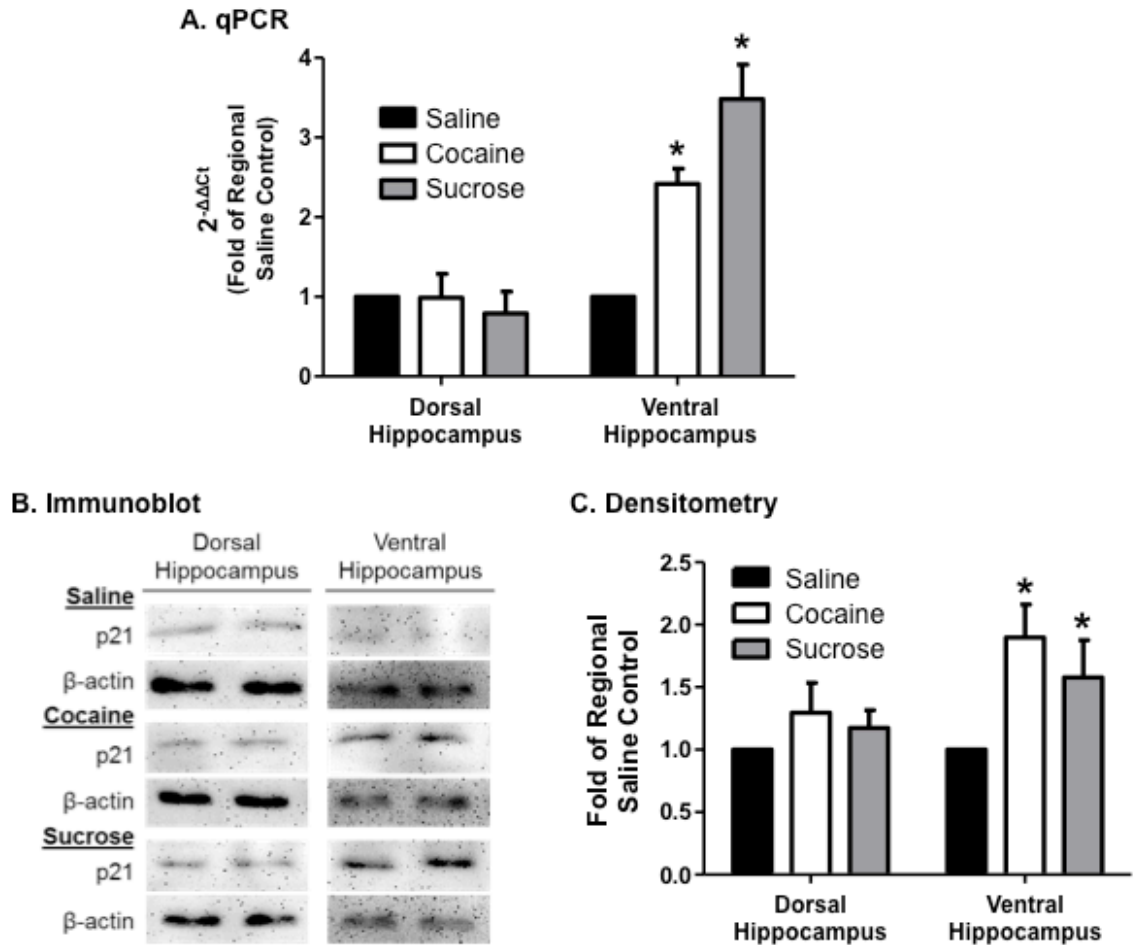


Figure 3.3. Effect of Self-Administration of Cocaine and Sucrose on p21 Expression in the Rat Hippocampus. Rats underwent a cocaine or sucrose self-administration assay as described above. Seven days after the final exposure, the indicated brain tissue was collected and assessed for p21 mRNA expression using qPCR (**A**) and for protein expression using immunoblot analysis (**B**, **C**). Data are indicative of at least 3 separate animals per group ($n \geq 3$) and are expressed as $2^{-(\Delta\Delta Ct)} \pm \text{SEM } \Delta Ct$ for each group in **A** and as mean $\pm$ SEM in **C**. (* $p < 0.05$ compared to saline-treated control).

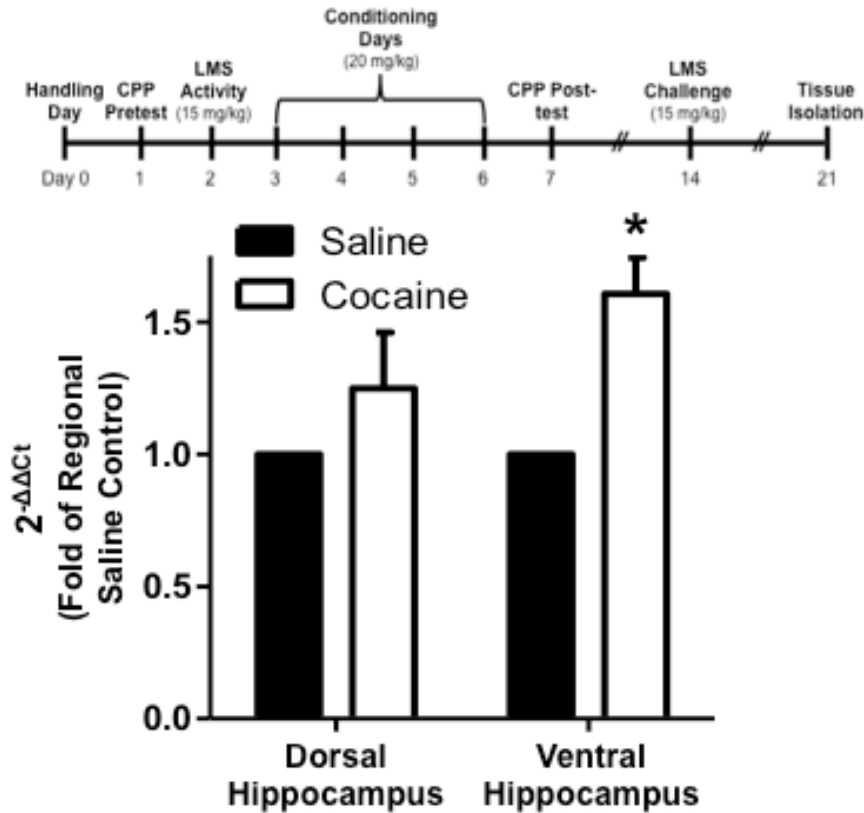


Figure 3.4. Effect of Cocaine on p21 mRNA Expression in Mouse Brain Tissue. Mice were injected i.p with either cocaine or saline under conditions shown to induce sensitization to cocaine. Seven days after the final exposure, the indicated brain tissue was collected and assessed for p21 mRNA expression using qPCR. Data are indicative of at least 3 separate animals per group ($n \geq 3$) and are expressed as $2^{-(\Delta\Delta Ct)} \pm \text{SEM } \Delta Ct$ for each group ($*p < 0.05$ compared to saline-treated control).

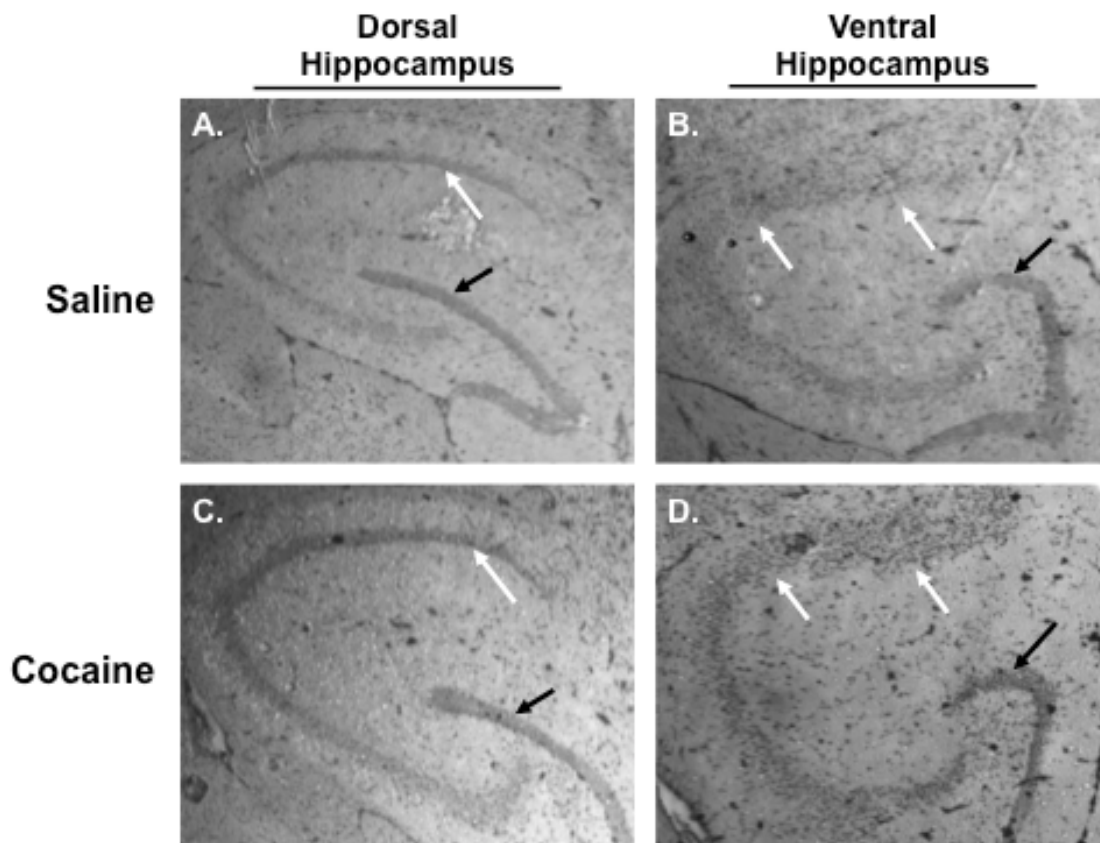
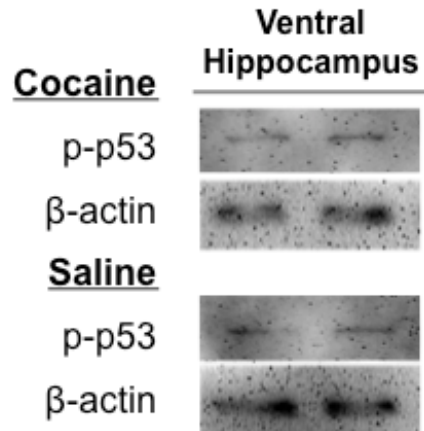


Figure 3.5. Effect of Cocaine on p21 Protein Expression in Wild-Type Mice. Wild-type mice were injected i.p with either cocaine or saline under conditions shown to induce sensitization to cocaine. Seven days following the final exposure, the left brain hemisphere was collected for cryosectioning and 10 μ m sagittal slices were obtained. p21 protein expression was assessed in the dorsal and ventral hippocampus of saline- (**A**, **B**) and cocaine-treated (**C**, **D**) mice using immunohistochemistry. Data are indicative of results from at least 3 separate animals per group ($n \geq 3$).

A. Immunoblot



B. Densitometry

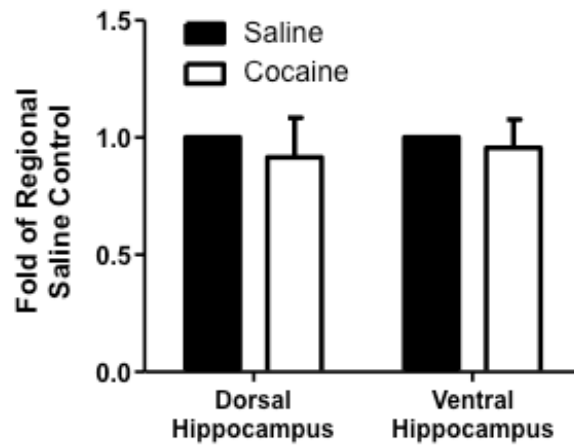
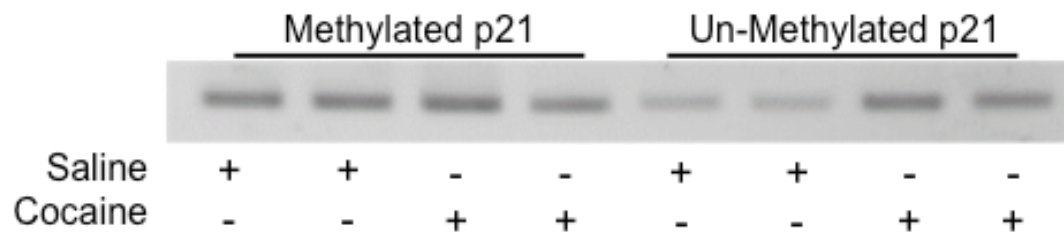


Figure 3.6. Effect of Cocaine on p-p53 (Ser15) Protein Expression in the Rat Hippocampus. Rats underwent a cocaine self-administration assay as described above. Seven days after the final exposure, the indicated brain tissue was collected and assessed for p-p53 protein expression using immunoblot analysis. Data are indicative of at least 3 separate animals per group ($n \geq 3$) and are expressed as mean $\pm$ SEM in **B**.

A. Rat Hippocampus



B. Mouse Hippocampus

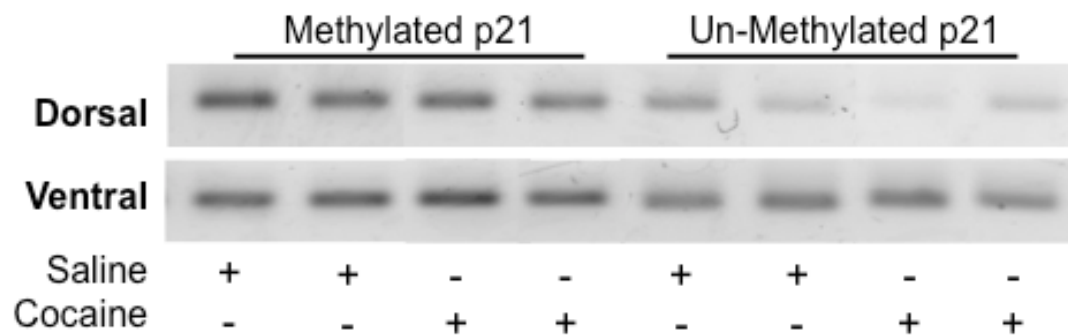


Figure 3.7. Effect of Cocaine on DNA Methylation of the p21 Coding Region in the Hippocampus of Rodents. Rats (**A**) and mice (**B**) were injected i.p with either cocaine or saline under conditions shown to induce sensitization to cocaine. Seven days after the final exposure, the indicated brain tissue was collected and assessed for p21 DNA methylation using bisulfite conversion and methylation specific PCR. Data are indicative of at least 3 separate animals per group ($n \geq 3$).

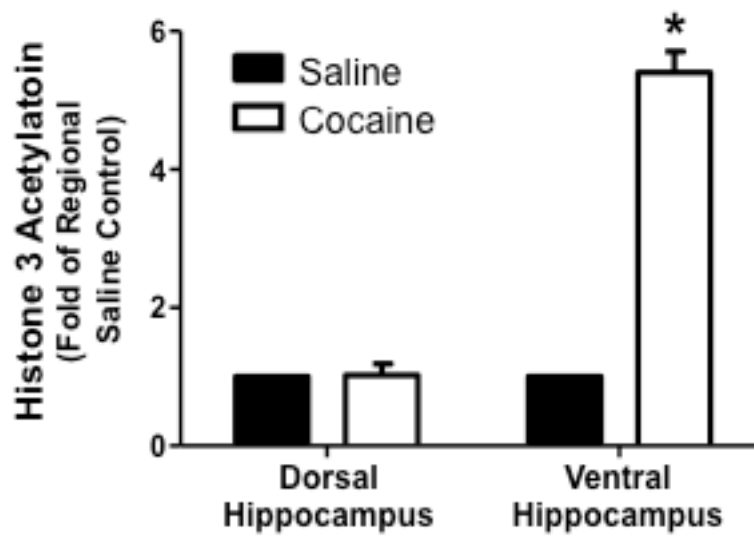


Figure 3.8. Effect of Cocaine on H3 Acetylation of the p21 Promoter Region. Rats underwent a self-administration assay for cocaine or sucrose as described above. Seven days after the final exposure, the indicated brain tissue was collected and assessed for histone 3 acetylation of the p21 promoter region using chromatin immunoprecipitation. Data are indicative of at least 3 separate animals per group ($n \geq 3$) and are expressed as $2^{-(\Delta\Delta Ct)} \pm \text{SEM } \Delta Ct$ compared to input DNA in ($*p < 0.05$ compared to regional saline-treated control).

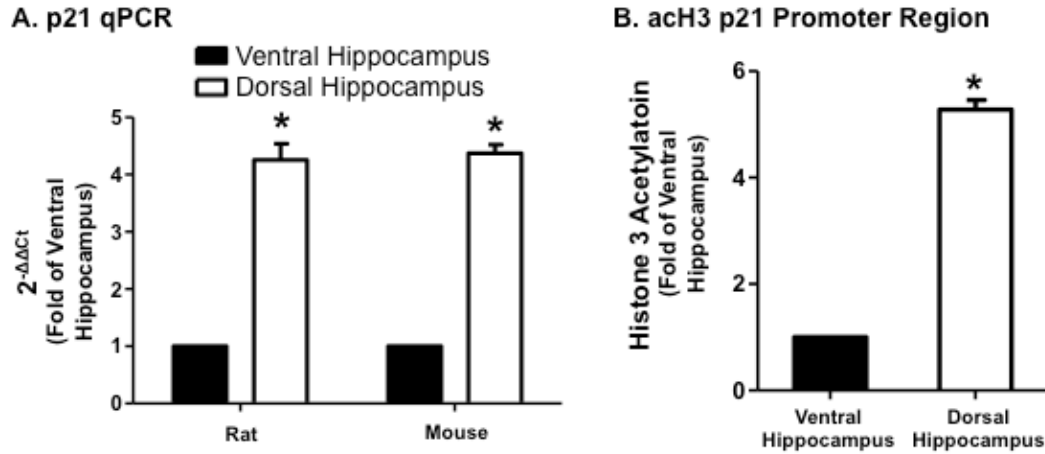


Figure 3.9. Region-Specific Basal Hippocampal p21 Expression in the Hippocampus of Rodents. The indicated brain tissue was collected from saline-exposed rats and mice and assessed for p21 mRNA expression using qPCR (**A**), or histone 3 acetylation of the p21 promoter using chromatin immunoprecipitation (**B**). Data are indicative of at least 3 separate animals per group ($n \geq 3$) and are expressed as $2^{-(\Delta\Delta Ct)} \pm \text{SEM } \Delta Ct$ for each group in **A** and as $2^{-(\Delta\Delta Ct)} \pm \text{SEM } \Delta Ct$ compared to input DNA in **B** (* $p < 0.05$ compared to ventral hippocampus).

Discussion

While the effects of cocaine and rewarding stimuli within the brain have been extensively studied, no studies exist assessing p21 expression in the hippocampus following cocaine exposure. In fact, many studies concerning the actions of addictive drugs have focused on the ventral striatum/nucleus accumbens (LaPlant and Nestler 2011; Larson et al. 2010; Meyer et al. 2009; Nestler 2012; Renthal et al. 2009; Robison and Nestler 2011). One such report performed a genome-wide analysis of cocaine-induced histone modifications in the mouse NAc and found an increase in histone acetylation of p21 (Renthal et al. 2009), but this study did not discuss the effect on p21 transcription or expression, nor did it contain data regarding the hippocampus.

Experimenter-administered cocaine treatment had a region-specific effect on the expression of p21, with increases observed in the rat hippocampus, but not in the ventral striatum, where the NAc is located. The hippocampus is involved in the formation of long-term memories and reinstatement to drug-seeking behavior (Fuchs et al. 2005; Lasseter et al. 2010; Rogers and See 2007; Scoville and Milner 1957). The selective increase in p21 observed in the ventral hippocampus, as opposed to the dorsal hippocampus or other brain regions, demonstrates that cocaine not only induces region-specific alterations in p21 expression, but also sector-specific alterations within a singular structure. The fact that cocaine-treatment increased p21 in both mice and rats, and that p21 was also increased by cocaine self-administration supports the conclusion that this effect is neither protocol- nor species-specific.

Not only do these data show that both p21 mRNA and protein expression is increased in response to cocaine in the ventral hippocampus, but also that epigenetic alterations may be responsible. Several studies show that both acute and chronic cocaine exposure alter gene expression in the limbic system via epigenetic mechanisms (LaPlant and Nestler 2011; Nielsen et al. 2012; Renthall et al. 2009; Robison and Nestler 2011; Schmidt et al. 2013; Wong et al. 2011; Zhou et al. 2011). In general, cocaine induces a more permissive transcription state through not only alterations in DNA methylation, but also histone modifications such as increased acetylation of histones 3 and 4 in brain regions in the mesolimbic reward pathway (LaPlant and Nestler 2011; Nielsen et al. 2012; Renthall et al. 2009; Schmidt et al. 2013; Wong et al. 2011). As mentioned previously, supplemental material in a study by Renthall et al. (2009) suggested an effect of cocaine on histone acetylation of p21 in the NAc. Our data indicates that this effect extends to the ventral hippocampus as well. Rewarding stimuli are known to induce dopamine release, and while the NAc is generally the region of focus for this effect, dopamine is also released in the hippocampus. Here, it can act via the D1/D5 class of dopamine receptors, resulting in an increase in histone 3 acetylation (Huang et al. 2014). This mechanism could explain the increase in histone 3 acetylation in the p21 promoter region observed in the hippocampus following cocaine administration. Interestingly, under basal conditions p21 expression was significantly lower in the ventral hippocampus than in the dorsal hippocampus, correlating with levels of histone acetylation of the p21 promoter region. In response to cocaine, p21 promoter histone acetylation within the

ventral hippocampus rose to levels comparable to that of the dorsal hippocampus. Therefore, the increase in hippocampal p21 expression observed in response to cocaine may be confined to the ventral hippocampus because of histone acetylation saturation in the dorsal sector.

Both sucrose self-administration and cocaine administration induced p21 expression, suggesting that this effect is not drug-specific. Rats maintained on a sugar-rich diet show behavioral and neurochemical changes similar to rats dependent on drugs of abuse (Hoebel et al. 1999). Further, the dopamine hypothesis of reward dictates that drugs of abuse and rewarding food substances activate certain common pathways (Hernandez and Hoebel 1988; Spangler et al. 2004). Thus, the observation that either sucrose self-administration or cocaine administration induces p21 expression can be understood. The occurrence of locomotor sensitization is common to the behavioral protocols that altered ventral hippocampal p21 expression. In addition to the animals from non-contingent studies demonstrating locomotor sensitization to cocaine, self-administration has also been found to augment behavioral and neurochemical responses to a challenge dose, resembling sensitization (Hooks et al. 1994). Thus these behaviors could be related to the changes in p21 expression observed in the ventral hippocampus.

As discussed, there is known to be extensive dichotomy between the function and physiological characteristics of the dorsal versus the ventral hippocampus, including incoming and outgoing projections as well as gene expression. In fact, genome-wide investigation has provided evidence for genetic

domains with discrete borders within the hippocampus (Thompson et al. 2008). Therefore, the inconsistency in p21 expression observed between the two sectors is reasonable. There is no evidence, however, that a clear boundary exists separating the hippocampus into just two portions: dorsal and ventral (Strange et al. 2014). The dentate gyrus, for example, can be segregated into three major domains: dorsal, intermediate and ventral, with subsequent subdomains (Dong et al. 2009). This tripartite model of the dorsoventral axis is gaining popularity and evidentiary support (Dong et al. 2009; Fanselow and Dong 2010; O'Reilly et al. 2014; Strange et al. 2014). Therefore, a limitation of this study is the lack of analysis of an intermediate sector of the hippocampus. Despite the aforementioned data regarding the differential expression of p21 along this dorsoventral axis, information regarding the functional role of this expression is still needed.

CHAPTER 4

ALTERATION IN HIPPOCAMPAL p21 EXPRESSION MODULATES COCAINE- INDUCED RESPONSE AND MOTIVATED BEHAVIORS¹

¹Scholpa N.E., Briggs S.B., Wagner J.J and Cummings B.S. To be submitted to
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Abstract

This study investigated the role of the cyclin-dependent kinase inhibitor p21 (Cdkn1a/WAF-1) in cocaine-induced response using a functional p21 knockout mouse model. Acute locomotor activity following cocaine administration (15 mg/kg, i.p.) was decreased in $p21^{-/-}$ mice, as compared to that induced in wild-type mice, yet both phenotypes became sensitized to cocaine. Additionally, cocaine-induced place preference was enhanced in $p21^{-/-}$ mice. While this could be an indicator of increased activation of the reward pathway, it could also be an indirect measure of cognitive ability. To address this, mice were exposed to the dysphoric κ -opioid U50,488 during a conditioned place aversion assay. Similar to cocaine, U50,488-induced conditioned place aversion was enhanced in $p21^{-/-}$ mice compared to wild-type mice, suggesting differences in learning and memory between the two models. These findings correlated to increased expression of the neurogenesis markers NeuN and doublecortin in the dorsal hippocampus of $p21^{-/-}$ mice. Interestingly, both cocaine and U50,488 increased dorsal hippocampal levels of NeuN in wild-type mice, indicating an effect of the behavior assay used and not of the stimuli. Collectively, these results show that genetic ablation of p21 expression altered acute locomotor response to cocaine, as well as conditioned responses to either rewarding or aversive stimuli. Increased neurogenesis in the dorsal hippocampus of $p21^{-/-}$ mice may attribute to the enhanced cognitive performance observed. These findings demonstrate previously un-reported involvement of p21 in modulating responses to cocaine and motivated behaviors.

Introduction

Studies investigating addiction often rely on various drug-induced behavioral effects for analyses. Animal models of drug-taking behavior, including both non-contingent experimenter-administered, and contingent self-administered exposure (Steketee and Kalivas 2011), are highly predictive of human responses (Griffiths et al. 1979; O'Brien and Gardner 2005). Depending on the drug administered and the pattern of administration, an animal can either develop tolerance or sensitization. Tolerance is an adaption by which repeated drug exposure over time results in attenuation of one or more of the drug-induced effects, and is seen following chronic high-dose drug administration (Lowinson 2005). Increased response to a stimulus after repeated exposure is known as sensitization, and occurs following intermittent low-dose administration (Lowinson 2005). In rodent models, sensitization to drugs of abuse is often determined by enhanced locomotor activity following repeated drug exposure (Steketee and Kalivas 2011) and can be termed locomotor sensitization (LMS). LMS occurs with multiple drugs including cocaine, amphetamine, morphine, nicotine and ethanol (Benwell and Balfour 1992; Cunningham and Noble 1992; Joyce and Iversen 1979; Post et al. 1991; Robinson and Becker 1986; Steketee and Kalivas 2011). Interestingly, cross-sensitization between drugs has been observed (Itzhak and Martin 1999; Lett 1989; Vezina et al. 1989), suggesting that common mechanisms underlie sensitization despite different drug classes and binding sites (Steketee and Kalivas 2011). Sensitization, while common following psychostimulant exposure in rodent models, does not seem to occur in humans.

The reason for this discrepancy could be the pattern by which humans use cocaine. In general, human cocaine-use tends to be in binges and not in the low-dose, intermittent pattern that is known to cause sensitization in rodents (O'Brien and Gardner 2005). Another potential explanation lies with the likelihood of developing addiction. Approximately 85% of people who experiment with cocaine do not become addicted, while 80-95% of rodents develop sensitization (Anthony et al. 1994). Therefore, it is possible that the majority of people do experience a form of sensitization, during which cocaine becomes unpleasant or aversive, while those that do not, become addicted (O'Brien and Gardner 2005).

The *Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition* (DSM-V) combines substance abuse and substance dependence into a single disorder known as Substance Use Disorder (Association 2013). This disorder is defined as a combination of cognitive, behavioral and physiological symptoms indicating continued use of a substance despite drug-related problems. Diagnosis of substance use disorders is based on a series of behaviors. Among these behaviors are drug-taking and drug-seeking. Though often assessed in rodents using a self-administration model, drug-seeking can also be measured using non-contingent models. One method for this is conditioned place preference (CPP). CPP is based off of Pavlovian stimulus learning, during which a previously neutral environmental stimulus acquires incentive salience by repeated pairing with rewarding drug administration (Bindra 1968; O'Brien and Gardner 2005). After the association between the environment and the reward state has been developed, the animal is allowed to choose between the drug-

paired environment and a non-drug-paired environment. An animal spending increased time in the drug-paired environment is exhibiting drug-seeking behavior, and the drug is presumed to be rewarding. The opposite is also true: if an animal spends less time in the drug-paired side, the drug is presumed aversive (O'Brien and Gardner 2005). In general, there is agreement between drugs that are considered rewarding in other assays and those found to be rewarding using CPP (Tzschentke 1998).

As discussed in Chapter 3, evidence suggests that the ventral and dorsal sectors have differing roles within the hippocampus and, as such, are responsible for different aspects of drug-induced behavioral response. Damage to the dorsal hippocampus impairs contextual reinstatement to cocaine, while ventral hippocampal disruption weakens drug-seeking in response to cocaine priming (Fuchs et al. 2005; Rogers and See 2007). Given the finding that cocaine and sucrose alter hippocampal p21 expression in the hippocampus, we utilized a p21 knockout ($p21^{-/-}$) mouse model to determine the effect of the absence of p21 on certain cocaine-induced behaviors, namely CPP and LMS.

Neurogenesis has been negatively correlated with drug-taking and drug-seeking behaviors (Noonan et al. 2010). Additionally, studies have suggested a role for p21 in neurogenesis, in that p21 decreases neural cell proliferation (Pechnick et al. 2008; Zonis et al. 2013). Further, there have been several studies investigating the effect of cocaine on neurogenesis; however, the findings have been controversial and variable depending on concentration and dosing regimen (Andersen et al. 2007; Garcia-Fuster et al. 2010; Sudai et al. 2011;

Yamaguchi et al. 2004). Although these published data suggest a connection between p21, neurogenesis and cocaine-induced behavior, this association has not yet been directly investigated or identified. As mentioned in Chapter 3, neurogenesis also appears to be segregated within the hippocampus along the dorsoventral axis, and drugs known to alter hippocampal neurogenesis are suggested to do so sector-specifically (O'Leary and Cryan 2014). These data led us to explore not only the relationship between p21, cocaine and hippocampal neurogenesis, but also the sector-specificity of this relationship.

Results

Genotyping of wild-type and p21^{-/-} mice

All mice were genotyped using primers and a PCR protocol (**Table 4.1, 4.2**) suggested by The Jackson Laboratory for wild-type p21 and the mutant p21 gene used for this strain. Products of ~900 bp with the wild-type (WT) primers indicate the presence of wild-type p21 (**Figure 4.1A**), while products of ~700 bp with the mutant (MT) primers indicate the presence of the mutated, functionally inactive form of p21, as found in the *p21^{-/-}* mice (**Figure 4.1B**). Products with both sets of primers indicate heterozygous mice. Fortunately, all mice were homozygous and as advertised.

Locomotor sensitization of wild-type versus p21^{-/-} mice

While the data presented in Chapter 3 show that p21 expression is increased in the ventral hippocampus of cocaine-treated animals compared to saline-treated animals, they do not indicate if this expression mediates behavioral response to the drug. To address this question, we utilized a p21 knockout (*p21^{-/-}*) mouse model in a behavioral protocol that combines locomotor activity and place preference assessments in the same animal (**Figure 3.4**). We first verified that wild-type mice respond to cocaine in a locomotor sensitization (LMS) assay similarly to rats (Seymour and Wagner 2008). Cocaine-treated mice exhibited increased locomotor activity post-injection (i.p 15 mg/kg) compared to saline-treated mice during the Activity Day (protocol day 2, **Figure 4.2A**). Additionally, during the Challenge Day (protocol day 14), the cocaine-treated wild-type mice

demonstrated significantly greater locomotor activity post-exposure than that seen during the Activity Day (i.e. sensitization).

The locomotor response to the initial cocaine exposure on the Activity Day of the protocol was significantly diminished in the $p21^{-/-}$ mice compared to the wild-type mice, with the activity of $p21^{-/-}$ mice being no greater with cocaine than with saline administration (**Figure 4.2C**). However, following conditioning with cocaine, the locomotor response to the drug was also enhanced in $p21^{-/-}$ mice, as assessed on the Challenge Day, and the magnitude of sensitization did not significantly differ between the two mouse models (**Figure 4.2B, D**). There was no difference in locomotor activity observed between saline-treated $p21^{-/-}$ and wild-type mice, nor was there a difference observed pre-injection between any groups (**Figure 4.2A, B**).

Conditioned place preference of wild-type versus $p21^{-/-}$ mice

Wild-type mice again acted similarly in response to cocaine as rat models during the conditioned place preference (CPP) assay (Seymour and Wagner 2008). Both cocaine-treated wild-type and $p21^{-/-}$ mice spent significantly more time in the designated drug-paired compartment during the CPP Post-test than during the Pretest (**Figure 4.3**). Interestingly, this preference for the drug-paired compartment during the Post-test was significantly greater for cocaine-treated $p21^{-/-}$ mice than wild-type mice. Saline-treated mice spent the same amount of time in the drug-paired compartment during the Pretest and Post-test.

Locomotor dose response in wild-type and $p21^{-/-}$ mice

We hypothesized that the diminished locomotor response to cocaine observed in the $p21^{-/-}$ mice might be due to a rightward shift in dose response in these animals. To test this, we exposed mice to varying concentrations of cocaine in an Activity Day setting, measuring their locomotor activity after injection. At 7.5 mg/kg, wild-type mice depicted movement no greater than that observed with saline, while at 25 mg/kg, wild-type mice had similar movement to that observed in response to 15 mg/kg, suggesting a plateau effect (**Figure 4.4A**). The $p21^{-/-}$ mice depicted significantly increased movement in response to 20 mg/kg and 25 mg/kg cocaine, compared to saline exposure, which was not observed following 15 mg/kg cocaine. The movement post-injection with these higher concentrations was comparable to that seen in wild-type mice in response to 15 mg/kg (**Figure 4.4B**).

Role of serotonin (5-HT) in cocaine-induced conditioned place preference of wild-type and $p21^{-/-}$ mice

A possible contributor to the diminished locomotor response observed in the $p21^{-/-}$ mice is a deficient dopamine pathway compared to wild-type mice, as dopamine is mainly responsible for locomotor movement in response to cocaine (Giros et al. 1996; Sora et al. 2001; Sora et al. 1998; Uhl et al. 2002; Zhuang et al. 2001). Dopamine deficient mice, however, still develop cocaine-induced conditioned place preference mediated by serotonin (Hnasko et al. 2007; Sora et al. 2001; Sora et al. 1998). To determine if serotonin was responsible for the cocaine-induced CPP observed in the $p21^{-/-}$ mice, we pretreated the mice with the serotonin receptor antagonist methiothepin maleate, which has been shown

to inhibit cocaine-induced CPP in mice with dopamine deficiencies (Hnasko et al. 2007), prior to cocaine conditioning. As expected, this pretreatment had no effect on cocaine-induced CPP in wild-type mice (data not shown). Additionally, $p21^{-/-}$ mice successfully displayed CPP with and without pretreatment with the serotonin antagonist, with no difference in time spent in the cocaine-paired side post-conditioning (**Figure 4.5**).

Locomotor sensitization and conditioned place aversion of wild-type versus $p21^{-/-}$ mice

Wild-type and $p21^{-/-}$ mice also underwent a conditioned place aversion assay using U50,488, a selective κ -opioid receptor agonist known to cause dysphoria and stress-like effects in rodents (Muschamp et al. 2012; Smith et al. 2012; Valdez and Harshberger 2012). To maintain consistency, locomotor sensitization was also investigated as part of this protocol. Similar to that observed by Ukai and Kameyama (1985), exposure to 5 mg/kg U50,488 did not alter locomotor activity compared to saline (**Figure 4.6A, B**).

In the wild-type mice, U50,488 exposure induced a decrease of approximately 150 sec in time spent in the drug-paired compartment during the Post-test, as compared to the Pretest; however, this value was not significantly different from that seen with saline treatment (**Figure 4.6C**). On the other hand, the U50,488-treated $p21^{-/-}$ mice did spend significantly less time (approximately 300 sec) in the drug-paired compartment during the Post-test compared to the Pretest (**Figure 4.6C**). Notably, U50,488 exposure had no effect on p21 mRNA

expression in either the dorsal or ventral hippocampus, as compared to saline treatment (**Figure 4.6D**).

Effect of p21 on hippocampal NeuN and DCX expression

To further investigate the enhanced place preference learning in the $p21^{-/-}$ mice, we examined the effect of p21 on doublecortin (DCX), a common marker for neurogenesis transiently expressed in immature neurons. To investigate more persistent effects, we also assessed NeuN, a marker of adult neurons, which increases in expression as DCX expression decreases (Brown et al. 2003). We observed increased expression of both DCX and NeuN in the dorsal hippocampus of $p21^{-/-}$ mice compared to wild-type mice using immunofluorescence (**Figure 4.7A**) and immunoblot analysis (**Figure 4.7B, C**). This effect was not observed in the ventral hippocampus (data not shown).

Effect of cocaine and U50,488 on hippocampal NeuN and DCX expression

We also investigated the effect of cocaine and U50,488 on NeuN and DCX expression in the hippocampus of wild-type mice. NeuN expression was increased in the dorsal hippocampus of cocaine-treated mice compared to saline-treated mice (**Figure 4.8A, C**), while there was no difference in DCX expression (data not shown). Mice exposed to U50,488 also displayed increased NeuN protein expression in the dorsal hippocampus and these mice had significantly decreased NeuN expression in the ventral hippocampus, compared to those exposed to saline (**Figure 4.8B, C**). NeuN expression was not altered in the hippocampus of cocaine-treated $p21^{-/-}$ mice (data not shown).

Table 4.1 Genotyping Reaction Mixture

Reaction Component	Final Concentration	Volume (μL)
ddH ₂ O	-	6.95
HotStarTaq Buffer	1X	2
MgCl ₂	2.5 mM	2
dNTPs	0.2 mM	0.4
Forward Primer (10 μM)	1.33 μM	2.66
Reverse Primer (10 μM)	1.33 μM	2.66
HotStarTaq	1.02 U/μM	0.33
cDNA	-	3
Total		20

Table 4.2 Genotyping PCR Protocol

Cycling Step	Temperature (°C)	Time
1	94	3 min
2	94	30 sec
3	64	1 min
4	72	1 min
Repeat Steps 2-4 for 35 Cycles		
5	72	2 min
6	10	Hold

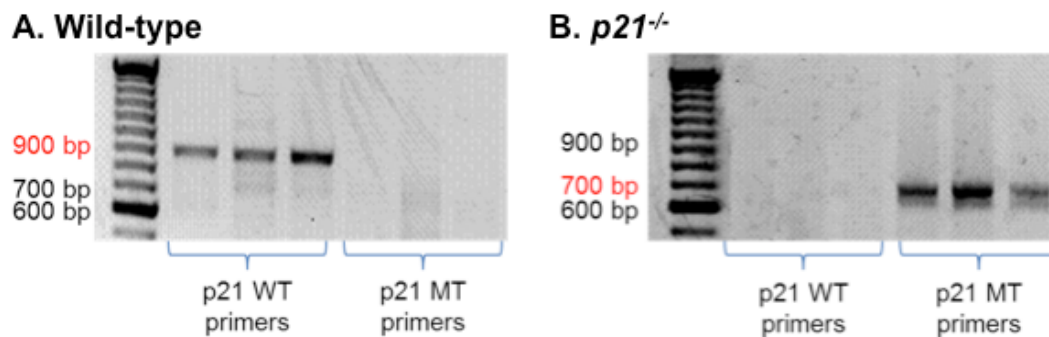


Figure 4.1. Genotyping of Wild-Type and *p21*^{-/-} Mice. cDNA from wild-type and *p21*^{-/-} mice was extracted and used for p21 genotyping using primers and a protocol suggested by The Jackson Laboratory. Animals positive for the wild-type p21 gene had products of ~900 bp when using the wild-type (WT) primers, and no products with the mutant (MT) primers (**A**). Animals positive for the mutant p21 gene, as expected in the *p21*^{-/-} mice, had products of ~700 bp with the mutant primers, but no products with the wild-type primers (**B**). Data for three animals per strain are presented, though all animals were tested.

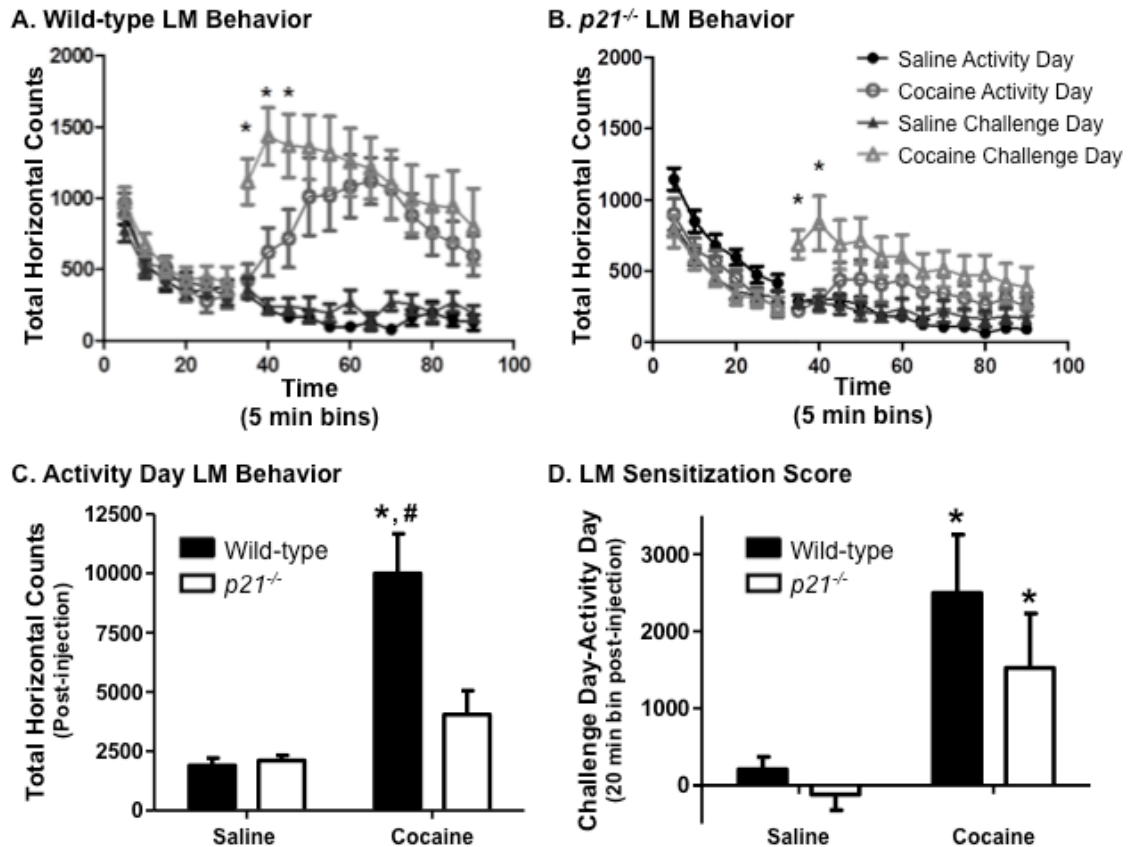


Figure 4.2. Effect of $p21$ on Cocaine-Induced Locomotor Activity. Mice were injected i.p. with either cocaine or saline under conditions shown to induce sensitization to cocaine. Locomotor activity was measured after the first exposure (Activity Day) and after the final injection (Challenge Day) in wild-type (**A**) and $p21^{-/-}$ (**B**) mice (* $p < 0.05$ compared to Activity Day). Total horizontal counts following the Activity Day injection were measured (**C**, * $p < 0.05$ compared to saline, # $p < 0.05$ compared to $p21^{-/-}$). The difference in total horizontal counts during the first 20 min post-injection during the Activity Day and after the final exposure (Challenge Day) was used to evaluate cocaine-induced sensitization (**D**, * $p < 0.05$ compared to saline). Behavioral data are indicative of a total of 14

saline-treated ($n=14$) and 12 cocaine-treated ($n=12$) wild-type and $p21^{-/-}$ mice, and are expressed as mean $\pm$ SEM.

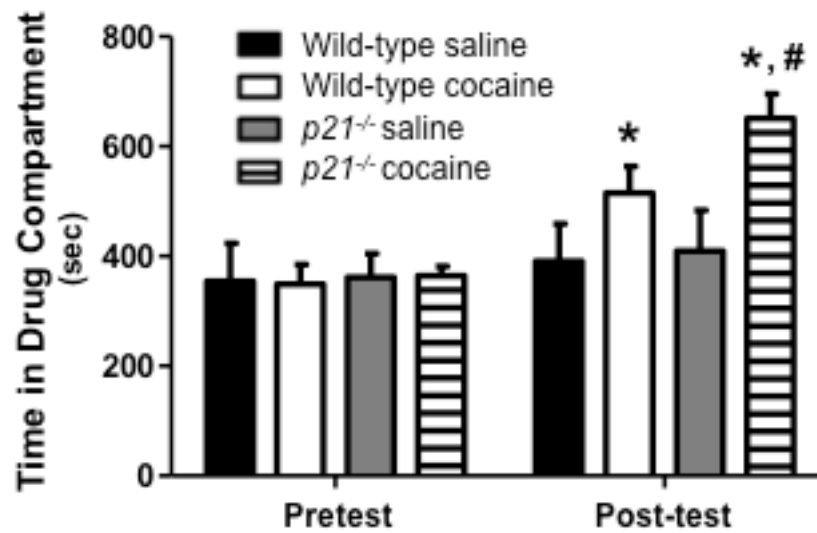


Figure 4.3. Effect of *p21* on Cocaine-Induced Conditioned Place Preference. Mice were injected i.p with either cocaine or saline under conditions shown to induce sensitization to cocaine. Cocaine-induced conditioned place preference was examined in wild-type and *p21*^{-/-} mice (**p* < 0.05 compared to Pretest, #*p* < 0.05 compared to wild-type). Behavioral data are indicative of a total of 14 saline-treated (*n*=14) and 12 cocaine-treated (*n*=12) wild-type and *p21*^{-/-} mice, and are expressed as mean ± SEM.

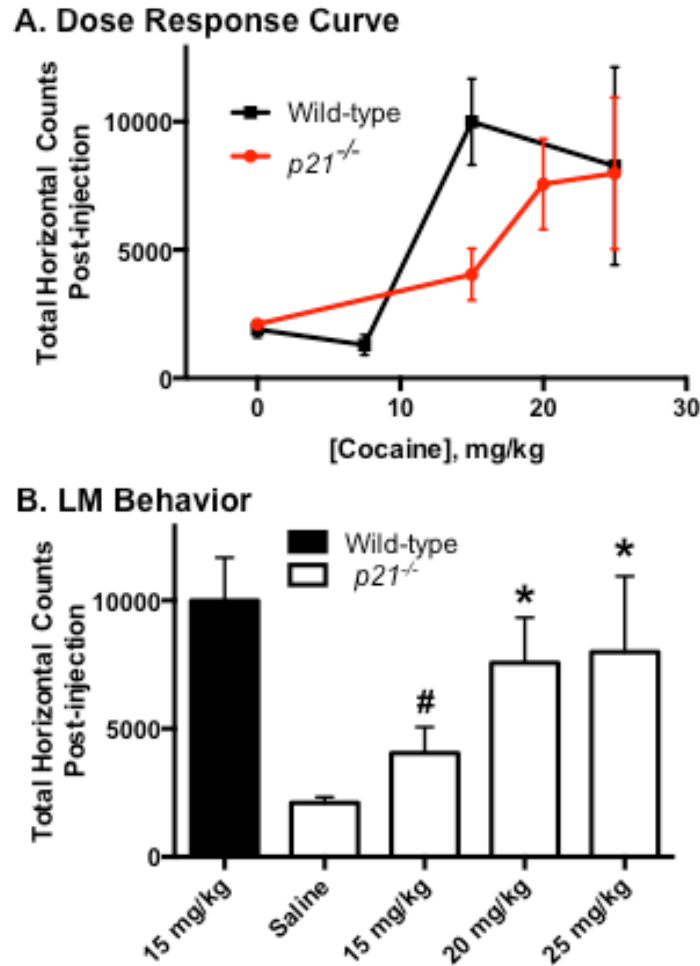


Figure 4.4. Effect of p21 on Cocaine-Induced Locomotor Dose Response. Mice were injected i.p with either saline or various concentrations of cocaine and locomotor activity measured post-injection. Wild-type mice were injected with 7.5, 15 or 25 mg/kg cocaine, and $p21^{-/-}$ mice were injected with 15, 20 or 25 mg/kg cocaine (**B**, * $p < 0.05$ compared to $p21^{-/-}$ saline-treated control, # $p < 0.05$ compared to wild-type 15 mg/kg). Behavior data are indicative of 14 saline-treated wild-type and $p21^{-/-}$ mice ($n=14$), 4 7.5 mg/kg cocaine-treated wild-type mice ($n=4$), 12 15 mg/kg cocaine-treated wild-type and $p21^{-/-}$ mice ($n=12$), 4 25 mg/kg cocaine-treated wild-type mice ($n=4$), 6 20 mg/kg cocaine-treated $p21^{-/-}$ mice ($n=6$) and 6 25 mg/kg cocaine-treated $p21^{-/-}$ mice ($n=6$).

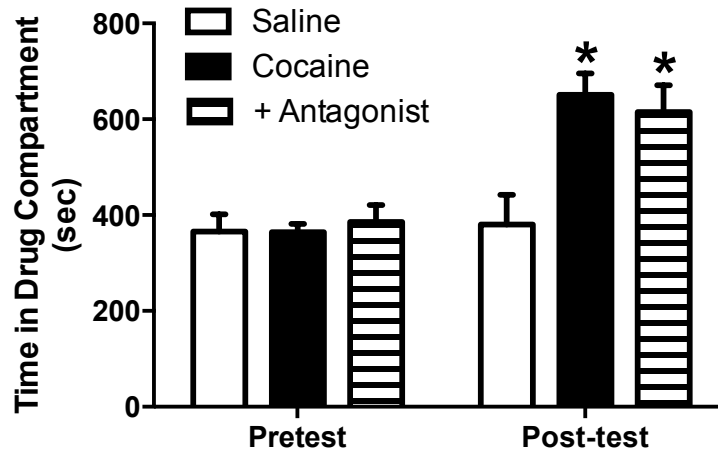


Figure 4.5. Role of Serotonin on Cocaine-Induced Conditioned Place Preference in $p21^{-/-}$ Mice. Mice were injected i.p with 50 $\mu\text{g/kg}$ of the 5-HT receptor antagonist methiothepin maleate 30 min prior to cocaine conditioning sessions on days 3-6, and with saline prior to same-day saline conditioning sessions. Cocaine-induced conditioned place preference was examined ($*p < 0.05$ compared to *Pretest*). Behavior data are indicative of 14 saline-treated animals ($n=14$), 12 cocaine-treated animals ($n=12$) and 9 pretreated cocaine-treated animals ($n=9$).

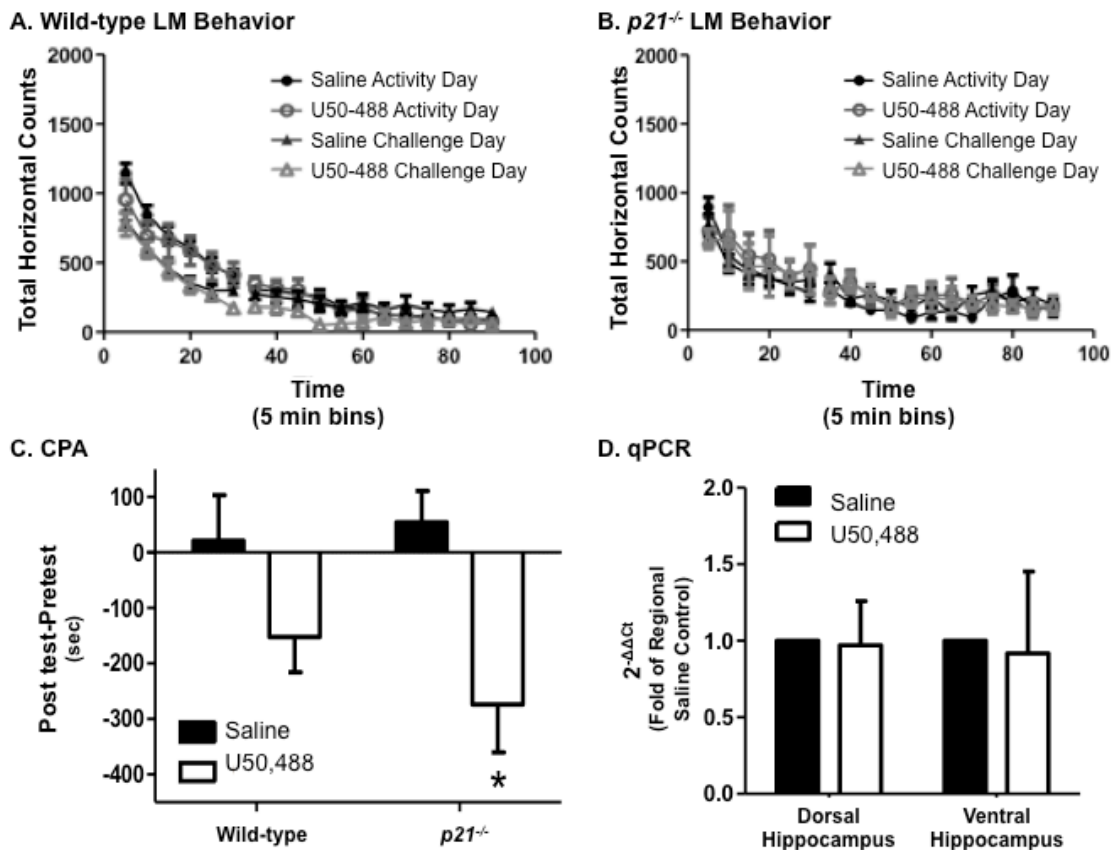


Figure 4.6. Effect of $p21$ on U50,488-Induced Locomotor Activity and Conditioned Place Aversion. Mice were injected i.p with either U50,488 or saline under conditions shown to induce sensitization to cocaine. Locomotor activity was measured after the first exposure (Activity Day) and after the final injection (Challenge Day) to evaluate U50,488-induced sensitization in wild-type (**A**) and $p21^{-/-}$ (**B**) animals. U50,488-induced conditioned place preference was also examined (**C**, $*p < 0.05$ compared to saline). Behavioral data are indicative of a total of 14 saline-treated ($n=14$) and 6 U50,488-treated ($n=6$) wild-type and $p21^{-/-}$ mice, and are expressed as mean $\pm$ SEM. The indicated tissues were assessed

for p21 mRNA expression and data are indicative of at least 4 separate animals per group ($n \geq 4$, ***D***).

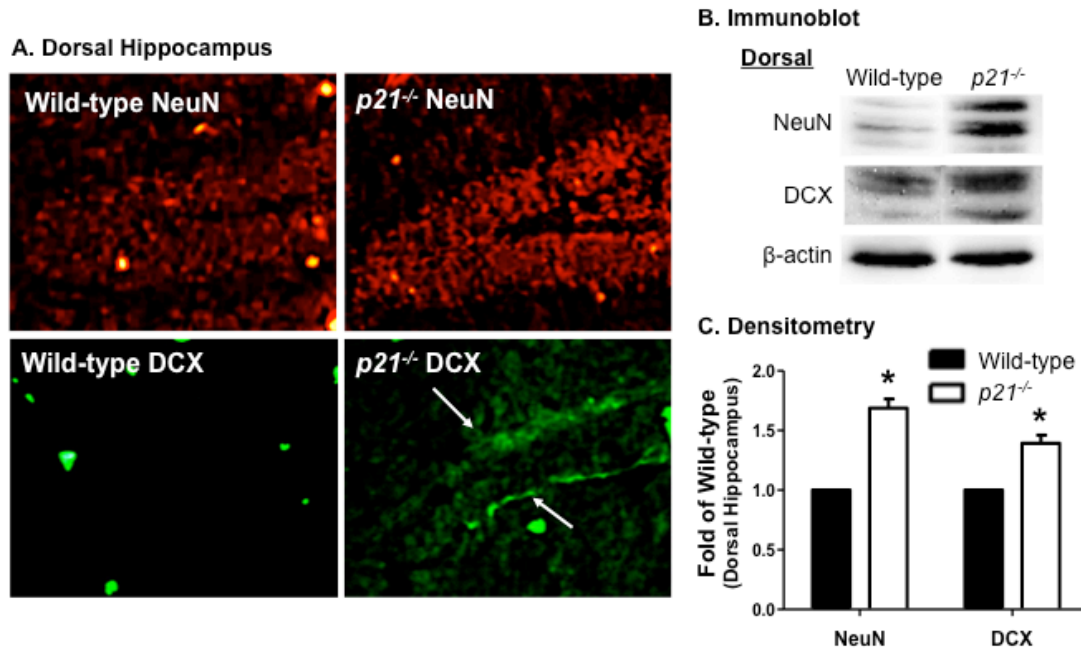


Figure 4.7. Effect of p21 on Dorsal Hippocampal NeuN and DCX Expression. The indicated brain tissue was collected and 10 μ m sagittal slices were obtained from the left hemisphere of wild-type and $p21^{-/-}$ mice. NeuN and DCX protein expression were assessed in the hippocampus using immunofluorescence (**A**) and immunoblot analysis (**B**, **C**). Data are indicative of at least 3 separate animals per group ($n \geq 3$) and are expressed as mean $\pm$ SEM in panel **C** (* $p < 0.05$ compared to saline-treated control).

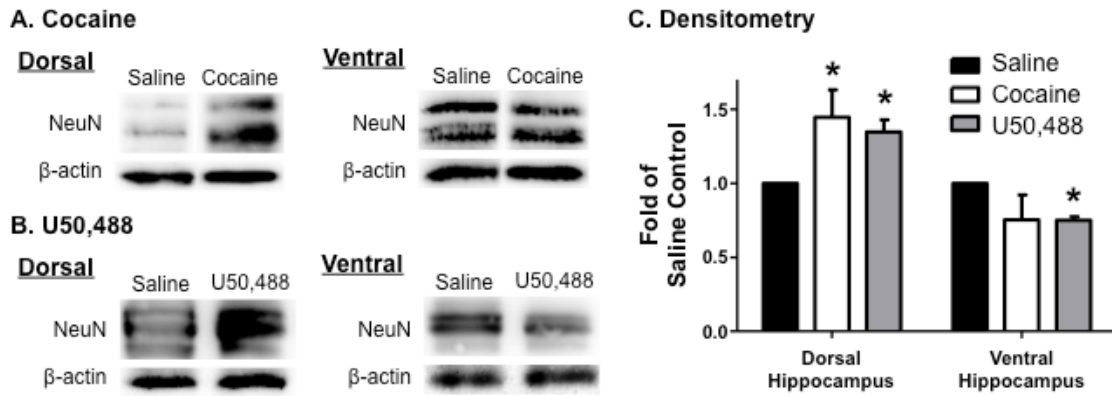


Figure 4.8. Effect of Cocaine and U50,488 on Hippocampal NeuN Expression. Wild-type mice were injected i.p. with either cocaine (**A**), U50,488 (**B**) or saline under conditions shown to induce sensitization to cocaine. Seven days after the final exposure, the indicated brain tissue was collected and assessed for NeuN protein expression using immunoblot analysis. Data are indicative of at least 3 separate animals per group ($n \geq 3$) and are expressed as mean $\pm$ SEM in **C** (* $p < 0.05$ compared to saline-treated control).

Discussion

The neurochemical mechanisms underlying the effects of cocaine are well studied; however, the role of p21 in these effects has not received much attention. Data demonstrating that genetic ablation of p21 alters locomotor activity and CPP in response to cocaine are exciting findings. While the enhanced cocaine-induced CPP performance in the *p21^{-/-}* mice could be due to increased salience of the drug reward, it could also be attributed to increased learning capacity. In support of the latter hypothesis, performance differences were also observed with CPA induced by U50,488 under the same protocol. Following treatment with U50,488, both mouse models exhibited an aversive trend; however the place aversion was significant only in the *p21^{-/-}* mice, suggesting enhanced associative learning capability. Major findings obtained from the behavior assays performed here are two-fold: first, altering p21 expression significantly diminishes the response to an initial cocaine exposure, demonstrating a previously unreported relationship between this cyclin-dependent kinase inhibitor and drug-induced locomotor behavior. Second, the absence of functional p21 significantly enhances place preference learning motivated by either a rewarding or an aversive stimulus.

Multiple studies suggest a correlation between adult neurogenesis and cognitive ability (Cameron and Glover, 2015; Deng et al. 2010; Raber et al., 2004; Shors et al., 2001; Shors et al., 2002; Snyder et al., 2009b; Winocur et al., 2006; Wojtowicz et al., 2008). *p21^{-/-}* mice have been found to have higher levels of baseline neurogenesis as suggested by increased expression of hippocampal

DCX and NeuN (Pechnick et al. 2011). Here we confirm that *p21*^{-/-} mice express higher levels of both DCX and NeuN, and we further report that these increases were specific to the dorsal hippocampus. As previously mentioned, the dorsal sector of the hippocampus is primarily involved in performing cognitive functions and spatial learning and memory (Moser et al. 1995). Thus, our data confirm that heightened ongoing neurogenesis occurs in the *p21*^{-/-} mice, and that this likely contributes to the enhanced place preference following conditioning with motivational stimuli. *p21*^{-/-} mice also had a blunted initial locomotor response to cocaine as compared to wild-type animals. The finding that the cocaine-induced CPP in the *p21*^{-/-} mice was not mediated by serotonin suggests that this effect is not due to dopamine deficiency, but could instead be attributed to neurogenesis. Noonan et al. showed that suppression of neurogenesis prior to drug-taking resulted in a leftward shift in dose response to cocaine (Noonan et al. 2010). Therefore, the effect we observed here may be due to a rightward shift in dose response in these *p21*^{-/-} mice due to the inherent increase in basal neurogenesis. Our dose response data support this hypothesis, as the locomotor response observed in the *p21*^{-/-} mice following 25 mg/kg cocaine was comparable to that observed in the wild-type mice following the initial 15 mg/kg.

The available data concerning the effect of cocaine on neurogenesis is variable (Andersen et al. 2007; Garcia-Fuster et al. 2010; Sudai et al. 2011; Yamaguchi et al. 2004). In the current work, cocaine-exposed wild-type mice had significantly increased NeuN expression in the dorsal hippocampus and a modest, insignificant decrease in the ventral hippocampus. If the effect of

cocaine on neurogenesis as measured in the earlier reports was based on the hippocampus in its entirety, the sector-specificity we observe could account for some of the inconsistencies in the literature. A significant decrease in NeuN expression in the ventral hippocampus was observed with U50,488-treated mice as compared to saline-treated wild-type mice. The ventral sector has been shown to be more susceptible to stress-induced decreases in neurogenesis (Jayatissa et al. 2006; Tanti et al. 2012), suggesting that our treatment with the κ -agonist may have evoked a stress-like effect. This occurred, however, in the absence of alterations in p21 expression. Interestingly, as with cocaine, we also observed an increase in NeuN in the dorsal hippocampus in response to conditioning with U50,488. Given the distinct pharmacological mechanisms and motivational properties of cocaine and U50,488, it is unlikely that the similar effects on NeuN are due to the direct effects of the stimuli themselves. Therefore, we hypothesize that this increase in NeuN in the dorsal hippocampus is due instead to the associative learning that occurs during the behavior assays, as the dorsal hippocampus has been previously implicated in conditioned place preference performance and learning has been shown to enhance adult hippocampal neurogenesis (Gould et al. 1999). If this hypothesis is correct, studies investigating the effect of a stimulus on neurogenesis will need to consider the method by which the stimulus is being introduced. If the assay requires an animal to learn, as with CPP and CPA, this behavior itself could alter neurogenesis. It should be noted that NeuN expression in $p21^{-/-}$ mice was not altered following the conditioning protocol (data not shown). A potential explanation for this

observation is that the inherent increase in neurogenesis in these animals may render them unresponsive to further activation.

The data presented here show that genetic ablation of p21 results in altered motivation-induced behavior and dorsal hippocampal neurogenesis, consistent with enhanced cognitive ability. Interestingly, the increase in p21 expression induced by cocaine and sucrose in the ventral hippocampus did not correspond with significant alterations in neurogenesis in that sector. Similarly, the decrease in neurogenesis in the ventral hippocampus following U50,488 treatment did not occur in tandem with alterations in p21. These data suggest that while p21 may not play a role in neurogenesis in the ventral hippocampus, alterations in p21 may affect the regulation of neurogenesis in the dorsal hippocampus, and subsequently impact cognitive ability and motivated behaviors.

CHAPTER 5

EFFECT OF COCAINE AND p21 EXPRESSION ON THE BLOOD LIPIDOME OF MICE¹

¹Scholpa N.E., Sahin S., Wagner J.J. and Cummings B.S. To be submitted to *Drug and Alcohol Dependence*.

Abstract

Cocaine induces a wide breadth of physiological effects, even at low, recreational doses, and studies suggest that these consequences encompass the lipidome. Lipids from the whole blood of mice were analyzed using the Bligh-Dyer extraction method, followed by direct-infusion lipidomics with electrospray ionization-mass spectrometry (ESI-MS). ESI-MS data was analyzed with multivariate analysis to determine changes in lipid profile between groups. Significant m/z features indicated alterations in four lipid categories: fatty acyls, glycerolipids, glycerophospholipids and sphingolipids, and features were further identified into classes. While the directionality of these changes varied, the greatest number of altered features were identified as sphingolipids, some of which were further characterized as ceramides. Remarkably, we found that the relative abundance of certain significantly altered features correlated to various drug-seeking behaviors in cocaine-treated animals. The molecular mechanisms by which cocaine-induced effects occur, including alterations in lipid profile, are not fully understood. Our data using $p21^{-/-}$ mice suggest a role for this cell cycle regulator in cocaine-induced response *in vivo*. Interestingly, the effects of genetic ablation of p21 were found to extend to the blood lipidome, as $p21^{-/-}$ mice had distinct lipidomic profiles compared to wild-type mice both inherently and following cocaine exposure. Further, m/z species distinct from those discerned in wild-type mice correlated to behavior in $p21^{-/-}$ mice. Taken together, these data suggest a role for p21 not only in behavioral responses to cocaine, but also in drug-induced alterations in the blood lipidome.

Introduction

Although cocaine is most often associated with its effects on the dopamine pathway within the brain, recreational doses are known to affect other biological systems as well. Depending on the route of exposure, these additional effects can incorporate the cardiovascular, respiratory and gastrointestinal systems, as well as certain organs of the excretory system (2010; Herculiani et al. 2009; Kanel et al. 1990; Schwartz et al. 2010; Tiwari et al. 2006; van der Woude 2000; Wanless et al. 1990). Recent data indicate that the biological consequences of cocaine also spread to the lipidome (Buydens-Branchey and Branchey 2003; Buydens-Branchey et al. 2003; Cummings et al. 2015). Lipids are a major class of naturally occurring biomolecules (Fahy et al. 2009) that play an important role in the physiology and pathophysiology of living things (Subramaniam et al. 2011). Lipids are found in all organ systems and have a wide breadth of cellular functions including, but not limited to, structure of the cell membrane, protein trafficking and energy storage (Watson 2006).

The era of the genome and the proteome has paved the way for similarly cataloging the structural diversity of other integral macromolecules, including lipids. Over the past few decades, the number of characterized lipid molecules has grown exponentially from a few hundred to thousands (Subramaniam et al. 2011; Watson 2006). Advances in mass spectrometric analysis have allowed for the identification of lipid species from a plethora of various cells and tissues. Lipidomics, however, is not only the study of lipid species, but also of the molecules with which they interact and their functional role in biological systems

(Subramaniam et al. 2011; Watson 2006). This type of evaluation allows for the development of a 'lipid profile,' which is a mass spectrum of the types of lipids, and their abundance, within a crude lipid extract from a given biological sample (Watson 2006). Using this analysis, changes in lipid composition can be monitored overtime, in response to various stimuli or during disease onset and progression.

Analytical technologies have allowed for the identification of lipids as a physiologically important class of macromolecules in a multitude of diseases, such as cancer, atherosclerosis, diabetes and, interestingly, some neuropathologies and psychiatric disorders (Buydens-Branchey and Branchey 2003; Buydens-Branchey et al. 2003; Gettler 1991; Glueck et al. 1994; Ho et al. 1977; Maccari et al. 1991; Mapstone et al. 2014; Morgan et al. 1993; Pratico et al. 1998; Virkkunen 1979; Virkkunen 1983; Watson 2006; Wenk 2005). Connections have been reported between cocaine use and lipids of various types. For example, relapse to drug-seeking and drug-taking in detoxified addicts has been correlated with blood cholesterol levels, as well as with polyunsaturated fatty acid levels (Buydens-Branchey and Branchey 2003; Buydens-Branchey et al. 2003).

The relationship between cocaine and lipids has not yet been fully characterized. Similarly, the mechanisms by which cocaine-induced alterations in lipid profiles occur are not wholly understood. Data presented in Chapter 3 clearly show that cocaine alters the expression of the cyclin-dependent kinase inhibitor p21. As discussed in detail in Chapter 1, lipids are also known to alter

p21 expression. For example, the sphingolipid metabolite S1P and short chain fatty acids inhibit HDACs, causing an increase in p21 transcription and expression (Chen et al. 2003; Hait et al. 2009). Additionally, inhibition of calcium-independent phospholipase A₂ decreases proliferation and phospholipid turn over via increased p21 expression and cell cycle arrest (Zhang et al. 2006). Despite these published data, there is no information linking cocaine, p21 and the lipidome.

The purpose of this study was to investigate the extent of the relationship between cocaine and p21 that was unearthed in the studies presented in Chapters 3 and 4. To accomplish this, we examined not only the effect of cocaine on the blood lipidome, but also the effect of genetic ablation of p21 on cocaine-induced alterations therein. We utilized shotgun-based electrospray-ionization mass spectrometry (ESI-MS), a soft ionization technique, to determine underlying differences in the lipidome of wild-type and *p21*^{-/-} mice, as well as alterations induced by cocaine exposure in both models. We then categorized significantly altered lipid species between various groups of comparison into fatty acids, glycerolipids, glycerphospholipids and sphingolipids, and tentatively identified features within each category. Further, we correlated the relative intensities of significantly altered *m/z* features to various cocaine-induced behaviors.

Results

Effect of Genetic Ablation of p21 on the Blood Lipidome in Saline-Treated Mice

To determine the role of p21 on the blood lipidome, lipids were first extracted from the trunk blood of control wild-type and $p21^{-/-}$ mice using a Bligh-Dyer extraction technique. Then, electrospray ionization-mass spectrometry (ESI-MS) was performed, followed by multivariate analysis using Partial Least Squares-Discriminant Analysis (PLS-DA) to determine the variance across groups of comparison in the positive and negative mode (**Figure 5.1**). Volcano plot analysis was used to determine the fold change of significantly altered m/z features in $p21^{-/-}$ mice compared to wild-type mice. The species were then classified into one of four categories: fatty acyls, glycerolipids, glycerophospholipids or sphingolipids (**Figure 5.2**, blue, black, red, and green bars, respectively) using SimLipid software (Premier Biosoft). While the directionality of the differences varied, the majority of significantly altered species in $p21^{-/-}$ mice compared to wild-type mice were identified as sphingolipids. These lipid features were further identified into classes within each category (**Table 5.1**).

Effect of Genetic Ablation of p21 on the Blood Lipidome of Cocaine-Treated Mice

ESI-MS was also used to investigate the role of p21 on the lipidome of cocaine-treated mice. Again, the variance between groups was established in both the positive and negative modes (**Figure 5.3**) and volcano plot analysis and SimLipid software used to determine the fold change and category of significantly altered species (**Figure 5.4**). We observed that, again, the majority of altered

species were identified as sphingolipids. In this case, however, SimLipid was unable to clearly identify certain m/z features, and instead gave multiple possible category identifications (**Figure 5.4**, white bars). The lipid features were further separated into classes and several sphingolipid species were identified as ceramides (**Table 5.2**).

Effect of Cocaine on the Lipidome of Wild-Type Mice

The above analyses were performed on the trunk blood of treated and control wild-type mice to identify the effect of cocaine exposure on the blood lipidome (**Figure 5.5**), as well as specific lipid species altered (**Figure 5.6**). Again, the majority of significantly altered features were classified as sphingolipids. Interestingly, these species depicted low fold changes in response to cocaine compared to species within other categories, such as glycerolipids and glycerophospholipids. It should be noted, however, that a greater difference does not necessarily mean a more biologically important, or even biologically relevant, effect. Ceramides were again identified as a class of significantly altered lipid species (**Table 5.3**).

Effect of Cocaine on the Lipidome of $p21^{-/-}$ Mice

To further investigate the relationship between cocaine and p21, the aforementioned analyses were performed to determine the effect of cocaine on the blood lipidome of $p21^{-/-}$ mice (**Figure 5.7**). Noticeably fewer individual species were altered by cocaine in the $p21^{-/-}$ mice, compared to the number altered in the wild-type mice, further advocating a difference in the blood lipidome of wild-type

and $p21^{-/-}$ mice (**Figure 5.8**). Regardless, again the majority of these altered species were identified as sphingolipids (**Table 5.4**)

Species Intensity Correlations to Cocaine-Induced Behaviors

To discern the physiological relevance of these alterations in the blood lipidome, we correlated the relative intensities of certain significantly altered m/z species with various cocaine-induced responses in $p21^{-/-}$ mice (**Figure 5.9**) and wild-type (**Figure 5.10**). These behavioral responses included Activity Day locomotor activity following an initial dose of cocaine, Challenge Day activity following a challenge dose of cocaine, cocaine-induced conditioned place preference and cocaine-induced locomotor sensitization. Of the many lipid features analyzed (**Appendix Table 1-4**), a handful did correlate ($R^2 > 0.65$) to these various drug-induced behaviors in either $p21^{-/-}$ mice or wild-type mice. There were no similarities between those which correlated with wild-type mice behavior and $p21^{-/-}$ mice behavior, nor were there species which correlated to more than one behavioral response.

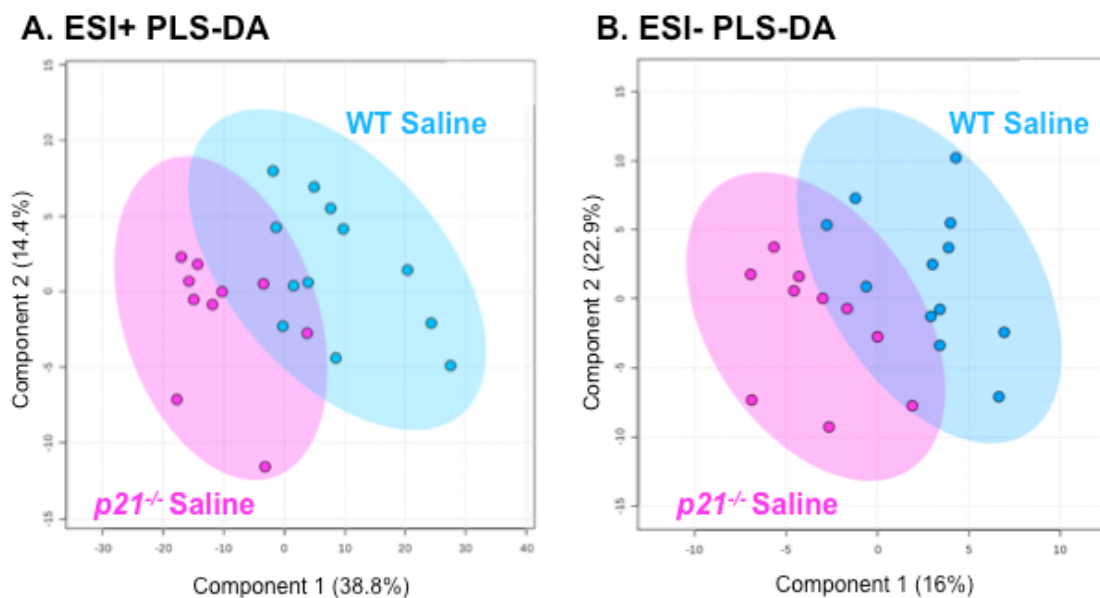


Figure 5.1. Effect of Genetic Ablation of p21 on the Blood Lipidome of Saline-Treated Mice. **A** depicts a PLS-DA scores plot comparing the lipid profiles of control wild-type mice (WT, blue) and control $p21^{-/-}$ mice (pink) in the positive mode (ESI+) with 53.2% of the total variance explained by Component 1 and Component 2. **B** depicts a PLS-DA scores plot comparing the lipid profiles of wild-type (WT) and $p21^{-/-}$ mice in the negative mode (ESI-) with 48.9% of the total variance explained by Component 1 and Component 2. The ovals in the scores plot indicate a 95% confidence interval.

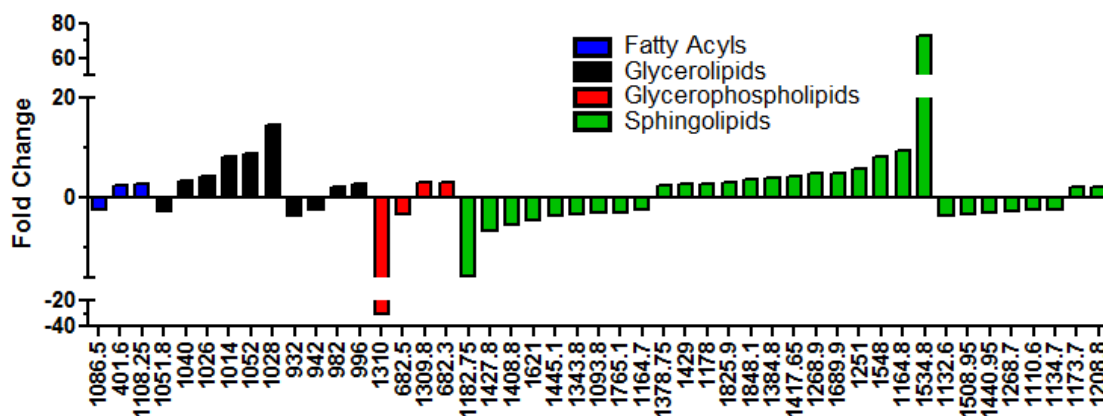


Figure 5.2. Lipid Categories of m/z Features Significantly Altered in $p21^{-/-}$ Mice Compared to Wild-Type Mice. Significant features were determined by volcano plot analysis based on a fold change of 1.5 and a p -value < 0.05 . Categories of altered lipid species were determined using SimLipid (Premier Biosoft), a comprehensive lipidome data analysis software that allows users to tentatively identify lipid species using m/z values from MS data based on a continuously updated database containing links from Kyoto Encyclopedia of Genes and Genomes (KEGG), Human Metabolome Database (HMDB), Chemical Entities of Biological Interest (CEBI), PubChem Substance database, LIPIDBANK and LIPIDAT.

Table 5.1 Types and Distribution of Lipids Shown to be Differentially Expressed in Saline-Treated Wild-Type and *p21^{-/-}* Mice ^a

Category	Classes Identified ^b (number of species)
Fatty Acyls	Acyl Coenzyme A (2) Unknown (1)
Glycerolipids	Triacylglycerol (10)
Glycerophospholipids	Cardiolipin (2) Phosphatidylethanolamine (2)
Sphingolipids	Unknown (30)
^a As determined by volcano plot analysis identifying those <i>m/z</i> values with significant ($p < 0.05$) differences in fold expression. ^b Lipid classes were identified by analysis of <i>m/z</i> values determined to be statistically significant using SimLipid (Premier Biosoft), a comprehensive lipidome data analysis software that allows users to tentatively identify lipid species using <i>m/z</i> values from MS data based on a continuously updated database containing links from Kyoto Encyclopedia of Genes and Genomes (KEGG), Human Metabolome Database (HMDB), Chemical Entities of Biological Interest (CEBI), PubChem Substance database, LIPIDBANK and LIPIDAT. This analysis was confirmed by screening of <i>m/z</i> values in Lipid Maps.	

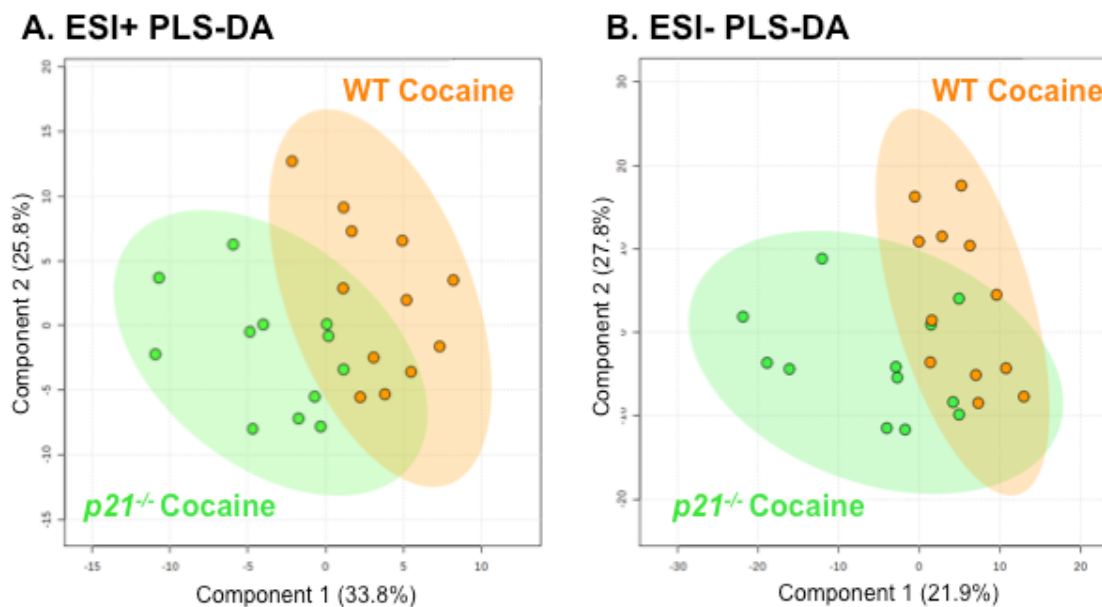


Figure 5.3. Effect of Genetic Ablation of p21 on the Blood Lipidome of Cocaine-Treated Mice. **A** depicts a PLS-DA scores plot comparing the lipid profiles of cocaine-treated wild-type mice (WT, orange) and cocaine-treated $p21^{-/-}$ mice (green) in the positive mode (ESI+) with 59.6% of the total variance explained by Component 1 and Component 2. **B** depicts a PLS-DA scores plot comparing the lipid profiles of wild-type (WT) and $p21^{-/-}$ mice in the negative mode (ESI-) with 49.7% of the total variance explained by Component 1 and Component 2. The ovals in the scores plot indicate a 95% confidence interval.

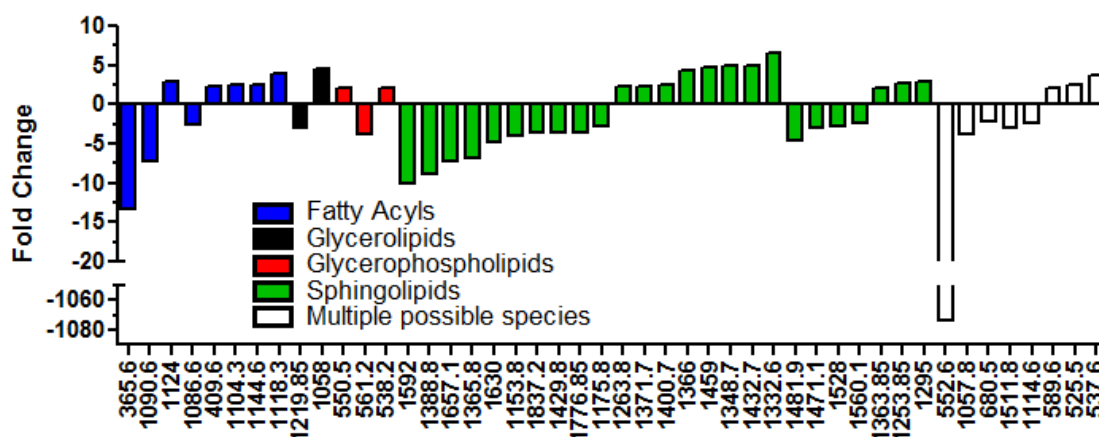


Figure 5.4. Lipid Categories of m/z Features Significantly Altered in Cocaine-Treated $p21^{-/-}$ Mice Compared to Cocaine-Treated Wild-Type Mice. Significant features were determined by volcano plot analysis based on a fold change of 1.5 and a p -value < 0.05 . Categories of altered lipid species were determined using SimLipid (Premier Biosoft), a comprehensive lipidome data analysis software that allows users to tentatively identify lipid species using m/z values from MS data based on a continuously updated database containing links from Kyoto Encyclopedia of Genes and Genomes (KEGG), Human Metabolome Database (HMDB), Chemical Entities of Biological Interest (CEBI), PubChem Substance database, LIPIDBANK and LIPIDAT.

Table 5.2 Types and Distribution of Lipids Shown to be Differentially Expressed
in Cocaine-Treated Wild-Type and *p21^{-/-}* Mice

Category	Classes Identified (number of species)
Fatty Acyls	Acyl coenzyme A (7) Mycolic acid (1) Carboceric acid (1) Mycocerosic acid (1) Montanyl alcohol (1) Linolenyl linoleate (1) Linoleyl linolenate (1) Palmitoleyl arachidonate (1) Tetracosanyl palmitoleate (1) Behenyl oleate (1) Oleyl behenate (1) Other fatty acyls (2) Unknown (3)
Glycerolipids	Diacylglycerol (3) Triacylglycerol (1) Digalactosyldiacylglycerol (1) Unknown (1)
Glycerophospholipids	Phosphatidylcholine (3) Phosphatidylserine (3) Archaetidylglycerol (1) Phosphatidylglycerol (1) Phosphatidic acid (2) Cardiolipin (1)
Sphingolipids	Ceramide (3) Unknown (26)

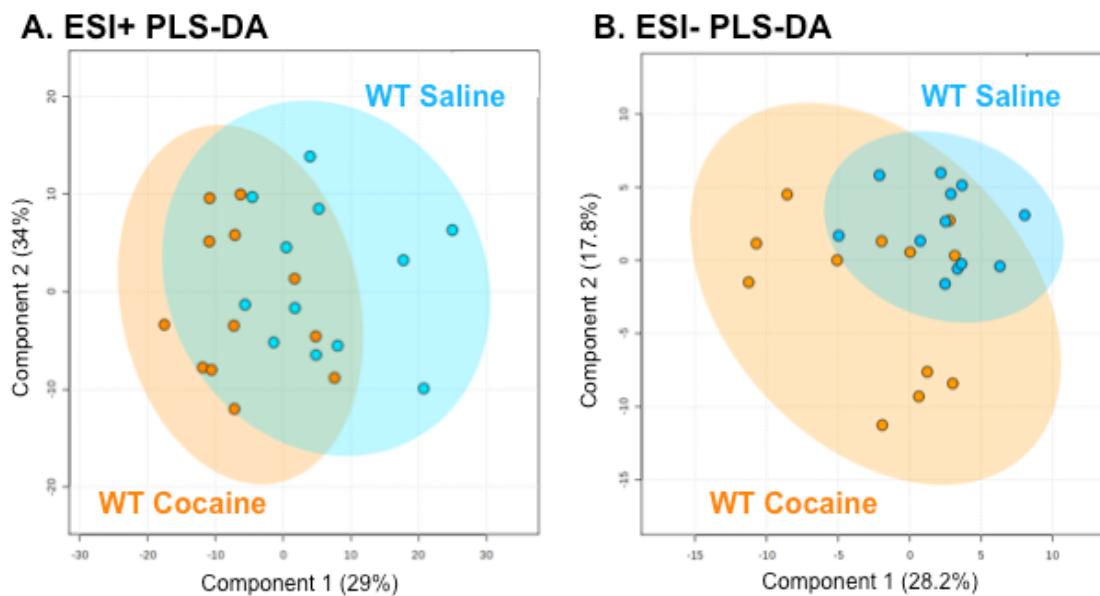


Figure 5.5. Effect of Cocaine on the Blood Lipidome of Wild-Type Mice. **A** depicts a PLS-DA scores plot comparing the lipid profiles of cocaine-treated wild-type mice (orange) and saline-treated wild-type mice (blue) in the positive mode (ESI+) with 63% of the total variance explained by Component 1 and Component 2. **B** depicts a PLS-DA scores plot comparing the lipid profiles of cocaine- and saline-treated wild-type mice in the negative mode (ESI-) with 46% of the total variance explained by Component 1 and Component 2. The ovals in the scores plot indicate a 95% confidence interval.

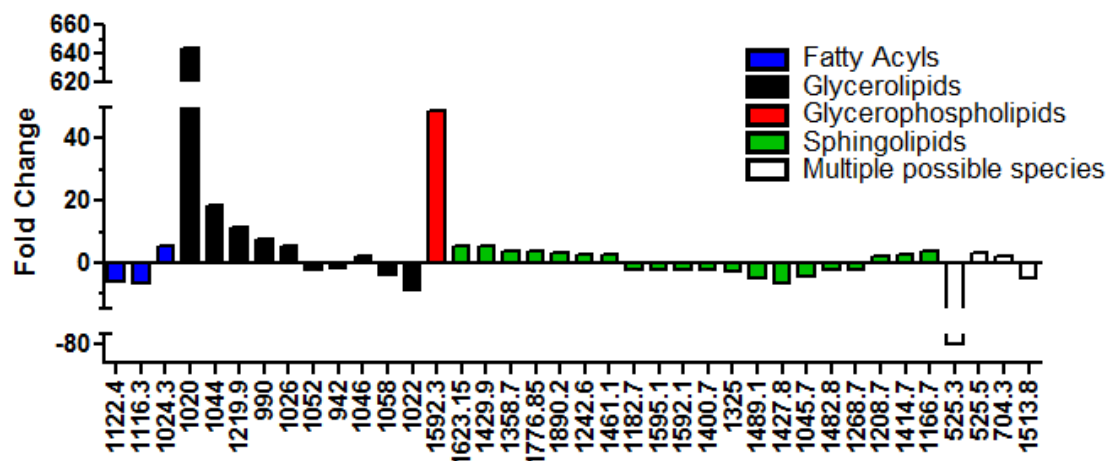


Figure 5.6. Lipid Categories of *m/z* Features Significantly Altered in Cocaine- Compared to Saline-Treated Wild-Type Mice. Significant features were determined by volcano plot analysis based on a fold change of 1.5 and a *p*-value < 0.05. Categories of altered lipid species were determined using SimLipid (Premier Biosoft), a comprehensive lipidome data analysis software that allows users to tentatively identify lipid species using *m/z* values from MS data based on a continuously updated database containing links from Kyoto Encyclopedia of Genes and Genomes (KEGG), Human Metabolome Database (HMDB), Chemical Entities of Biological Interest (CEBI), PubChem Substance database, LIPIDBANK and LIPIDAT.

Table 5.3 Types and Distribution of Lipids Shown to be Differentially Expressed
in Cocaine- and Saline-Treated Wild-Type Mice

Category	Classes Identified (number of species)
Fatty Acyls	Acyl coenzyme A (3) Linolenyl linoleate (2) Palmitoleyl arachidonate (2) Other fatty acyls (2) Unknown (1)
Glycerolipids	Triacylglycerol (10) Diacylglycerol (2) Digalactosyldiacylglycerol (1)
Glycerophospholipids	Phosphatidylglycerol (2) Phosphatidylserine (1) Cardiolipin (1) Bisphosphatidylglycerol (1)
Sphingolipids	Ceramide (1) Fumonisin (2) Unknown (20)

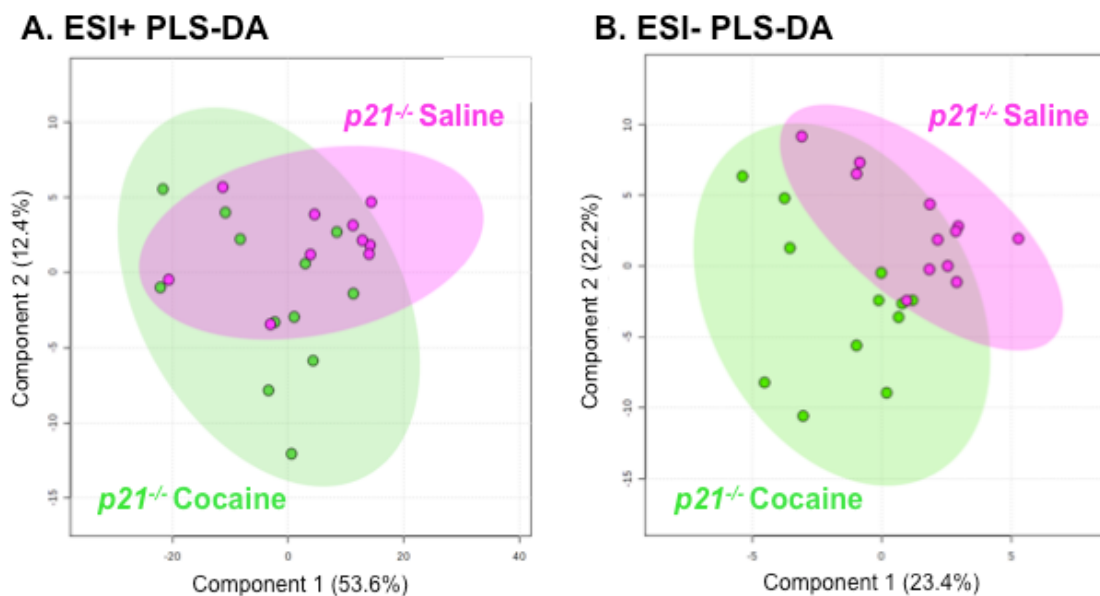


Figure 5.7. Effect of Cocaine on the Blood Lipidome of *p21*^{-/-} Mice. **A** depicts a PLS-DA scores plot comparing the lipid profiles of cocaine-treated *p21*^{-/-} mice (green) and saline-treated *p21*^{-/-} mice (pink) in the positive mode (ESI+) with 66% of the total variance explained by Component 1 and Component 2. **B** depicts a PLS-DA scores plot comparing the lipid profiles of cocaine- and saline-treated *p21*^{-/-} mice in the negative mode (ESI-) with 45.6% of the total variance explained by Component 1 and Component 2. The ovals in the scores plot indicate a 95% confidence interval.

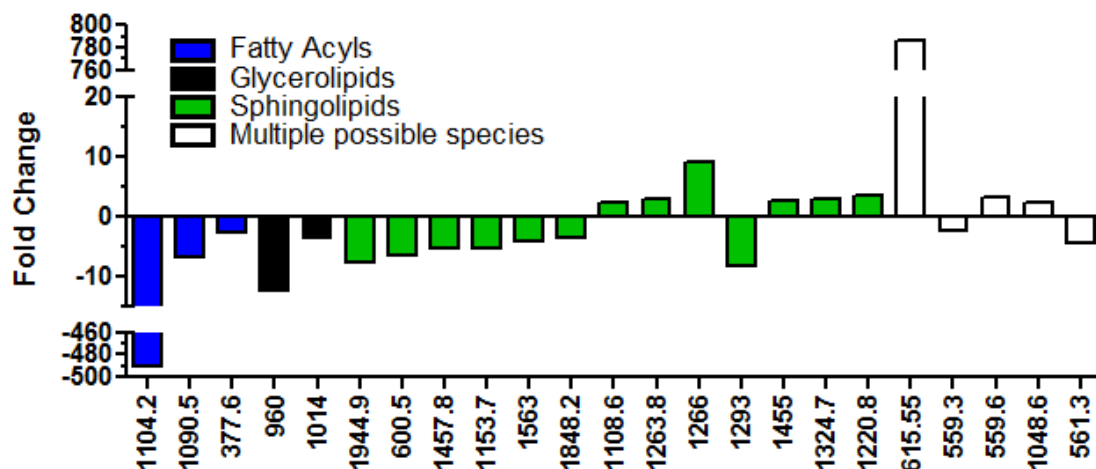
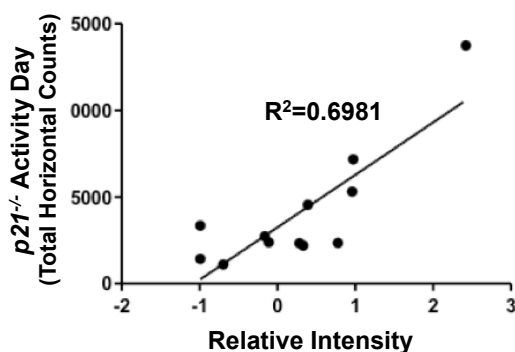


Figure 5.8. Lipid Categories of *m/z* Features Significantly Altered in Cocaine-Compared to Saline-Treated *p21*^{-/-} Mice. Significant features were determined by volcano plot analysis based on a fold change of 1.5 and a *p*-value < 0.05. Categories of altered lipid species were determined using SimLipid (Premier Biosoft), a comprehensive lipidome data analysis software that allows users to tentatively identify lipid species using *m/z* values from MS data based on a continuously updated database containing links from Kyoto Encyclopedia of Genes and Genomes (KEGG), Human Metabolome Database (HMDB), Chemical Entities of Biological Interest (CEBI), PubChem Substance database, LIPIDBANK and LIPIDAT.

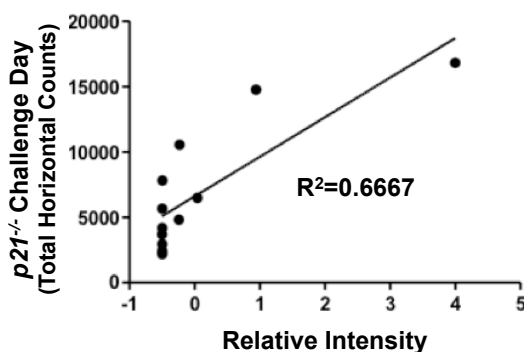
Table 5.4 Types and Distribution of Lipids Shown to be Differentially Expressed
in Cocaine- and Saline-Treated *p21^{-/-}* Mice

Category	Classes Identified (number of species)
Fatty Acyls	Acyl coenzyme A (2) Mayolene (2) Linoleyl arachidate (1) Unknown (1)
Glycerolipids	Triacylglycerol (2) Diacylglycerol (2) MfGL-II Unknown (1)
Glycerophospholipids	Phosphatidic acid (2) Phosphatidylinositol (1) Phosphatidylethanolamine (1) Phosphatidylglycerol (1)
Sphingolipids	Unknown (13)

A. Species 1513.8



B. Species 1392.75



C. Species 1429.9

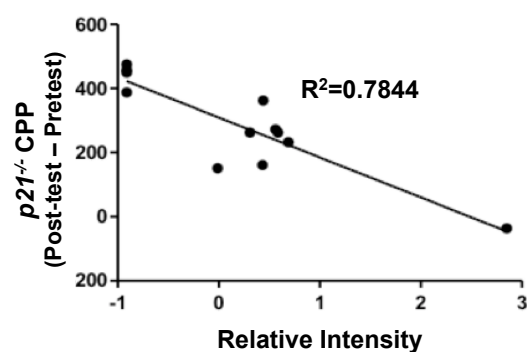
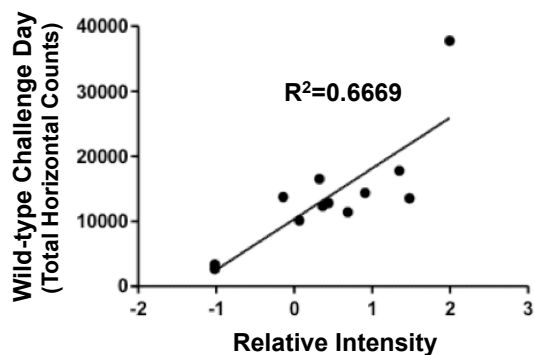


Figure 5.9. Lipid Features in the Blood of *p21*^{-/-} Mice Correlated to Cocaine-Induced Behaviors. Behaviors include LMS Activity and Challenge Day locomotor movement, Conditioned Place Preference and Sensitization. Species and behaviors with correlations over 0.65 are shown. Data include analyses of 12 animals per group ($n=12$).

A. Species 1129.6



B. Species 1551.15

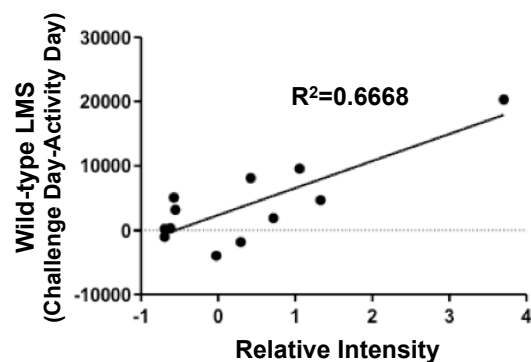


Figure 5.10. Lipid Features in the Blood of Wild-Type Mice Correlated to Cocaine-Induced Behaviors. Behaviors include LMS Activity and Challenge Day locomotor movement, Conditioned Place Preference and Sensitization. Species and behaviors with correlations over 0.65 are shown. Data include analyses of 12 animals per group ($n=12$).

Discussion

Indicative of the wide range of drug-induced physiological effects, several studies report alterations in select blood lipids in response to drugs of abuse (Buydens-Branchey and Branchey 2003; Buydens-Branchey et al. 2003; Wannasirindr et al. 2000). The data reported here further support this phenomenon. We observed the existence of multiple significantly altered m/z features in the blood of wild-type mice exposed to cocaine compared to those exposed to saline. There was no general trend, however, as the directionality of the changes observed between the two treatments varied, suggesting m/z -specific effects. The altered species could be divided into fatty acids, glycerolipids, glycerophospholipids and sphingolipids, with the majority being identified as sphingolipids, and lipid species within each category could be further divided into classes. While many sphingolipids could not be further characterized, some were identified as ceramides. Ceramides, metabolites of the phospholipid sphingomyelin, are present in high concentrations in the central nervous system, where they are produced via synthesis or sphingomyelin hydrolysis (Car et al. 2012). Interestingly, ceramide has been suggested to be a participant in the neuronal cell death that accompanies several different pathologies, including Alzheimer's disease and ischemia-induced brain damage, as it plays a role in numerous cellular functions, such as growth, differentiation and apoptosis (Car et al. 2012; Phillips et al. 2007). Ceramide has also been shown to alter the dopamine pathway by modifying the function of transporters and decreasing dopamine uptake (Riddle et al. 2003).

The majority of brain lipids play functional and structural roles within neuronal membranes (Agranoff et al. 1999), and while sphingolipids, including ceramide, are highly expressed, over 30% of brain lipids are classified as polyunsaturated fatty acids (Buydens-Branchey et al. 2003). In this study, multiple fatty acids were found to be altered in response to cocaine. Interestingly, deficiencies in certain lipids within this category have been shown to negatively affect synaptic outgrowth, signal transduction and other neuronal processes, correlating to learning deficits and impaired neurodevelopment (Salem et al. 2001). As discussed in depth in Chapter 1, low levels of fatty acids have been connected to lower levels of endogenous dopamine, reduction of dopamine release and various neurological disorders, including substance abuse (Buydens-Branchey et al. 2003; L'Hirondel et al. 1995; Wannasirindr et al. 2000; Zhang and Reith 1996). Given the known involvement of ceramides and fatty acids in this multitude of neurological processes, cocaine-induced alterations in the abundance of these lipid classes is a plausible result.

The observed differences between the blood lipidome of wild-type and *p21*^{-/-} mice were exciting findings. While studies have shown that certain lipid species can alter p21 expression, to our knowledge, this is the first examining the effect of p21 itself on lipid abundance. The majority of species were found to differ between wild-type and *p21*^{-/-} mice were once again sphingolipids. Considering the importance of sphingolipids in cellular functions including proliferation and survival, it is not surprising that altering the expression of a protein integral to cell cycle might alter this particular category of lipids (Fyrst and

Saba 2010; Lee et al. 2011). As mentioned, ceramide has been identified as an inductor of apoptosis in various disorders including Alzheimer's disease, and this may be occurring via p21-induced cell cycle arrest (Car et al. 2012; Phillips et al. 2007). Therefore, knocking out of p21 could alter ceramide-induced neuronal cell death, which may have therapeutic potential for multiple neuropathologies extending beyond the realm of substance abuse.

Interestingly, the blood lipidome also differed between cocaine-treated wild-type and cocaine-treated *p21*^{-/-} mice, further supporting the dissimilarity in drug-induced effects between the two models. In this case, sphingolipids and fatty acids were the categories with the greatest number of identified altered species. This finding agrees with several studies suggesting that cocaine induces alterations in fatty acids and sphingolipids, and that there is potential for these categories to be correlated to drug-induced behavior and neurological processes (Buydens-Branchey et al. 2003; L'Hirondel et al. 1995; Riddle et al. 2003; Salem et al. 2001; Wannasirindr et al. 2000; Zhang and Reith 1996). Based on this hypothesis, altered expression of lipids within these key categories could be connected to the different drug-induced responses observed between wild-type and *p21*^{-/-} mice. Interestingly, fewer lipids were altered in response to cocaine in *p21*^{-/-} mice than observed in wild-type mice. One possible explanation for this finding is that p21 is involved in the mechanism of alteration of certain lipid species following cocaine exposure, so these specific features are not affected with genetic ablation of p21. Further, perhaps these p21-mediated species influence drug-induced behaviors, contributing to the dissimilarities observed in

response to cocaine between the two models. It is also possible: however, that these lipid species are altered in response to the behaviors themselves, and because wild-type and *p21^{-/-}* mice depicted different cocaine-induced behaviors, the lipid species effected were also different. Although potentially powerful, the full meaning behind the variations observed within the blood lipidome of wild-type versus *p21^{-/-}* mice, both naturally and in response to cocaine, remains to be determined.

It should be emphasized that the analyses for this study were performed on lipids obtained from the blood as opposed to brain tissue, so while the data could be indicative of effects within the brain, they could also result from changes elsewhere within the body. That being said, a recent study by Mapstone et al. (2014) found parallels between lipids in the plasma of Alzheimer's patients and lipids known to be altered in cerebrospinal fluid with the disease, suggesting that alterations in blood lipids may be reflective of changes in cell membrane integrity within the brain (Mulder et al. 2003; Walter et al. 2004). These data indicate that blood and brain lipids may be connected and perhaps comparable, and that the fact that the lipids in this study were analyzed from blood samples should not negate the significant findings that cocaine alters the lipidome of both wild-type and *p21^{-/-}* mice. In support of this, studies have indicated correlations between blood lipids and drug-induced behaviors in humans (Buydens-Branchey and Branchey 2003; Buydens-Branchey et al. 2003)

Although data demonstrating cocaine-induced alterations in the blood lipidome are interesting, the potential therapeutic relevance of these findings is

weak without an association to behavior. To address this, various drug-induced behaviors were correlated to lipid abundance. As outlined in Chapter 3, and in parallel to what is observed in humans, rodents have multiple cocaine-induced behaviors, including movement, drug-taking and drug-seeking. In general, drug-induced behaviors do not all occur via the same mechanisms. For this reason, it is not surprising to find that different lipid species correlate to different behavioral responses to cocaine in mice. Similarly, given the underlying differences in the blood lipidome of wild-type and *p21*^{-/-} mice, it would be expected that different lipids contribute to these various behaviors. We observed correlations between certain features and movement following the first exposure to the drug, movement following the final exposure, preference for the drug-paired compartment and locomotor sensitization. It is important to note that the lipid species that were found to correlate to behavior were not necessarily those with the greatest fold change between saline- and cocaine-treated mice, indicating that a greater difference in abundance does not necessarily constitute a more biologically significant effect. These findings are similar to those observed in a previous study from our laboratory using a rat model (Cummings et al. 2015). Despite the data presented here, the biological roles of the specific species identified and of the alterations induced in response to cocaine, if any, remain to be seen. Regardless, this study lays the foundation for future work and supports the idea that certain blood lipids could be utilized as biomarkers for cocaine use or as indicators of potential drug-induced effects and behaviors.

CHAPTER 6

SUMMARY

Despite published data describing alterations in hippocampal neurogenesis following drugs of abuse, few studies have assessed drug-induced changes in cell cycle related proteins, such as p21 (Faustman et al. 2002; Marqués-Torrejón et al. 2013; Pechnick et al. 2011; Pechnick et al. 2008; Zonis et al. 2013). In fact, to our knowledge, the only such study that investigated cocaine alongside p21 *in vivo* focused on the nucleus accumbens, where neurogenesis does not take place (Renthal et al. 2009). The series of studies presented here attempted to bridge this gap in knowledge by characterizing the relationship between p21 and cocaine-induced response. The overall hypothesis of this work was that cocaine induces alterations in hippocampal p21 expression via epigenetic mechanisms, and these alterations mediate cocaine-induced response. To address this hypothesis, we first confirmed the effect of cocaine on p21 expression in various brain regions, targeting structures of the limbic system, and found that cocaine exposure increases p21 expression specifically in the ventral sector of the hippocampus (**Figure 6.1A**). Interestingly, we observed this outcome in both mice and rats following both non-contingent, experimenter-administered and contingent, self-administered cocaine exposure, indicating that this effect is neither species- nor protocol specific. Sucrose exposure also increased ventral hippocampal p21, suggesting that this phenomenon is also not

specific to drugs of abuse. A commonality among the treatments found to induce ventral hippocampal p21 expression is the occurrence of sensitization, suggesting that this behavior could be connected to the alterations in p21 expression observed. In support of this, non-contingent administration of the dysphoric U50,488 induced neither locomotor sensitization nor alterations in hippocampal p21 expression.

Initially, we postulated that increased p21 expression, and subsequent cell cycle arrest, could be a means by which cocaine is inhibiting adult hippocampal neurogenesis. The actual effect of cocaine on neurogenesis, however, remains controversial. For this reason, we first assessed markers of hippocampal neurogenesis in our own drug-exposed animals, and obtained exciting and somewhat unexpected results. We found that both cocaine- and U50,488-treated mice actually had enhanced neurogenesis in the dorsal sector of the hippocampus. This finding was noteworthy for multiple reasons: first, the levels of ventral hippocampal p21 expression did not correlate with neurogenesis as we expected; second, this effect was likely not due to the direct activity of the drugs themselves, as they have distinctly different pharmacological mechanisms. The common element between these experiments was the drug exposure protocols used; therefore, we believe that this increased neurogenesis is a product not of the stimuli, but of the learning that the mice experience during the behavior assays performed. This discovery could potentially revolutionize the methods by which investigators determine the effect of a given stimulus on neurogenesis.

With regard to developing a therapeutic treatment for addiction, the effects of drugs of abuse on gene expression, while interesting, are irrelevant without a connection to behavior. We observed that genetic ablation of p21 alters cocaine-induced response in two different behavior assays: conditioned place preference and locomotor sensitization (**Figure 6.1B**). The *p21^{-/-}* mice depicted enhanced drug-induced CPP, which could be attributed to the increased neurogenesis and cognitive ability detected in this model (**Figure 6.1C**). We also observed decreased locomotor response to an initial cocaine exposure in these *p21^{-/-}* mice (**Figure 6.1B**), which is likely credited to a rightward shift in dose response also resulting from increased neurogenesis (Noonan et al. 2010). This finding suggests the potential for targeting and inhibiting p21 expression to prevent cocaine-induced response. Ideally, this could be exploited to prevent relapse to drug-seeking and taking behavior in cocaine addicts, as, based on the data presented here, this inhibition could result in a blunted response to drug exposure, which may prevent further drug-taking. Future studies could begin to address this hypothesis using a self-administration assay. Such a study would not only directly investigate the role of p21 in drug-taking behavior, but would also be more comparable to the human condition than the non-contingent assays performed here. Interestingly, the role of p21 in cocaine-induced response extended beyond behavior, as the blood lipidome of *p21^{-/-}* mice and wild-type mice following drug exposure also differed. The full implication of these differences remains to be elucidated; however, this finding clearly corroborates the dissimilarity in cocaine-induced effects between these two models.

Not only did we demonstrate the novel finding that cocaine increases p21 expression exclusively in the ventral hippocampus of rodents, but we also demonstrated that this effect correlates to alterations in epigenetic regulation, specifically histone 3 acetylation of the p21 promoter region (**Figure 6.1A**). Studies have shown that treatment with inhibitors of epigenetic events can alter drug-seeking and taking behaviors in response to cocaine (LaPlant and Nestler 2011; Nielsen et al. 2012; Renthall et al. 2007; Wong et al. 2011). It is plausible that treatment with these inhibitors prevents cocaine-induced increases in p21 expression, which, given the link between p21 and drug-induced behavior presented here, could contribute to the results observed in these published studies. Although additional studies are necessary to test this hypothesis, the mechanistic information depicting cocaine-induced alterations in the epigenetic status of p21 is both promising and integral to the potential exploitation of this protein as a therapeutic target for addictive behaviors.

While the nature of this particular study focused on the relationship between p21 and cocaine, the inherent differences observed between wild-type and *p21^{-/-}* mice could have a much wider range of implications. The enhanced cognitive performance of *p21^{-/-}* mice, coupled with the increased neurogenesis observed in the dorsal hippocampus of these mice, speaks to the likely importance of p21 in neurological processes outside of addiction (**Figure 6.1C**). Further supporting this idea, lipidomic analysis revealed novel differences between wild-type and *p21^{-/-}* mice in the abundance of several lipid species connected with various neurological functions, including neuronal cell death

(Buydens-Branchey et al. 2003; Car et al. 2012; Fyrist and Saba 2010; L'Hirondel et al. 1995; Lee et al. 2011; Riddle et al. 2003; Salem et al. 2001; Zhang and Reith 1996). That being said, the data presented here regarding the relationship between p21 and drugs of abuse could be merely skimming the surface of the significance of p21 in neurological systems. The findings of this study could therefore act as the groundwork for numerous analyses regarding the full extent of the involvement of this cyclin dependent kinase inhibitor in neuronal processes.

Although at present, there are no pharmacological inhibitors for p21, treatment with various FDA approved antidepressants *in vivo*, including norepinephrine- and serotonin-selective reuptake inhibitors, has been shown to decrease p21 expression, correlating with increased hippocampal neurogenesis (Pechnick et al. 2011). Therefore, while targeting p21 directly may not be possible, a conceivable next step for this study is treatment with these antidepressants prior to measuring cocaine-induced behavior. Data depicting a decreased response to cocaine following such treatment could be very exciting findings, as these drugs are already FDA approved and available for prescription. Alternatively, there are several anti-cancer drugs in clinical trials that act via inhibition of various cyclin-dependent kinases, thereby mimicking the effects of p21 (<https://clinicaltrials.gov>, PD0332991, AT-7519, AG-024322). Thus, an additional avenue of experimentation could be treatment of wild-type mice with these drugs and investigating changes in cocaine-induced behavior. Given the parallels between the actions of these drugs and p21, this analysis could be

representative of the effect of upregulating p21 on cocaine-induced response. Unfortunately, the ability of these compounds to cross the blood-brain barrier is not yet known, which may hinder their ability to alter drug response. One side effect of many anti-cancer drugs is a condition of mental cloudiness, including thinking and memory problems, commonly referred to as chemo brain (Aluise et al. 2010; Andres et al. 2014; Hermelink 2015; Porter 2013; Sorokin et al. 2014; Staat and Segatore 2005). As many chemotherapeutics act by altering cell cycle and cell proliferation, such as the aforementioned cyclin-dependent kinase inhibitors, a possible explanation for this effect could be alterations in adult neurogenesis. Therefore, targeted inhibition of p21 within the hippocampus could not only be a possible avenue for therapeutic treatment of addiction and prevention of relapse, but also a mechanism of preventing chemotherapeutic-induced adverse effects.

Future studies should expound upon the findings presented here, while attempting to address the restriction of the whole-animal knockout model. The *p21^{-/-}* mice utilized for these studies were bred with a mutated gene globally coding for a non-functional p21. Because of the all-encompassing nature of this knockout, differences in cocaine-induced response between *p21^{-/-}* and wild-type mice cannot be unequivocally attributed to the lack of p21 specifically within the hippocampus of these animals. Additionally, while the drug-induced behavioral differences observed between the two models are exciting and novel findings, the goal of targeting p21 as a therapeutic treatment for addiction would undoubtedly benefit from the development of an inducible p21 knockout model.

This technology would allow us to investigate the behavioral effect of acutely altering p21 expression, for example after an initial drug exposure or conditioning, as opposed to the result of lacking p21 throughout development. Utilizing a hippocampal-targeted inducible knockout model would address both of these limitations and substantially add to the validity and strength of this work. While such future studies are necessary to unearth the full potential for p21 as a prospective therapeutic target for addictive behaviors, the data presented here not only confirm a role for p21 in drug-induced effects, but also suggest ramifications for p21 well beyond addiction.

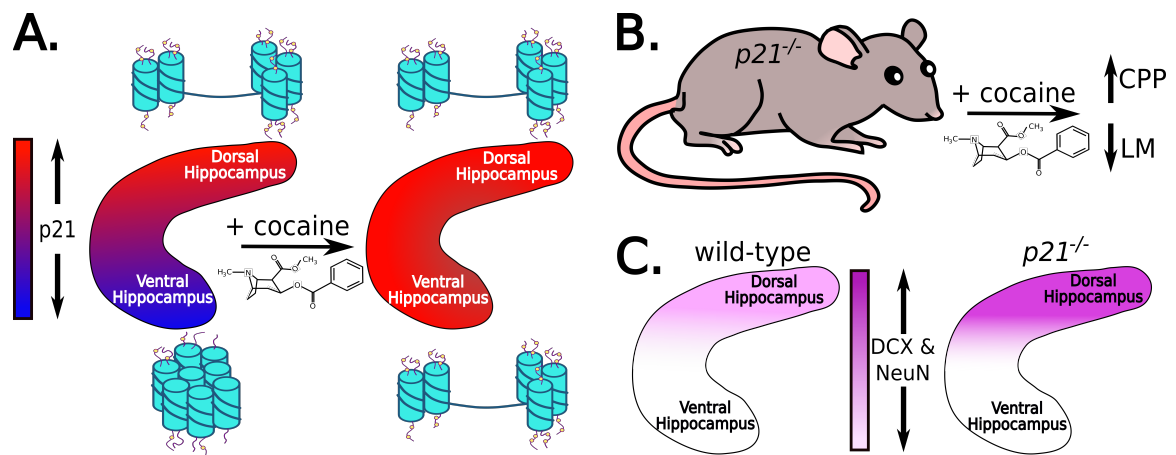


Figure 6.1 Pictorial Representation of the Major Findings of the Study. Image derived using Inkscape (www.inkscape.org). Cocaine was found to increase the expression of p21 solely in the ventral hippocampus, which correlated with increased histone 3 acetylation (yellow circles) of the promotor region in this sector. Additionally, the dorsal hippocampus showed inherently higher acetylation and p21 expression compared to the ventral hippocampus of control animals (**A**). Mice lacking p21 portrayed increased cocaine-induced conditioned place preference and decreased locomotor activity in response to cocaine compared to wild-type animals (**B**). Both of these behavioral differences could be attributed to increased neurogenesis, as measured by the neurogenesis markers DCX and NeuN, in the dorsal hippocampus of these $p21^{-/-}$ mice (**C**).

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APPENDIX

Appendix Table 1. Correlation between various drug-induced behaviors in wild-type mice and intensity of *m/z* features shown to be significantly altered between saline- and cocaine-treated wild-type mice

<i>m/z</i>	R^2			
	Activity Day	Challenge Day	CPP	LMS
947.2	0.05127	0.02721	0.01557	0.0004037
1420	0.04695	0.00005776	0.01629	0.03335
1225.8	0.06269	0.2279	0.05859	0.1751
1323.85	0.01836	0.1588	0.09465	0.1719
1489.1	0.00009915	0.04386	0.06827	0.07422
1105.8	0.04337	0.0005709	0.05424	0.02348
1592.1	0.04888	0.131	0.0003823	0.08372
1186.4	0.03325	0.2224	0.003552	0.222
1653.4	5.747E-07	0.04802	0.1106	0.04218
1345.8	0.05335	0.2271	0.1734	0.1889
1835.7	0.007226	0.009676	0.04844	0.00319
1022	0.1602	0.7301	0.000217	0.6264
1392.75	0.1416	0.1049	0.0104	0.01005
1020	0.001317	0.04219	0.1096	0.05834
1109.4	0.009602	0.001422	0.04129	0.019
1346.1	0.1732	0.000009999	0.3165	0.1403
1399.2	0.07905	0.01944	0.01787	0.003955
1763.5	0.1485	0.1188	0.01039	0.0144
1155.95	0.03433	0.001281	0.05808	0.01364
1589.1	0.4135	0.02168	0.1972	0.1402
758	0.1261	0.1535	0.05462	0.04423
1614.2	0.01349	0.01196	0.04162	0.001897
1804.35	0.0008568	0.1366	0.05038	0.2729
1217.1	0.00634	0.02187	0.00009358	0.01633
1720.4	0.1513	0.1977	0.003415	0.06301
1427.8	0.1329	0.001384	0.001318	0.07529
1829.3	0.03125	0.004419	0.003992	0.004626
1477.2	0.0009126	0.1289	0.02771	0.2073
1639.5	0.05363	0.00003885	0.02289	0.03909
1026	0.004402	0.04224	0.1086	0.1123

1559.6	0.00219	0.0008705	0.2275	0.006605
1429.9	0.04007	0.001185	0.1658	0.0174
1044	0.1966	0.1304	0.00145	0.008186
1419.8	0.0858	0.00005165	0.01303	0.063
1325	0.2746	0.000008516	0.09993	0.2211
1058	0.0001924	0.03741	0.0103	0.06124
1800.3	0.0002182	0.00181	0.05015	0.00494
1483.6	0.03246	0.02859	0.112	0.1501
1129.6	0.3499	0.6669	0.007731	0.3255
1122.4	0.1417	0.0001638	0.006954	0.124
1513.8	0.000137	0.0002677	0.08476	0.0001337
1470.7	0.221	0.438	0.08308	0.2215
1219.9	0.1496	0.02339	0.1849	0.01926
1358.7	0.1476	0.048	0.002805	0.002268

Appendix Table 2. Correlation between various drug-induced behaviors in *p21^{-/-}* mice and intensity of *m/z* features shown to be significantly altered between saline- and cocaine-treated wild-type mice (± 0.5)

<i>m/z</i>	R²			
	Activity Day	Challenge Day	CPP	LMS
947.2	0.08295	0.00162	0.09726	0.0188
1420.3	0.008289	0.02223	0.2206	0.03051
1225.8	0.2423	0.03595	0.0156	0.1972
1323.8	0.0004542	0.3575	0.4578	0.2479
1489	0.009319	0.1694	0.05937	0.1528
1105.8	0.00792	0.03479	0.04675	0.04156
1592	0.00551	0.3536	0.1344	0.1926
1186.6	0.1902	0.2532	0.3561	0.4429
1653.4	0.08871	0.00832	0.08214	0.0623
1345.8	0.1405	0.03074	0.1741	0.1319
1836.2	0.07596	0.1624	0.004615	0.02712
1021.8	0.001884	0.06722	0.05928	0.03417
1392.4	0.01089	0.6667	0.2595	0.3615
1020	0.0004686	0.004867	0.03421	0.001924
1109.2	0.02631	0.3053	0.195	0.296
1109.6	0.03801	0.01075	0.05728	0.03963
1346.4	0.02282	0.1669	0.1623	0.177
1399.2	0.5239	0.00643	0.009717	0.2418
1763.15	0.06088	0.06252	0.03575	0.003307
1155.6	0.06262	0.125	0.001102	0.01946
1589.05	0.05125	0.0001435	0.02114	0.0205
1614.2	0.0452	0.01972	0.004948	0.001283
1804.3	0.08645	0.1772	0.03732	0.02834
1217.1	0.07609	0.05063	0.1491	0.0004013
1720.3	0.4779	0.0946	0.1167	0.4319
1427.4	0.01519	0.02734	0.06684	0.04279
1428.2	0.01419	0.04737	0.02597	0.06095
1829.1	0.005851	0.3551	0.1104	0.1924
1477	0.1958	0.04685	0.08888	0.1906
1639.6	0.0632	0.004249	0.03332	0.04044
1026	0.004471	0.2419	0.2943	0.1924
1560	0.07414	0.04404	0.1464	0.00009649
1430.1	0.00315	0.3878	0.7844	0.2232
1044	0.007836	0.1153	0.05945	0.1075
1419.7	0.05427	0.1425	0.001329	0.0286
1419.95	0.0554	0.3112	0.25	0.09865

1325.1	0.01753	0.0494	0.002902	0.0668
1058	0.03123	0.1673	0.01084	0.05189
1800.1	0.07988	0.001516	0.003289	0.01823
1800.5	0.539	0.01706	0.0006599	0.286
1484	0.008427	0.4336	0.1419	0.3464
1129.6	0.04583	0.1571	0.03834	0.2006
1122	0.05717	0.03649	0.1905	0.0001986
1514.1	0.6981	0.0003123	0.0005842	0.2569

Appendix Table 3. Correlation between various drug-induced behaviors in $p21^{-/-}$ mice and intensity of m/z features shown to be significantly altered between saline- and cocaine-treated $p21^{-/-}$ mice

m/z	R^2			
	Activity Day	Challenge Day	CPP	LMS
1267.8	0.1676	0.06611	0.3594	0.2025
1448	0.02371	0.01426	0.0005662	0.03522
1313.8	0.001163	0.00198	0.008474	0.003161
1090.5	0.644	0.0002049	0.001188	0.2129
1838.4	0.01071	0.08815	0.03133	0.03239
1037.5	0.04169	0.0002376	0.01122	0.01122
1014	0.06888	0.0001033	0.1652	0.02656
1383.8	0.04708	0.1618	0.0123	0.03945
1104.2	0.05968	0.002592	0.1739	0.01054
1560	0.07414	0.04404	0.1464	0.00009649
1274.8	0.075	0.0007868	0.07465	0.03393
1306	0.06521	0.03833	0.2032	0.00007032
1415.7	0.02034	0.4849	0.3149	0.2315
1666.8	0.0003823	0.001658	0.000003881	0.0004634
1268	0.02249	0.012198	0.0162	0.001021
909.2	0.04212	0.07766	0.03174	0.01107
1848.2	0.004644	0.01961	0.001383	0.005402
1234.8	0.02336	0.09335	0.06831	0.02493
1355.8	0.0594	0.002386	0.1732	0.01082
1437.1	0.111	0.07324	0.006545	0.0005396
997.2	0.1331	0.06789	0.03885	0.000013
1537.35	0.05951	0.002447	0.174	0.01074
672	0.02609	0.1677	0.001748	0.1829
1212.75	0.1229	0.01417	0.06726	0.092
1278.8	0.0372	0.04959	0.1772	0.004496
1944.9	0.0595	0.002475	0.174	0.174
975.2	0.09328	0.1541	0.2154	0.2486
921.2	0.09935	0.02873	0.0336	0.002326
1408.45	0.0154	0.06638	0.002416	0.07968
1339.6	0.05985	0.002498	0.1744	0.01074
514.8	0.1283	0.001405	0.04016	0.03266
1327.25	0.003413	0.005924	0.0004436	0.0007859
1247.1	0.2752	0.006589	0.02715	0.1407
1470.7	0.0685	0.2661	0.1571	0.3284
488.8	0.02032	0.2066	0.005138	0.2052
1164.6	0.002029	0.03141	0.01207	0.02904

1455	0.03447	0.03155	0.01651	0.001205
1275	0.02277	0.09158	0.2373	0.1119
644.8	0.1088	0.04212	0.1177	0.0007776
1563	0.1439	0.008331	0.0375	0.02236
273.55	0.02286	0.2625	0.3853	0.255
1394.6	0.1462	0.1235	0.3851	0.003573
1192.8	0.05905	0.002663	0.1755	0.01028
1607	0.05868	0.01164	0.01712	0.00305

Appendix Table 4. Correlation between various drug-induced behaviors in wild-type mice and intensity of *m/z* features shown to be significantly altered between saline- and cocaine-treated *p21*^{-/-} mice (± 0.5)

<i>m/z</i>	R²			
	Activity Day	Challenge Day	CPP	LMS
1267.8	0.41	0.09363	0.07884	0.02522
1448.25	0.05256	0.06544	0.1971	0.01949
1313.9	0.03064	0.178	0.1712	0.1688
1090.5	0.2484	0.004521	0.1374	0.1247
1091.2	0.007975	0.004952	0.02954	0.0002265
1838	0.0008431	0.0009195	0.06903	0.0002218
1037.2	0.0003727	0.004495	0.03968	0.0115
1013.2	0.01271	0.09169	0.102	0.2571
1383.8	0.09321	0.154	0.0004038	0.06522
1103.85	0.03886	0.06905	0.01242	0.03152
1560.1	0.001525	0.004714	0.00001045	0.01613
1274.7	0.00741	0.01719	0.08317	0.06387
1306.4	0.04115	0.002063	0.01551	0.05833
1415.7	0.5435	0.4438	0.1214	0.05703
1667.2	0.1556	0.02315	0.005247	0.02153
1268.2	0.1936	0.07157	0.1924	0.00104
909.2	0.226	0.0001138	0.679	0.167
1234.7	0.001421	0.09924	0.1246	0.1518
1355.45	0.009038	0.09147	0.02607	0.2409
1356.2	0.002094	0.001286	0.00001865	0.00005558
1436.4	0.01788	0.05658	0.1412	0.04021
1437.8	0.06921	0.03205	0.03918	0.2252
997.2	0.1354	0.03729	0.2084	0.3444
1537.15	0.003089	0.001399	0.07222	0.009941
672	0.2244	0.7037	0.0106	0.4973
1212.6	0.08675	0.04833	0.0767	0.3107
1278.8	0.05865	0.08564	0.008232	0.03153
1944.6	0.258	0.3734	0.01536	0.136
975.2	0.3283	0.03437	0.219	0.06806
1408.2	0.06242	0.00108	0.001899	0.03175
1339.75	0.02422	0.2315	0.005737	0.2578
514.8	0.2124	0.1391	0.4753	0.008254
1327	0.006602	0.1257	0.1705	0.1631
1247.1	0.05712	0.2925	0.1234	0.2639
1470.7	0.221	0.438	0.08308	0.2215
488.8	0.05335	0.003611	0.009697	0.01558

1164.1	0.0554	0.1336	0.1699	0.07923
1455.1	0.2453	0.01703	0.01361	0.07047
1275.1	0.3456	0.5898	0.04044	0.2585
644.8	0.2164	0.02112	0.177	0.04784
1563.2	0.01182	0.002006	0.01353	0.02462
273.6	0.04861	0.0176	0.0166	0.0003242
1394.4	0.07202	0.4473	0.05481	0.435
1192.7	0.009262	0.09753	0.01211	0.2551

EPIGENETIC CHANGES IN p21 EXPRESSION IN RENAL CELLS AFTER
EXPOSURE TO BROMATE¹

¹This is a pre-copyedited, author-produced PDF of an article accepted for publication in *Toxicological Sciences* following peer review. The version of record Scholpa N.E., Zhang, X., Kolli, R.T. and Cummings B.S. (2014) *Toxicological Sciences*. 141(2) 432-440. is available online at: <http://toxsci.oxfordjournals.org/cgi/reprint/kfu138?ijkey=pPyG1GeSJ8cRS9R&keytype=ref>.

Abstract

This study tested the hypothesis that bromate (KBrO₃)-induced renal cell death is mediated by epigenetic mechanisms. Global DNA methylation, as assessed by 5-methylcytosine staining, was not changed in normal rat kidney cells treated with acute cyto- toxic doses of KBrO₃ (100 and 200 ppm), as compared with controls. However, KBrO₃ treatment did increase p38, p53 and histone 2AX (H2AX) phosphorylation, and p21 expression. Treatment of cells with inhibitors of DNA methyltransferase (5-azacytidine or 5-Aza) and histone deacetylase (trichostatin A or TSA) in addition to KBrO₃ increased cytotoxicity, as compared with cells exposed to KBrO₃ alone. 5-Aza and TSA co-treatment did not alter p38 or p53 phosphorylation, but slightly decreased H2AX phosphorylation and significantly decreased p21 expression. We also assessed epigenetic changes in cells treated under sub-chronic conditions with environmentally relevant concentrations of KBrO₃. Under these conditions (0–10ppm KBrO₃ for up to 18 days), we detected no increases in cell death or DNA damage. In contrast, slight alterations were detected in the phosphorylation of H2AX, p38, and p53. Sub-chronic low-dose KBrO₃ treatment also induced a biphasic response in p21 expression, with lower concentrations increasing expression, but higher concentrations decreasing expression. Methylation-specific PCR demonstrated that sub-chronic KBrO₃ treatment altered the methylation of cytosine bases in the p21 gene, as compared with controls, correlating to alterations in p21 protein expression. Collectively, these data show the novel finding that KBrO₃-induced

renal cell death is altered by inhibitors of epigenetic modifying enzymes and that KBrO_3 itself induces epigenetic changes in the p21 gene.

Introduction

Chlorination, ozonation or the use of ultraviolet light on public water protects an estimated 260 million Americans from water-borne pathogens that would otherwise be present in drinking, cooking or bathing water. Unfortunately, these processes also oxidize organic and inert elements into disinfection byproducts (DBPs). Some of these DBPs are known to cause risks to human health (Richardson et al. 2007). For example, exposure to high levels of select DBPs can cause cancer in rodents (Bull and Cottruvo 2006; Umemura et al. 2006; Wolf et al. 1998), as well as increases in miscarriages and onset of select cancers in humans (Sedlak and von Gunten 2011).

Bromate (BrO_3^-) is a DBP formed after the ozonation of water and the oxidation of bromide (Br^-), an anthropogenic contaminant that naturally exists in ground water (von Gunten and Hoigne 1994). KBrO_3 is known to induce renal cancers in male and female rats, as well as thyroid cancer and testicular cancer in male rats (DeAngelo et al. 1998; Kurokawa et al. 1990; Wolf et al. 1998). Studies assessing the tumorigenic effect of high levels of KBrO_3 showed that renal neoplasias correlated to the induction of 8-hydroxyguanosine (8-OHdG), a mutagenic oxidative DNA lesion (Kasai et al. 1987). However, subsequent studies showed that knocking out 8-OHdG glycosylase activity did not increase renal neoplasia in comparison to wild-type litter mates, despite a large increase in 8-OHdG adducts (Arai et al. 2002). This observation indicates that KBrO_3 genotoxicity does not correlate with tumor susceptibility, suggesting that other mechanisms may mediate its nephrotoxicity and/or carcinogenesis. Given the

ability of KBrO₃ to alter DNA, we hypothesized that this DBP may also induce epigenetic changes, and that such changes may mediate KBrO₃-induced nephrotoxicity.

Our lab previously reported on the non-epigenetic mechanism of KBrO₃ nephrotoxicity using both *in vitro* and *in vivo* models (Kolisetty et al. 2013a; Kolisetty et al. 2013c; Zhang et al. 2011; Zhang et al. 2010). Our studies suggested that KBrO₃-induced nephrotoxicity was mediated by oxidative stress- and DNA damage-dependent mechanisms. Our *in vitro* studies showed that KBrO₃ treatment induced the expression of the tumor suppressor protein p53, and the cyclin-dependent kinase inhibitor p21 (Zhang et al. 2010). Our *in vivo* studies showed that KBrO₃ treatment increased p21 expression in the proximal tubule region of the rat kidney (Kolisetty et al. 2013a; Kolisetty et al. 2013b).

p21, also known as cyclin-dependent kinase inhibitor 1a (Cdkn1a), is a critical signaling protein that mediates several cellular processes including cell cycle, cell death and senescence (Jung et al. 2010; Price et al. 2009). Activation of p21 typically protects against numerous nephrotoxic events (Jiang and Dong 2008; Megyesi et al. 2001; Nowak et al. 2003; Price et al. 2009); however, p21 has also been hypothesized to be a driver of carcinogenesis via its ability to inhibit apoptosis and facilitate DNA repair (Abbas and Dutta 2009; Gartel 2009). While studies have shown that p21 is regulated via epigenetic pathways (Jung et al. 2010; Yoon et al. 2012), the ability of KBrO₃, or any other DBP, to epigenetically alter p21 expression has never been reported.

DNA methylation is one epigenetic event that can alter gene expression, protein expression and cell function (Phillips 2008). Essentially, it is the covalent addition of a methyl group, catalyzed by DNA methyltransferase (DNMT), to the 5-carbon of a cytosine residue in a CpG dinucleotide (Jaenisch and Bird 2003). This methyl group impedes the binding of transcription proteins, silencing a gene's transcription (Yu and Wang 2008). A number of tumor suppressor genes are silenced by methylation, including several cell cycle regulatory genes, p14, p15, p16, p57kip2 and Rb (Mathews et al. 2009).

Another type of epigenetic event is histone modification. Eukaryotic DNA is wrapped around histone octamers, which consist of four different histones: H2A, H2B, H3 and H4 (Luger et al. 1997). The N-terminal tail of each histone can be post-transcriptionally modified by lysine acetylation, lysine and arginine methylation, serine and threonine phosphorylation and lysine ubiquitination and sumoylation (Vaquero et al. 2003). Promoter-specific histone modifications can result in improper silencing or transcription of a gene, which can lead to cellular transformation, carcinogenesis or cancer progression (Fraga et al. 2005; Shiio and Eisenman 2003). Generally, histone acetylation induces gene activation by decreasing the histone's affinity for DNA, resulting in a more open conformation, referred to as euchromatin. Conversely, loss of an acetyl group, catalyzed by histone deacetylase (HDAC), results in the formation of heterochromatin and gene silencing. Similar to DNA methylation, it is not known if histone acetylation can mediate KBrO_3 toxicity.

This study tested the hypothesis that KBrO₃-induced renal cell death is mediated by epigenetic pathways. To test this hypothesis we determined the effect of inhibitors of both DNA methylation and histone deacetylation on the nephrotoxicity of KBrO₃ *in vitro*. Additionally, we determined the ability of these inhibitors to alter KBrO₃-induced changes in the expression of stress related proteins such as p38, p53 and especially p21. Finally, we assessed the ability of KBrO₃ to induce DNA methylation of p21.

Materials and Methods

Materials

Normal rat kidney (NRK) and human embryonic kidney (HEK293) cells, DMEM media, F-12K media and fetal bovine serum were purchased from American Type Culture Collection (Mannasas, VA). Antibiotic antimycotic solution was purchased from Sigma-Aldrich (St. Louis, MO). Potassium bromate (KBrO_3), 5-azacytidine (5-Aza), trichostatin A (TSA), and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). KBrO_3 was dissolved in deionized water and all other chemicals were dissolved in dimethyl sulfoxide (DMSO) from Fisher Scientific (Pittsburg, PA). Mouse anti-human p21 and phospho-H2AX antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-rat phospho-p38, phospho-p53 and p38 antibodies were purchased from Cell Signaling Technology (Danvers, MA). Secondary antibodies were obtained from Promega (Madison, WI). According to the manufacturer's notes, most of these antibodies cross-react with both rat and human tissue, and we verified the cross-reactivity of several of these antibodies prior to this study.

MTT Assay

The staining of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was used as an indicator of toxicity and corroborated with phase contrast microscopy. All cells were seeded in 48-well plates at 100,000 cells/mL and were grown for 48 h. After this time, cells were treated with varying

concentrations of epigenetic inhibitors (5-Aza, TSA) or vehicle control in the presence or absence of KBrO_3 for 48 h, and never above 1% of the total volume per well. After 48 h, 0.25 mg/mL of MTT was added to each well and the plates were incubated for 2 h. The media was then aspirated and replaced with DMSO. The plates were shaken at 100 rpm for 15 minutes to dissolve all precipitates and the absorbance of each well, including control and blank wells, was measured at 544 nm with a FLUOstar OPTIMA plate reader (BMG Lab Technologies, Inc., Durham, NC).

Immunoblot Analysis

Cells were seeded in 12 well plates at a concentration of 150,000 cells/mL and allowed to grow for 24 h (90% confluent) prior to addition of epigenetic inhibitors and/or KBrO_3 . After treatment, cells were washed three times and removed using a rubber policeman in immunoblot buffer (0.25 M Tris-HCl (pH 6.8), 4% SDS, 10% glycerol, 1 mg/ml bromophenol blue and 0.5% (v/v) 2-mercaptoethanol). Protein levels were determined in duplicate wells using lysis buffer that contained 1% (v/v) Triton X-100. Cell lysates containing 20 μg total protein were heated to 70°C for 10 min. Protein was separated under reducing conditions on a 12% SDS-polyacrylamide gel, and transferred to a nitrocellulose membrane. Non-specific binding was blocked by incubating the membrane with 3% (w/v) bovine serum albumin in TBS buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl) overnight at room temperature. Membranes were then incubated with the indicated primary antibody for 2 h, followed by incubation in the appropriate

secondary antibody for 1.5 h. Bands were detected by enhanced chemiluminescence (GE Healthcare, Chalfont St. Giles, UK).

Immunofluorescence

Cells (64,000-128,000 cells/mL) were grown on glass coverslips for 24 h and then treated. Following KBrO_3 treatment, cells were fixed using 10% buffered formalin/4% formaldehyde and washed with PBS. Cells were then permeabilized, washed with PBS, and non-specific binding was blocked by incubation in 8% (w/v) bovine serum albumin in PBS for 30 min. After washing, cells were incubated overnight at 4°C with either primary antibody against 8-OHdG, 5-methylcytosine (5 $\mu\text{g/mL}$) or an IgG control. Cells were then washed three times and incubated with a secondary antibody conjugated to FITC for 2 h. Samples were counterstained with DAPI and washed three times with PBS. Mounting medium was applied and the cover slips inverted onto glass slides. Visualization was performed using a Nikon AZ100 fluorescence microscope (Nikon, Melville, NY) with excitation and emission filters of 488 and 520 nm, respectively, for 8-OHdG and 5-methylcytosine, and 350 and 486 nm, respectively, for DAPI.

Methylation-Specific PCR

Cells were seeded in 6 well plates (32,000 cells/mL) and grown for 24 h. Cells were then exposed to low doses of KBrO_3 (0-10 ppm) for up to 28 days. Cells were passaged every 3 days and at least 1/2 of the cells were preserved for analysis, while another 1/2 was sub-cultured. Cell aliquots were passaged and treated again for 3 days. At each passage, 1/2 of the cells were

homogenized and genomic DNA extracted using a DNeasy tissue kit (Qiagen, Valencia, CA) following the manufacturer's protocol. Bisulfite conversion of DNA was performed using the CpGenome DNA Modification kit (Chemicon International, Billerica, MA) following the manufacturer's protocol. Converted DNA was amplified via PCR with primers specific for a CpG rich region of p21. The methylated and un-methylated primers amplified coding region residues 9577-9903 and 9577-9906, respectively, of the rat p21 gene. The products were confirmed by electrophoresis on a 2% agarose gel. The primers used are as follows:

Methylated Rat p21 sense: 5'-TGTATTAGTTATAGGTATTATGTTCTGA-3'

anti-sense: 5'-ACCCCTACAACAAAACCGAA-3'

Un-methylated Rat p21 sense: 5'-TGTATTAGTTATAGGTATTATGTTTGA-3'

anti-sense: 5'-CTAACCCCTACAACAAAACCAAA-3'

Statistical Analysis

Cells isolated from a distinct passage represented one experiment (n = 1). Unless otherwise indicated, data are represented as the mean $\pm$ SEM of at least 3 separate experiments (n = 3). An analysis of variance (ANOVA) followed by Tukey post hoc test was performed for each data set using GraphPad Prism software with $P < 0.05$ being considered indicative of a statistically significant difference.

Results

Effect of KBrO₃ on global DNA methylation

Global DNA methylation can be detected using several methods including immunohistochemistry, enzyme-linked immunosorbent assays (ELISAs), liquid chromatography tandem mass spectrometry (LC-MS) and so on (Vassar et al. 2009). Immunofluorescence approaches use a 5-methylcytosine antibody in tandem with a nuclear counter stain such as DAPI. We used this technique to demonstrate that neither 100 ppm nor 200 ppm KBrO₃ increased 5-methylcytosine staining in NRK cells after 3 days of treatment (**Figure 1**), compared to controls. This suggests that these doses of KBrO₃ may not alter global DNA methylation.

Effect of 5-Azacytidine and trichostatin A on KBrO₃-induced cytotoxicity

Our lab previously reported that KBrO₃ treatment alters both mRNA and protein expression *in vitro* and *in vivo* (Kolisetty et al. 2013c; Zhang et al. 2010). It is unknown if these alterations in expression occur via epigenetic mechanisms, or if epigenetic regulation is involved in KBrO₃ toxicity. 5-Azacytidine (5-Aza) and trichostatin A (TSA) are DNMT and HDAC inhibitors, respectively (Christman 2002; Yoshida et al. 1995). DNA methylation and histone deacetylation are modes of epigenetic regulation that generally result in a decrease in gene transcription and expression. Inhibition of these catalyzing enzymes, therefore, inhibits this silencing of expression.

To study the role of epigenetic regulation on KBrO₃-induced cytotoxicity, we treated NRK cells with various concentrations of TSA or 5-Aza, in the presence and absence of KBrO₃. The concentrations of KBrO₃ used were based on our previous *in vitro* and *in vivo* studies (Kolisetty et al. 2013a; Kolisetty et al. 2013c; Zhang et al. 2010). The concentrations of 5-Aza and TSA used were based on literature studies investigating DNMT and HDAC inhibitory activity (Creusot et al. 1982; Vigushin et al. 2001), as well as our own preliminary toxicity studies (data not shown). Treatment of cells with KBrO₃ alone induced a slight, but significant, decrease in MTT staining compared to controls (**Figure 2**). Treatment of cells with 5-Aza also resulted in a slight decrease in MTT staining compared to controls (**Figure 2A**). In contrast, TSA treatment had a greater effect on MTT staining (**Figure 2B**). Treatment of cells with 5-Aza and KBrO₃ resulted in greater decreases in MTT staining compared to cells exposed to KBrO₃ alone. Similar results were seen with TSA (**Figure 2B**) and in HEK293 cells (**Supplemental Figure 1**). These data were confirmed using cell and nuclear morphology (**Figure 2C-F**).

Effect of 5-Aza and TSA on KBrO₃-induced protein expression

We next assessed the effect of 5-Aza and TSA on the expression of proteins we previously demonstrated to be altered by KBrO₃, including p38, p53, H2AX and p21 (Zhang et al. 2010). 5-Aza and TSA alone had a minimal effect on p-p38 expression, compared to control cells. In contrast, 5-Aza treatment induced a marked increase in p-p53 and p21 expression. Interestingly, TSA

alone had a minimal effect on p-p53 levels, but still slightly increased p21 expression.

Treatment of NRK cells with 20 μ M 5-Aza and 100 ppm KBrO₃ for 3 days did not significantly alter the expression of p-p38 compared to cells treated with KBrO₃ or the inhibitor alone (**Figure 3**). Treatment with 5-Aza and KBrO₃ did not appear to increase p-p53 expression, but did decrease p21 and p-H2AX expression compared to KBrO₃ alone. Similar to 5-Aza, treatment with TSA did not appear to alter p-p38 expression, but did lower p-p53, p21 and p-H2AX expression compared to KBrO₃ alone. Similar results were seen in HEK293 cells, with the exception that changes in p21 and p-H2AX were not as large (**Supplementary Figure 2**).

Effect of low doses of KBrO₃ on cell morphology and cell count

A limitation of the above studies is that the doses of KBrO₃ used were much higher than those encountered within the environment. Therefore, we studied the effect of low doses of KBrO₃ on cell death and protein expression after sub-chronic exposure. These doses include 10 ppb (or 0.01 ppm), which is the recommended maximum containment limit (MCL) for BrO₃⁻ suggested by the United States Environmental Protection Agency (EPA 2012). We could not study the sub-chronic effect of KBrO₃ in combination with 5-Aza and TSA due to the toxicity seen with the inhibitors after long-term exposure (data not shown).

Treatment of NRK cells with 50 ppm KBrO₃ induced significant alterations in cell morphology starting after 9 days of treatment (3 passages); on the other

hand, concentrations of KBrO_3 lower than 50 ppm did not significantly alter cell morphology (**Figure 4A-F**). Similar results were seen in HEK293 cells (**Supplementary Figure 3**). KBrO_3 treatment started to decrease NRK cell number after exposure to 10 ppm for 15 days (5 passages, **Figure 4G**). Treatment of NRK cells with doses of KBrO_3 lower than 50 ppm did not decrease MTT staining at any dose studied (data not shown). These doses of KBrO_3 also did not result in detectable increases in 8-OHdG staining, a marker of KBrO_3 -induced DNA damage (**Figure 5**). Similarly, we did not detect any changes in nuclear morphology that would indicate the occurrence of apoptosis (i.e. nuclear fragmentation, chromatin condensation; **Figure 5**).

Effect of low doses of KBrO_3 on the expression of stress response proteins

Treatment of cells with low doses of KBrO_3 cells for 3 days (1 passage) caused a general increase in the expression of p-p38 and appeared to increase p21 expression compared to control at all doses tested (**Figure 6A**). KBrO_3 treatment for 6 days (2 passages) increased the expression p-H2AX at doses as low as 10 ppb (**Figure 6B**). Expression of p-p38 and p-p53 were also increased after 6 days of exposure to the lowest concentration of KBrO_3 tested (**Figure 6B**); however, treatment of cells with KBrO_3 for 9 days (3 passages) resulted in a return of p-p38 and p-p53 expression to control levels (**Figure 6C**), and this trend was maintained after 18 days of treatment (data not shown).

As mentioned above, treatment of NRK cells for 3 days with KBrO_3 altered p21 protein expression at doses as low as 0.01 ppm (10 ppb; **Figure 6A**). Unlike

p-p38 and p-p53, p21 protein expression continued to differ from control cells after 6 and 9 days of treatment (**Figure 6B and C**). Interestingly, KBrO₃ treatment induced a biphasic response in p21 expression after 6 and 9 days, with lower concentrations increasing protein expression, but higher concentrations (10 ppm) decreasing expression. To our knowledge, these are the lowest concentrations of KBrO₃ demonstrated to alter protein expression in any model.

Effect of KBrO₃ on DNA methylation of the p21 gene

DNA methylation plays a crucial role in carcinogenesis through methylating cytosine residues of various tumor suppressor genes, silencing their transcription. (Yu and Wang 2008). With this in mind, we tested the hypothesis that KBrO₃-induced changes in p21 protein expression correlate to changes in methylation of cytosine residues of p21. Treatment of NRK cells with 0.01-1 ppm KBrO₃ increased the level of un-methylated p21 after 18 days of treatment, while treatment of cells with 10 ppm KBrO₃ did not induce alterations in methylated or un-methylated p21 expression compared to control (**Figure 6D**). In contrast, treatment with the same concentration of KBrO₃ resulted in slight decreases in methylated p21 at 0.01 ppm. These data are in agreement with the protein expression data presented in **Figure 6C**.

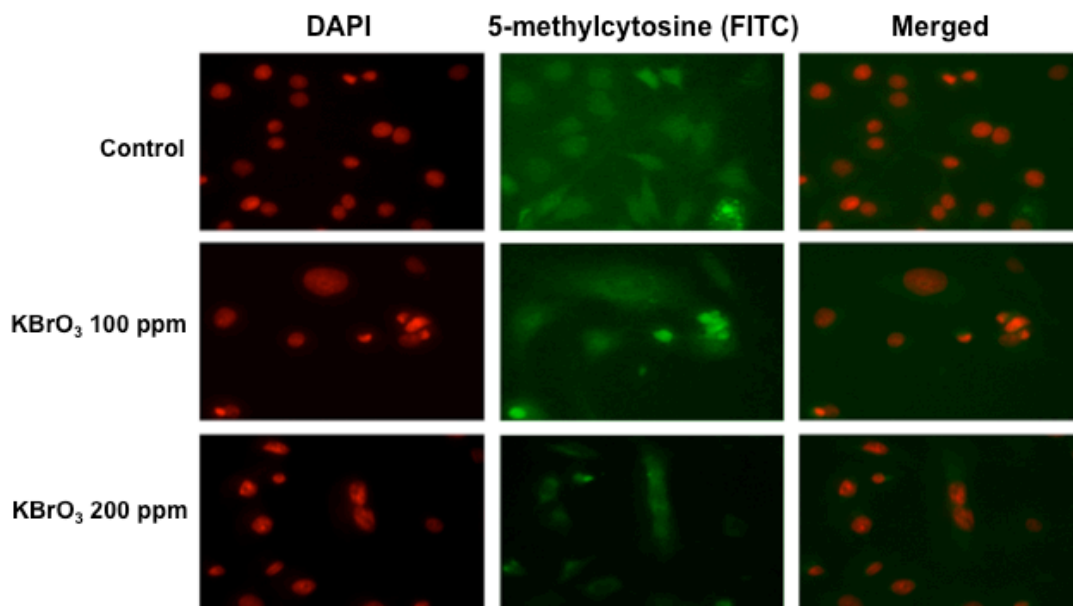


Figure 1. Effect of KBrO₃ on global DNA methylation in NRK cells. Cells were exposed to 100 ppm or 200 ppm KBrO₃. After 3 days, cells were fixed for analysis of 5-methylcytosine (FITC) and nuclear morphology (DAPI) using fluorescence microscopy. DAPI staining is pseudo-colored red and 5-methylcytosine is shown in green. Images were merged in the third column. Data are representative of at least 3 independent experiments ($n=3$).

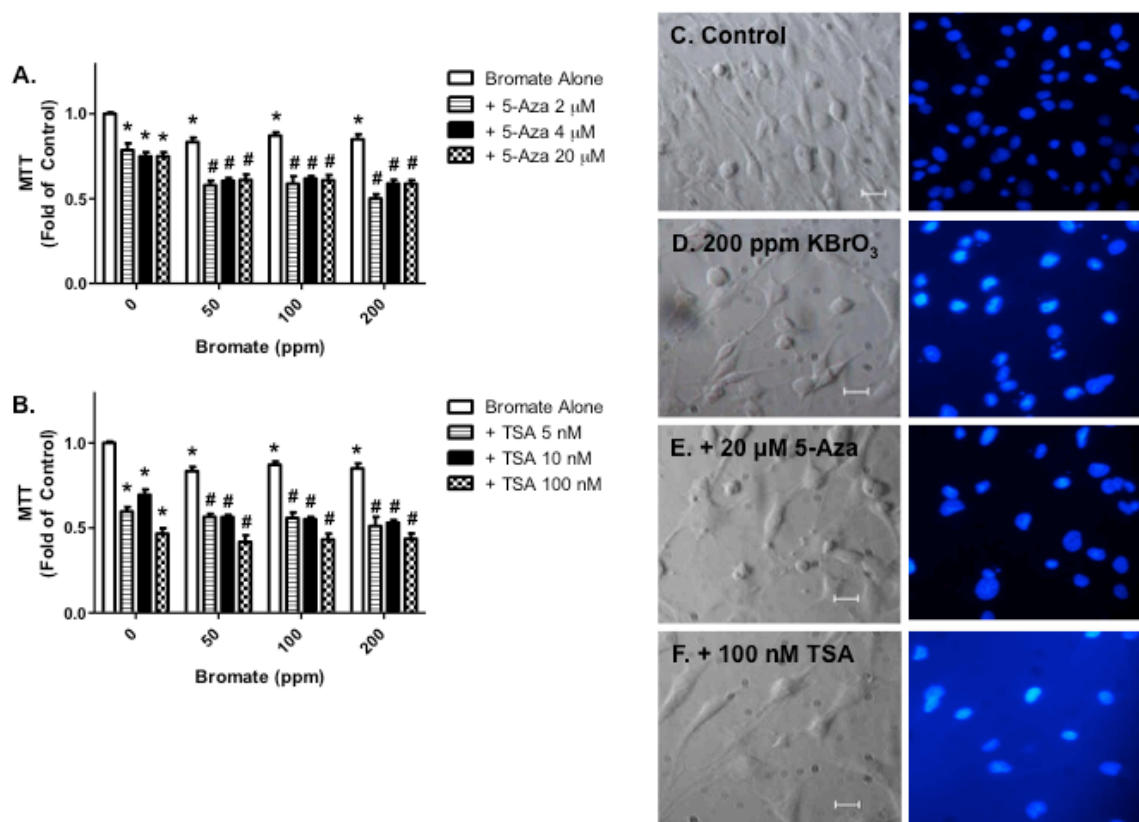


Figure 2. Effect of epigenetic inhibitors on KBrO₃-induced toxicity in NRK cells. Cells were treated with 5-Aza (**A**) or TSA (**B**) in the presence or absence of KBrO₃ for 48 h and then assessed for MTT staining (**A** and **B**) or cell and nuclear morphology (**C-F**). Data in A and B are represented as the mean $\pm$ the standard error (SE) of at least 3 separate passages ($n=3$). * $p < 0.05$ compared to control; # $p < 0.05$ compared to respective KBrO₃ treatment alone. Data in **C-F** are representative of multiple analysis and the scale bar represents 10 μ m.

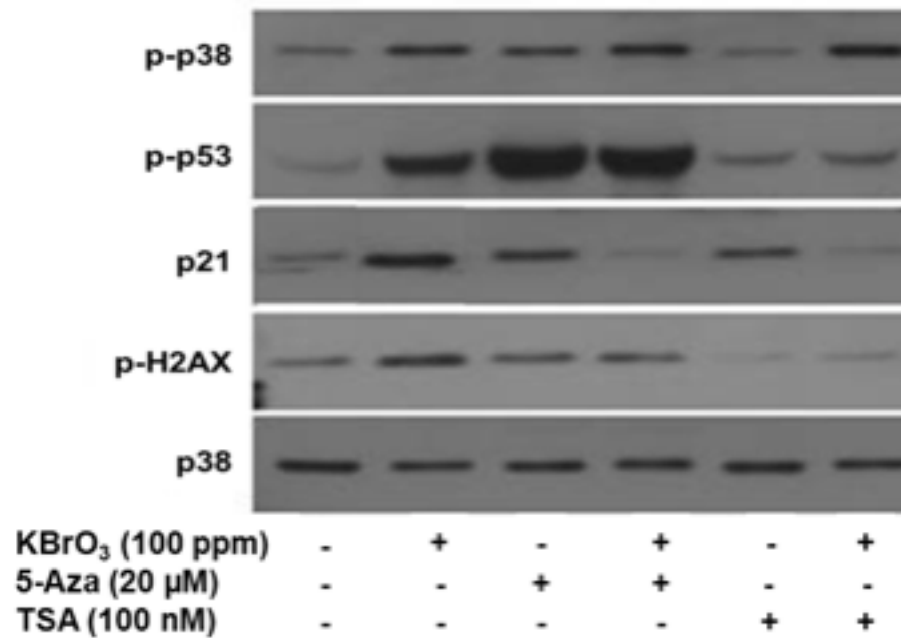


Figure 3. Effect of epigenetic inhibitors and KBrO₃ on the expression of stress response and cell cycle related proteins in NRK cells. Cells were treated with 5-Aza or TSA in the presence or absence of KBrO₃ for 3 days. Whole cell lysates were collected for immunoblot analysis. Total p38 is shown as the loading control. Data are representative of at least 5 independent experiments.

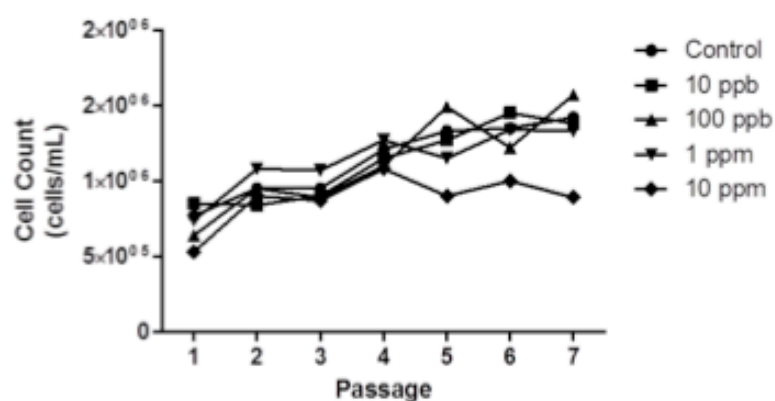
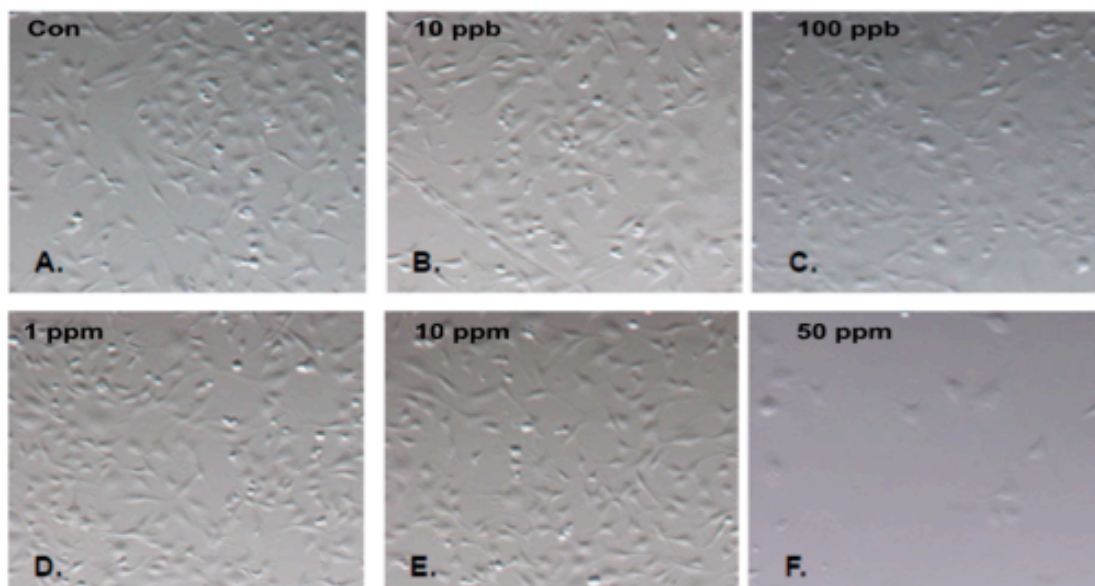


Figure 4. Effect of low doses of KBrO_3 on NRK cell morphology and number. NRK cells were exposed to 0-50 ppm KBrO_3 for 3 days and then passaged and retreated for an additional 3 days for seven total passages. Cell morphology (**A**) and cell number (**B**) was assessed after each passage. Data in **A** are representative of at least 5 independent experiments and are cells at passage 7. Data in **B** are representative of at least 5 separate passages ($n=5$).

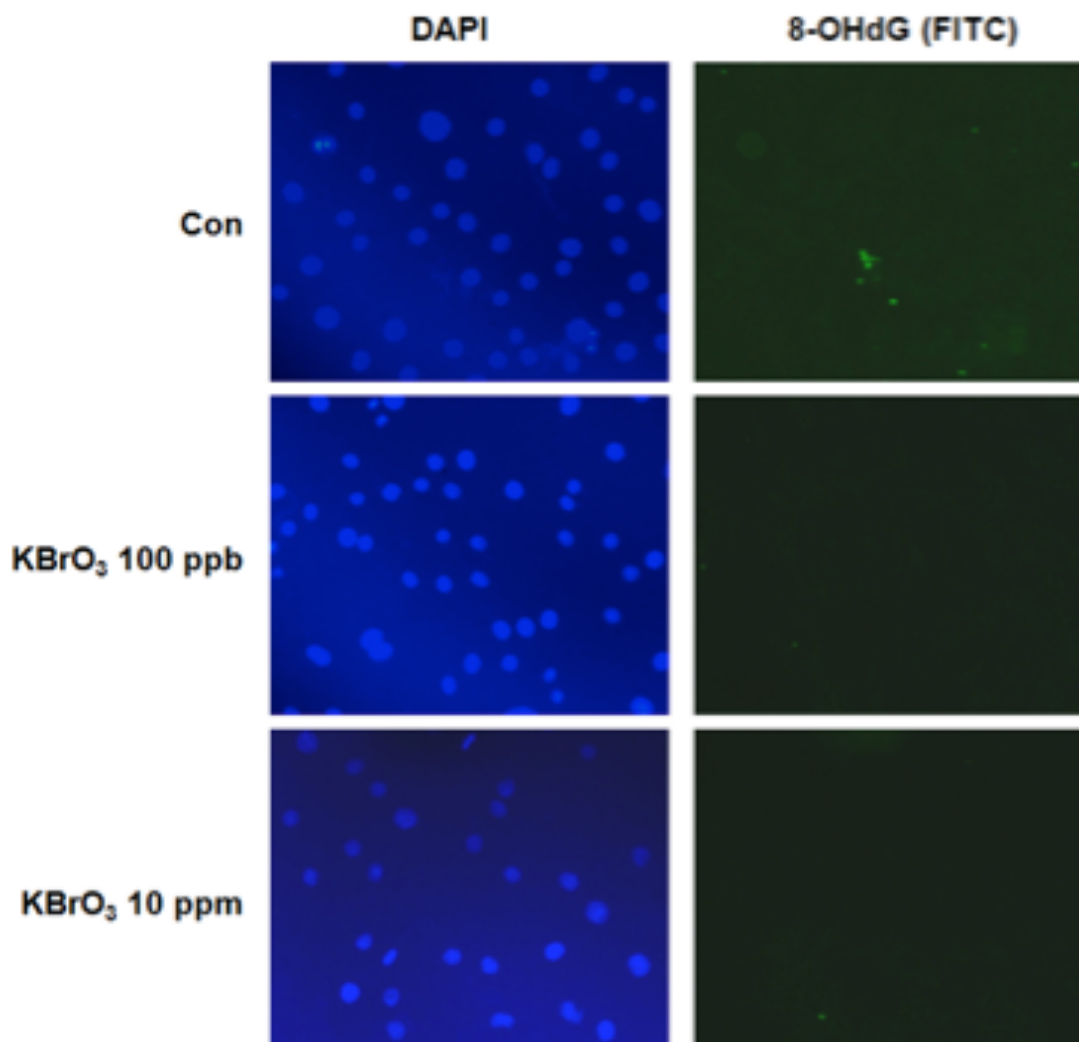


Figure 5. Effect of low doses of KBrO₃ on NRK cell nuclear morphology and 8-OHdG formation. NRK cells were treated with 100 ppb or 10 ppm KBrO₃ for 9 days prior to analysis of nuclear morphology and 8-OHdG staining using fluorescence microscopy. DAPI staining is shown in blue and 8-OHdG staining is shown in green. Data are representative of at least 3 independent experiments ($n=3$).

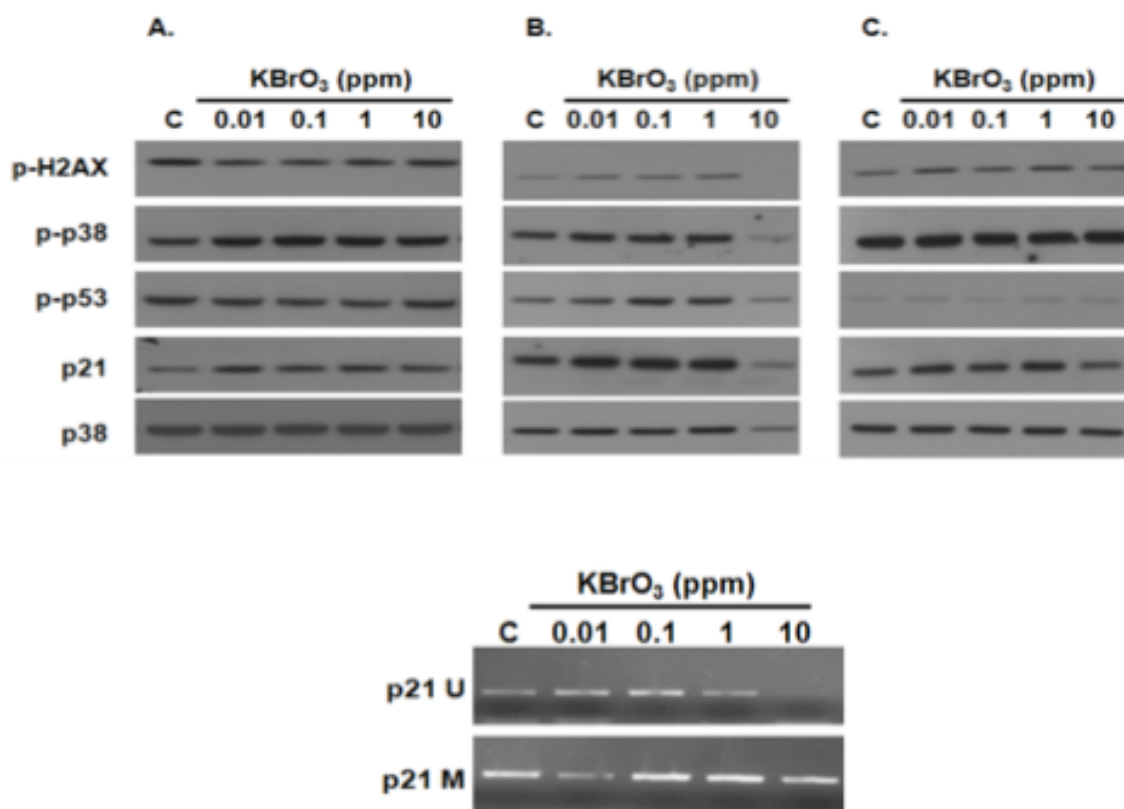
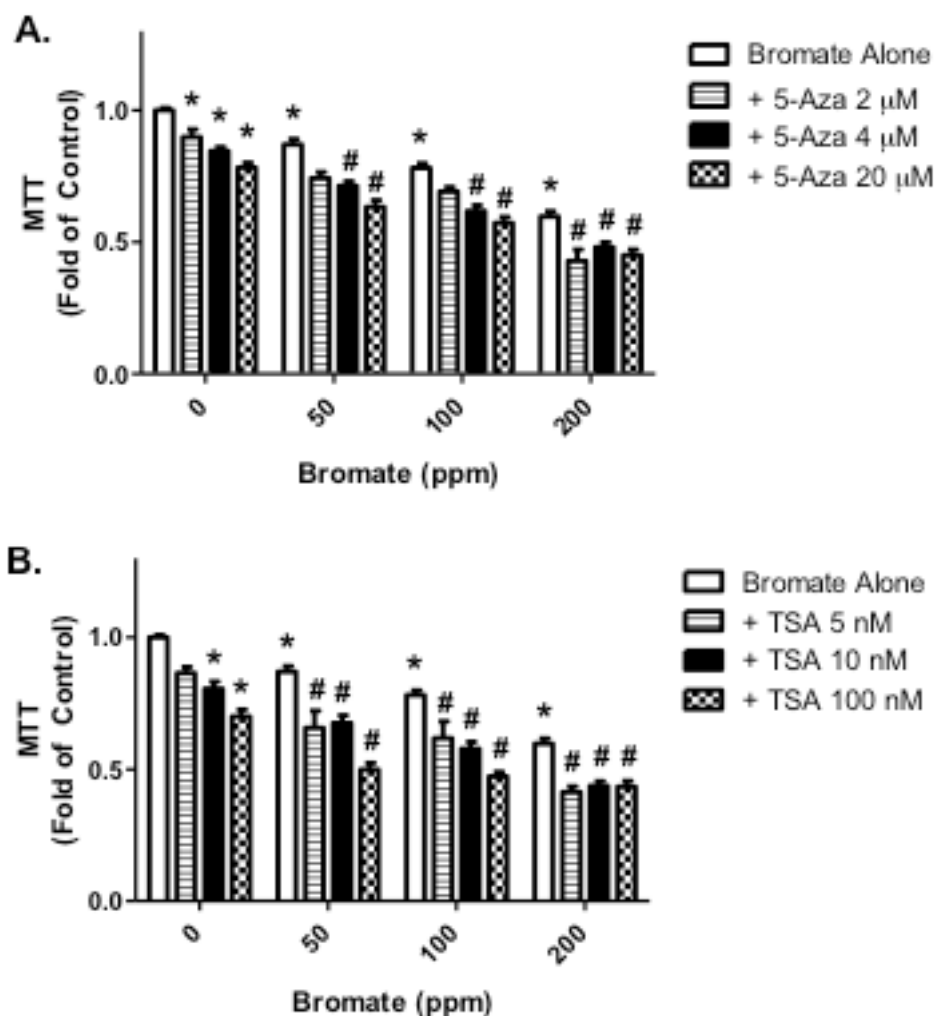
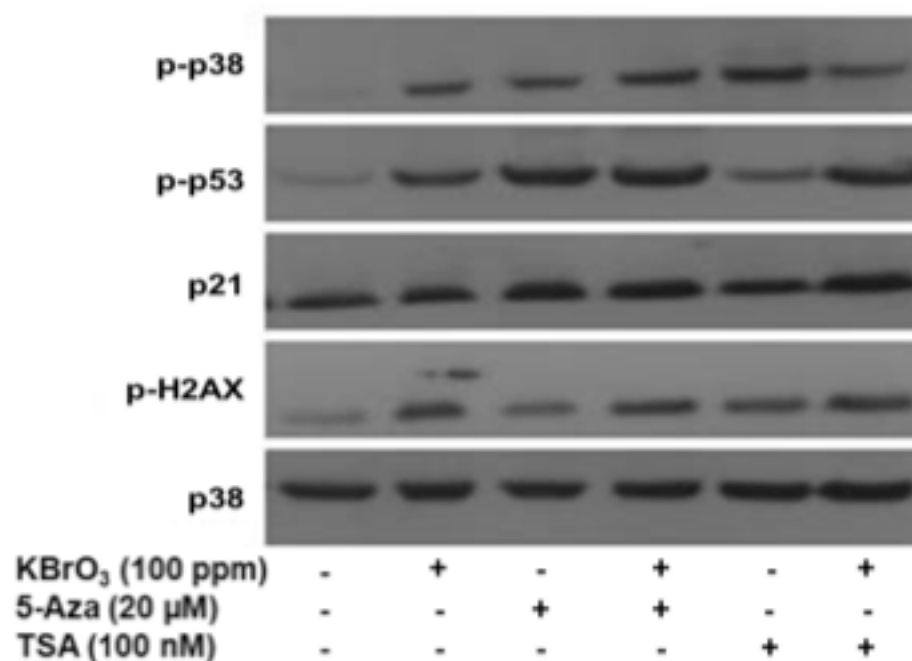


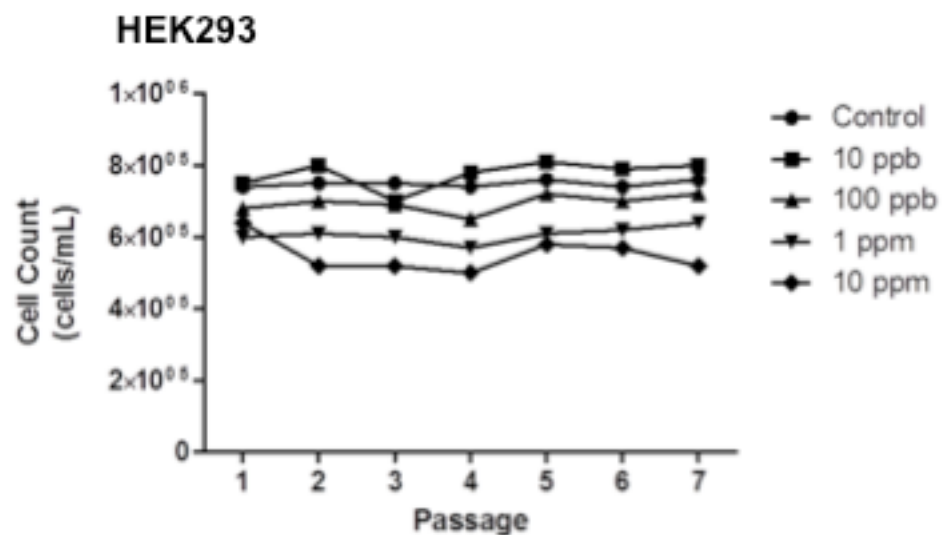
Figure 6. Effect of low doses of KBrO₃ on the expression and methylation of stress response and cell cycle related proteins in NRK cells. Cells were exposed to 0-50 ppm KBrO₃ for 3 days (**A**), then cells were sub-cultured and retreated for an additional 3 days (**B**), and sub-cultured and retreated again (**C**), prior to immunoblot analysis. p38 is shown as a loading control. After the final treatment (day 18) total DNA was extracted, subjected to bisulfite conversion followed by methylation-specific PCR for p21, and analyzed on 2% agarose gels stained with ethidium bromide and visualized under UV light (**D**). Data are representative of at least 3 independent experiments ($n=3$).



Supplemental Figure 1. Effect of epigenetic inhibitors on KBrO_3 -induced toxicity in HEK293 cells. HEK293 cells were treated with 5-Aza (**A**) or TSA (**B**) in the presence or absence of KBrO_3 for 48 h. Data are represented as the mean $\pm$ the standard error (SE) of at least 3 separate passages ($n=3$). * $p < 0.05$ compared to control; # $p < 0.05$ compared to respective bromate treatment alone.



Supplemental Figure 2. Effect of epigenetic inhibitors and KBrO₃ on the expression of stress response and cell cycle related proteins in HEK293 cells. Cells were treated with 5-Aza or TSA in the presence or absence of KBrO₃ for 3 days. Whole cell lysates were collected for immunoblot analysis. Total p38 is shown as the loading control. Data are representative of at least 5 independent experiments.



Supplemental Figure 3. Effect of low doses of KBrO₃ on HEK293 cell number. Cells were exposed to 0-50 ppm KBrO₃ for 3 days and then passaged and retreated for an additional 3 days, for seven total passages. Cell number was determined at each passage using a hemocytometer. Data are represented as the mean of at least 5 separate passages ($n=5$).

Discussion

This study showed that inhibitors of DNMT and HDAC altered cell death induced by BrO_3^- , a common water DBP and possible human carcinogen. As mentioned above, DNMT and HDAC are important epigenetic regulators, and mediate the transcription of numerous genes (Unoki et al. 2009). Thus, these data suggest that KBrO_3 -induced renal cell death may be mediated, in part, by epigenetic mechanisms. This conclusion is supported by data demonstrating that DNMT and HDAC inhibitors also alter the effect of KBrO_3 on the expression of several stress-related proteins. Further, KBrO_3 induced alterations in the methylation of p21 DNA.

To our knowledge, this is one of the first reports demonstrating that the toxicity of any DBP is altered by DNMT or HDAC inhibitors. However, this is not the first report to demonstrate that DBPs can alter DNA methylation. In fact, Tao et al (2005) showed that DNA hypomethylation was induced in mouse and rat kidneys after treatment with several different DBPs, such as dibromoacetic acid, dichloroacetic acid and trichloroacetic acid (Ge et al. 2001; Tao et al. 2005). None of these studies, however, included KBrO_3 . It should also be noted that the above studies used high doses of DBPs (500 mg/kg), which may not be environmentally relevant.

While no studies exist assessing if DNMT or HDAC inhibitors mediate KBrO_3 -induced cell death, a few do exist testing the effect of TSA or 5-Aza on cisplatin-induced nephrotoxicity (Arany et al. 2008; Dong et al. 2010; Sakao et al.

2011; Tikoo et al. 2009). In contrast to our data with KBrO_3 , these studies suggest that these inhibitors were protective against cisplatin-induced nephrotoxicity (Dong et al. 2010; Tikoo et al. 2009). Thus, the effect of DNMT or HDAC inhibitors on chemical-induced nephrotoxicity may be chemical-specific.

A limitation of many studies assessing the mechanisms of DBP toxicity, including our own, is that the concentrations of DPBs used are often high compared to the environmental levels. For example, our previous studies used concentrations of KBrO_3 in the range of 15-400 ppm (Kolisetty et al. 2013a; Zhang et al. 2010). However, KBrO_3 levels in most drinking water ranges from 10-100 ppb. We addressed this limitation in this study by using low-dose and sub-chronic treatments. Similar to data seen with high-dose acute exposures, exposure of NRK cells to KBrO_3 at concentrations ranging from 0.01 to 10 ppm altered the expression of p-H2AX, p-p53, p-p38 and p21 after 3, 6 and 9 days. These data suggest that the mode of action of low doses of KBrO_3 may be similar to that seen at higher doses if allowed sufficient treatment time. An important aspect of these findings is that increases in 8-OHdG staining were not detected at these doses, nor were changes in nuclear morphology, suggesting that KBrO_3 can induce alterations in cell signaling that occur in the absence of wide-spread DNA damage.

This study also investigated the effect of KBrO_3 on the epigenetic status of p21. p21 is a cell cycle regulator whose protein and mRNA expression have been shown by us to be altered both *in vitro* and *in vivo* after KBrO_3 treatment (Kolisetty et al. 2013a; Zhang et al. 2010). Our data shows that KBrO_3 alters the

level of p21 DNA methylation at doses as low as 0.01 ppm and after only 6 days of exposure and that these alterations persist after 9 days of exposure. The inability of KBrO₃, even at high doses, to increase global DNA methylation compared to control cells suggests that KBrO₃ exposure may not alter the overall activity of DNA methyltransferases and that this effect is gene-specific.

No studies could be found demonstrating that any DPB, let alone KBrO₃, can alter p21 gene methylation. Our data shows a biphasic response of p21 methylation to KBrO₃ exposure. Low doses (0.01-1 ppm) of KBrO₃ increased the level of un-methylated p21 in kidney cells, indicating hypomethylation; however, exposure to 10 ppm KBrO₃ decreased the expression of un-methylated p21, indicating hypermethylation. The shift in favor of un-methylated p21 seen with lower doses correlates to the increase in p21 protein expression observed under similar conditions. As previously mentioned, p21 is a tumor suppressor and the increases in p21 expression after low-dose KBrO₃ treatment may be a protective response, as p21 is known to protect against nephrotoxicity induced by several compounds (Abukhdeir and Park 2008; Jiang and Dong 2008; Megyesi et al. 2001; Nowak et al. 2003; Price et al. 2009). This hypothesis is supported by our data showing that the increase in cell death induced by 5-Aza and TSA after acute exposure to KBrO₃ correlates to decreases in p21 expression. It should also be noted that increases in cell death correlated to increases in p53 expression as well.

The biphasic response in p21 expression observed after sub-chronic treatment to low doses of KBrO₃ is puzzling. The decrease in un-methylated p21

seen with 10 ppm could be indicative of an increase in DNA damage that was not detectable using the methods employed herein, and may represent a threshold for cell death, which was observed at 50 ppm. These doses are nearing the range of KBrO_3 shown to induce carcinogenesis *in vivo* after long-term treatments (DeAngelo et al. 1998; Kurokawa et al. 1990; Wolf et al. 1998). Interestingly, studies suggest that hypermethylation of the p21 gene is associated with increased risk of developing certain cancers, as well as with decreased cancer survival rate (Askari et al. 2013; Roman-Gomez et al. 2002). Thus, the decrease in un-methylated p21 and p21 protein expression at 10 ppm may represent initial mechanisms of transformation in these cells. Future studies are needed to address this hypothesis. The ability of KBrO_3 to alter p21 methylation *in vivo* also needs to be assessed.

It is important to note that the alterations in gene methylation and protein expression induced by KBrO_3 doses near the MCL (0.01 ppm or 10 ppb) may not be “toxic.” Rather, these changes may be more “adaptive” in nature. This assertion is supported by the fact that we detected no increases in cell death using a variety of assays (cell morphology, nuclear morphology, 8-OHdG staining, etc.). Additionally, our previous studies showed that 10 ppm KBrO_3 did not increase ROS formation, annexin or PI staining, or decrease ATP levels after 72 h (Zhang et al. 2010).

Even though the doses of KBrO_3 used here are among the lowest ever shown to induce altered cell signaling or molecular changes, it is still possible that renal cells *in vivo* might not be exposed to concentrations of KBrO_3 as high

as 10 ppb. This hypothesis is supported by our recent kinetic analysis of KBrO_3 in blood and urine of rats after both acute and sub-chronic exposures, which suggested KBrO_3 levels below 1 mg/kg would almost be completely converted to bromide (Br^-) after oral ingestion (Bull et al. 2012).

The concentrations of TSA and 5-Aza used for our study have been previously shown to inhibit HDAC and DNMT activity, respectively (Creusot et al. 1982; Vigushin et al. 2001); however, both TSA and 5-Aza can have non-specific effects, especially at high concentrations. For example, TSA can alter the acetylation of non-histone proteins, particularly transcription factors, thereby affecting their function (Adcock 2007). Further, 5-Aza can be incorporated into RNA, in addition to DNA, causing disruption of nucleic acid and protein metabolism and leading to apoptosis (Aimiwu et al. 2012; Kiziltepe et al. 2007; Kuykendall 2005). Before determining the specificity of TSA and 5-Aza for epigenetic mediators, we first wanted to ensure that these inhibitors altered the toxicity of BrO_3^- . The importance of the individual HDACs and DNMTs inhibited and the specificity

In conclusion, data reported in this study demonstrate that treatment of renal cells with inhibitors of enzymes important for epigenetic regulation alters KBrO_3 -induced renal cell death. These data also demonstrate that KBrO_3 treatment itself alters DNA methylation of p21 and that such alterations are manifested phenotypically, based on changes in protein expression. These findings yield novel insights into the mechanism of KBrO_3 -induced cytotoxicity. The data also beg the question if other DBPs also induce epigenetic changes,

what other genes may be altered and if such changes can be detected *in vivo*. Finally, it is known that epigenetic regulation of gene expression is species-specific, so studies in human models of renal cell death are still needed.

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NEPHROTOXICITY OF EPIGENETIC INHIBITORS USED FOR THE TREATMENT OF CANCER¹

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Abstract

This study determined the anti-neoplastic activity and nephrotoxicity of epigenetic inhibitors *in vitro*. The therapeutic efficacy of epigenetic inhibitors was determined in human prostate cancer cells (PC-3 and LNCaP) using the DNA methyltransferase inhibitor 5-azacytidine (5-Aza) and the histone deacetylase inhibitor trichostatin A (TSA). Cells were also treated with carbamazepine (CBZ), an anti-convulsant with histone deacetylase inhibitor-like properties. 5-Aza, TSA or CBZ alone did not decrease MTT staining in PC-3 or LNCaP cells after 48 hr. In contrast, docetaxel, a frontline chemotherapeutic, induced concentration-dependent decreases in MTT staining. Pretreatment with 5-Aza or TSA increased docetaxel-induced cytotoxicity in LNCaP cells, but not PC-3 cells. TSA pretreatment also increased cisplatin-induced toxicity in LNCaP cells. Carfilzomib, a protease inhibitor approved for the treatment of multiple myeloma, had minimal effect on LNCaP cell viability, but reduced MTT staining 50% in PC-3 cells compared to control, and pretreatment with 5-Aza further enhanced toxicity. Treatment of normal rat kidney (NRK) and human embryonic kidney 293 (HEK293) cells with the same concentrations of epigenetic inhibitors used in prostate cancer cells significantly decreased MTT staining in all cell lines after 48 hr. Interestingly, we found that the toxicity of epigenetic inhibitors to kidney cells was dependent on both the compound and the stage of cell growth. Collectively, these data show that combinatorial treatment with epigenetic inhibitors alters the efficacy of chemotherapeutics in cancer cells in a compound- and cell-specific

manner; however, this treatment also has the potential to induce nephrotoxic cell injury.

Introduction

DNA methylation is an epigenetic event that affects cell function by altering gene expression (Phillips 2008). Essentially, it is the covalent addition of a methyl group, catalyzed by DNA methyltransferase, to the 5-carbon of a cytosine in a CpG dinucleotide (Jaenisch and Bird 2003). This methyl group impedes the binding of transcription proteins, thereby decreasing transcription and expression. DNA methylation plays a crucial role in carcinogenesis through methylation of cytosines at promoter regions of various genes, silencing their transcription (Yu and Wang 2008). A number of tumor suppressor genes can also be silenced via methylation (Mathews et al. 2009).

Histone modifications can also affect gene expression. Histone acetylation generally induces gene activation through post-transcriptional acetylation of lysine residues at the N-terminal tail of each of four histones: H2A, H2B, H3, and H4 (Luger et al. 1997; Vaquero et al. 2003). Histone acetyltransferase catalyzes the transfer of an acetyl group from acetyl-coenzyme A to the histone. The addition of an acetyl group decreases the interaction of the histone with the negatively charged phosphate groups of DNA. This results in less condensed chromatin, referred to as euchromatin, which is more readily and easily transcribed. Histone deacetylases remove these acetyl groups. Alterations in promoter-specific histone modifications can result in improper silencing or transcription, which could lead to cellular transformation, carcinogenesis or cancer progression (Fraga et al. 2005; Shiio and Eisenman 2003).

Growing evidence indicates that epigenetic abnormalities contribute to the dysregulation in gene expression associated with cancer formation and progression (Jones and Baylin 2007). Prostate cancer is the second leading cause of cancer death among men in the United States. Although the mechanisms underlying prostate cancer formation are not well understood, irregular epigenetic events including alterations in DNA methylation and histone modifications appear to contribute to its development (Bhatia and Agarwal 2001; Dobosy et al. 2007; Li et al. 2005; Majid et al. 2008; Yu et al. 2010). This is one reason why epigenetic inhibitors are being proposed as potential treatments for prostate cancer, among other cancer types, both alone in combination with established chemotherapeutics (Juergens et al. 2011; Li et al. 2014; Ramachandran et al. 2009; Silverman et al. 2002).

Several studies have investigated the role of epigenetic alterations on the expression of specific genes and how these genes contribute to prostate cancer growth (Bhatia and Agarwal 2001; Majid et al. 2008; Yu et al. 2010). In contrast, fewer studies have examined the effect of concomitant treatments of epigenetic inhibitors and known chemotherapeutics on prostate cancer cell growth. One such study assessed the effect of pretreatment of prostate cancer cells with the DNA methyltransferase inhibitor 5-azacytidine followed by the chemotherapeutic docetaxel, and found that this co-treatment increased docetaxel-induced apoptosis in DU-145 cells (Ramachandran et al. 2009). Another study found that combinatorial treatment with a histone deacetylase inhibitor and androgen

receptor antagonist synergistically reduced prostate cancer cell proliferation and increased cell death (Marrocco et al. 2007).

A limitation of the above studies, as well as other studies, is that the anti-cancer activities of these treatments were not compared directly to their non-target organ toxicity. The kidney is often subject to adverse effects by chemotherapeutic treatments (Kintzel 2001; Ries and Klastersky 1986). Further, epigenetic inhibitors are known to induce cell- and compound-specific nephrotoxicity. For example, 5-azacytidine induces renal tubular dysfunction in patients with advanced acute leukemia (Kintzel 2001; Peterson et al. 1981). Dong et al. (2008) found that histone deacetylase inhibitors induce apoptosis in renal tubular cells. DNA methyltransferase inhibitors and histone deacetylase inhibitors have also been shown to induce nephrotoxicity via increased oxidative stress (Nadasi et al. 2009). These studies support the need to examine the effect of concomitant treatment schedules of epigenetic inhibitors with established chemotherapeutics on nephrotoxicity. This study determined the cytotoxicity of DNA methylation and histone deacetylation inhibition alone and in combination with various chemotherapeutics on prostate cancer and kidney cells.

Materials and Methods

Materials

Normal rat kidney (NRK) cells, human embryonic kidney (HEK293) cells, human prostate cancer (PC-3, LNCaP) cells, DMEM media, F-12K media, RPMI 1640 media and fetal bovine serum were purchased from American Type Culture Collection (Mannasas, VA). Antibiotic antimycotic solution was purchased from Sigma-Aldrich (St. Louis, MO). Docetaxel, cisplatin, carfilzomib (CFZ), 5-azacytidine (5-Aza), carbamezapine (CBZ), trichostatin A (TSA) and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). All compounds were dissolved in dimethyl sulfoxide (DMSO) from Fisher Scientific (Pittsburg, PA).

Cell Lines and Treatments

The human PC-3 cell line was initiated from bone metastasis of a prostatic adenocarcinoma. These cells are p53-null, androgen insensitive and malignant. LNCaP cells are derived from a human lymph node metastasis of prostate carcinoma and are p53-positive, androgen sensitive and less aggressive. Docetaxel is a frontline chemotherapeutic for prostate cancer and acts through tubulin disturbance (Omlin et al. 2014). Cisplatin is a common chemotherapeutic that acts through DNA alkylation. Carfilzomib is a selective proteasome inhibitor approved for the treatment of myeloma (Siegel et al. 2012). 5-Aza is a DNMT inhibitor, TSA is an HDAC inhibitor and CBZ is a clinically used anticonvulsant

with HDAC inhibitor-like properties (Beutler et al. 2005; Christman 2002; Yoshida et al. 1995).

MTT Staining

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) staining was used as an indicator of toxicity and was corroborated with examination of cellular morphology using phase contrast microscopy. All cell lines were seeded in 48-well plates at 100,000 cells/mL. The prostate cancer cells were incubated 24 hr to reach log phase growth. The kidney cells were grown for 48 hr to obtain confluence. After this time, kidney cells and prostate cancer cells were pretreated with varying concentrations of epigenetic inhibitors (5-Aza, CBZ, TSA) or vehicle control for 30 min, followed by treatment with chemotherapeutics or vehicle control for an additional 48 hr. The total dosing amount was never above 1% of the total volume per well. After 48 hr, 0.25 mg/mL of MTT was added to each well and the plates were incubated for 2 hr. The media was then aspirated and replaced with DMSO. The plates were shaken at 100 rpm for 15 min to dissolve all precipitates and the absorbance of each well at 544 nm, including control and blank wells, was measured with a FLUOstar OPTIMA plate reader (BMG Lab Technologies, Inc., Durham, NC).

Statistical Analysis

Cells isolated from a distinct passage represented one experiment (n = 1). Data are represented as the mean $\pm$ SEM of at least 3 separate experiments (n = 3). A one-way analysis of variance (ANOVA) was performed for each data set

followed by Bonferonni post-hoc test using GraphPad Prism software with $p < 0.05$ being considered indicative of a statistically significant difference between mean values.

Results

Effect of epigenetic inhibitors on prostate cancer and kidney cytotoxicity

Both kidney and prostate cancer cells were treated with epigenetic inhibitors for 48 hr. The DNA methyltransferase inhibitor 5-azacytidine (5-Aza) was used at concentrations ranging from 0-20 μ M, the HDAC inhibitor trichostatin A (TSA) was used at concentrations of 0-100 nM and carbamezapine (CBZ) was used at 0-100 μ M. These concentrations were chosen based on literature studies investigating DNMT and HDAC activity (Beutler et al. 2005; Creusot et al. 1982; Vigushin et al. 2001). 5-Aza had minimal effects on MTT staining in PC-3 or LNCaP cells as compared to controls. In contrast, 5-Aza treatment decreased MTT staining in NRK and HEK293 cells at concentrations as low as 2 mM (**Figure 1A**). TSA treatment decreased MTT staining in kidney cells at concentrations of 10 nM and higher, and decreased MTT staining in LNCaP cells at a concentration of 100 nM. Similar to 5-Aza, TSA had no effect on MTT staining in PC-3 cells at any concentration (**Figure 1B**). On the other hand, CBZ treatment only altered MTT staining at concentrations above 100 μ M, where it significantly decreased MTT staining in LNCaP, HEK293 and NRK cells compared to control (data not shown).

Effect of chemotherapeutics on prostate cancer and kidney cytotoxicity

Docetaxel is a frontline chemotherapeutic for the treatment of prostate cancer and acts via tubulin disruption (Omlin et al. 2014). Unlike 5-Aza and TSA, docetaxel decreased MTT staining in all cell lines tested, with initial decreases

being detected at concentrations as low as 1 nM (**Figure 1C**). As anticipated, docetaxel had a greater effect in prostate cancer cells than in kidney cells.

We also examined the effects of carfilzomib (CFZ), a selective proteasome inhibitor approved for the treatment of myeloma, on kidney and prostate cancer cytotoxicity at concentrations from 0-20 nM. Interestingly, treatment of cells with only 10 nM, the lowest concentration used for this study, caused an approximate 50% decrease in MTT staining in PC-3 cells, but had minimal effects on MTT staining in LNCaP cells. CFZ also had a minimal effect on MTT staining in kidney cells (**Figure 1D**). Cisplatin, another prominent chemotherapeutic and well-known nephrotoxicant, was also studied at concentrations ranging from 0-10 μ M, and induced approximately a 25% decrease in MTT staining in each cell line at the highest concentration used (**Supplemental Figure 1**).

Effect of epigenetic inhibitors on chemotherapeutic-induced cytotoxicity in prostate cancer cells

Prostate cancer cells were treated with epigenetic inhibitors for 30 min prior to 48 hr exposure to chemotherapeutics. Pretreatment with 5-Aza, TSA or CBZ had no effect on docetaxel-induced cytotoxicity in PC-3 cells (data not shown). 5-Aza and TSA treatment alone had little to no effect on MTT staining or cell number and morphology in LNCaP cells, while docetaxel induced a concentration-dependent decrease. Pretreatment with any concentration of 5-Aza tested further decreased MTT staining by approximately 20% compared to

10 nM docetaxel alone in LNCaP cells (**Figure 2A**). Similar effects were observed with TSA, as combinatorial treatments with docetaxel and TSA at 1 nM and 10 nM, respectively, decreased MTT staining to levels comparable to 10 nM docetaxel (**Figure 2B**). These data were confirmed using phase contrast microscopy (**Figure 2B-F**).

We further explored the ability of epigenetic inhibitors to augment chemotherapeutic efficacy in prostate cancer cells by studying the effect of TSA and 5-Aza on cisplatin- and CFZ- induced toxicity. Similar to the results reported above, pretreatment of LNCaP cells with TSA decreased MTT staining compared to cells treated with cisplatin alone (**Figure 3A**). However, these same results were not seen with 5-Aza or CBZ (data not shown). While CFZ induced a concentration-dependent decrease in MTT staining in PC-3 cells, neither TSA nor CBZ altered these decreases (data not shown). In contrast, pretreatment of PC-3 cells with 5-Aza resulted in modest, but significant, decreases in MTT staining, compared to cells exposed to only CFZ (**Figure 3B**). The results of these combinatorial treatments are summarized in **Table 1**.

Effect of epigenetic inhibitors on chemotherapeutic-induced cytotoxicity in kidney cells

While epigenetic inhibitors may increase cancer cell death on their own, or even augment the effect of other chemotherapeutics, these beneficial clinical results may be for naught if they also induce nephrotoxicity. To address this possibility, we exposed both rat and human renal cell lines to the same treatment

protocols used for prostate cancer cells. As reported above, the epigenetic inhibitors alone did prove to be more toxic to kidney cells than to the prostate cancer cells (**Figure 1**). Treatment of HEK293 cells with either 5-Aza or TSA prior to exposure to docetaxel or CFZ did not further decrease MTT staining, compared to cells treated with either chemotherapeutic alone (**Figure 4**). Similar results were seen in NRK cells exposed to TSA and 5-Aza in combination with docetaxel and CFZ (**Supplemental Figure 2**) and in kidney cells treated with CBZ and either chemotherapeutic (**Supplemental Figure 3**). In contrast, treatment of HEK293 and NRK cells with 5-Aza prior to exposure to cisplatin significantly decreased MTT staining, compared to cells treated with cisplatin alone (**Figure 5**). Treatment with TSA or CBZ prior to cisplatin had no effect on MTT staining in HEK293 or NRK cells (**Supplemental Figure 4**).

Effect of stage of cell growth on chemotherapeutic- and epigenetic inhibitor-induced toxicity in kidney cells

For the experiments discussed above, kidney cells were exposed to treatment at confluence to more closely mimic the biological state of the kidney. We also exposed kidney cells to epigenetic inhibitors and chemotherapeutics during log phase growth to maintain consistency with the experiments performed on prostate cancer cells. As shown in **Figure 6A-C**, decreases in MTT staining in HEK293 cells were similar at almost all concentrations of epigenetic inhibitors, with slight differences being seen at the highest concentrations tested. As expected, the toxicity of the chemotherapeutics was dependent on the stage of cell growth (data not shown). In contrast to HEK293 cells, the stage of cell growth

had a more significant effect on epigenetic inhibitor-induced decreases in MTT staining in NRK cells. Of even greater interest was the fact that decreases in MTT staining were greatest in confluent cells. For example, 5-Aza exposure resulted in a 20% greater decrease in MTT staining in confluent NRK cell cultures, as compared to cells exposed during log phase growth (**Figure 6D**). This same trend was seen with TSA, but not with CBZ (**Figure 6E and F**).

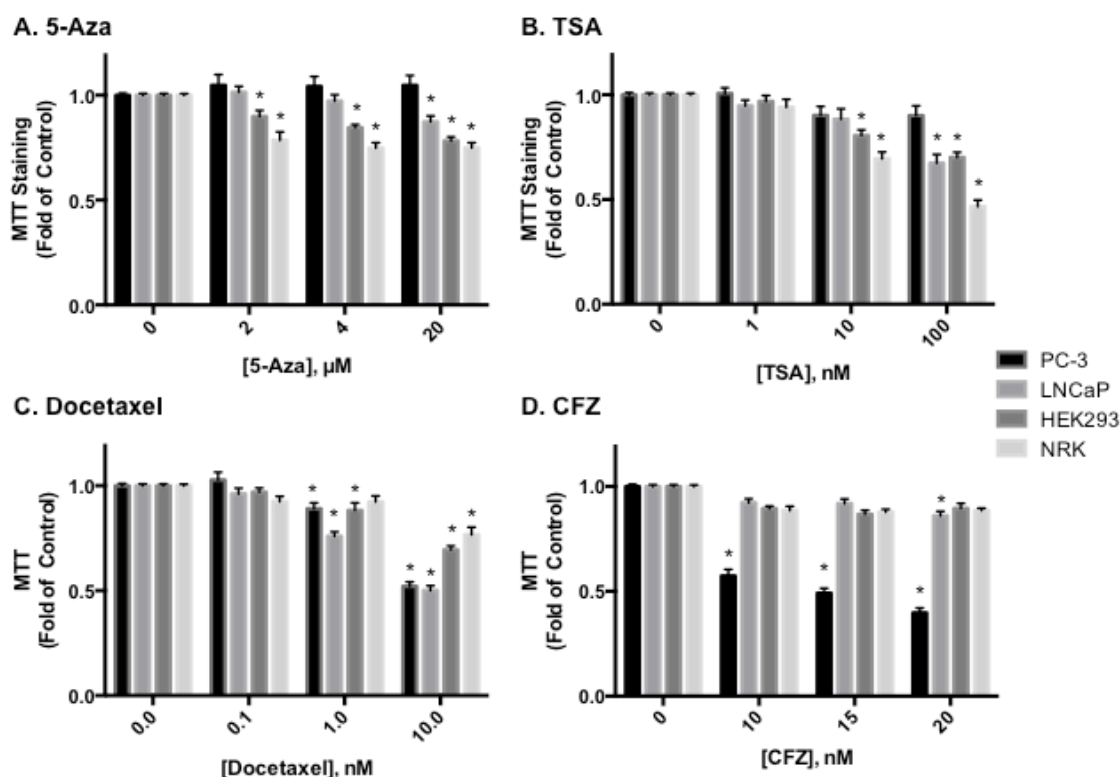


Figure 1. Effect of epigenetic inhibitors and chemotherapeutics on prostate cancer and kidney cytotoxicity. Cells were treated with 5-Aza (**A**), TSA (**B**), docetaxel (**C**) or CFZ (**D**) for 48 hr and cytotoxicity was assessed using MTT staining. Data are indicative of results from at least 3 separate passages per cell line (n=3) and are expressed as mean $\pm$ SEM. (* $p < 0.05$ compared to respective control by One-way ANOVA followed by Bonferonni post-hoc test).

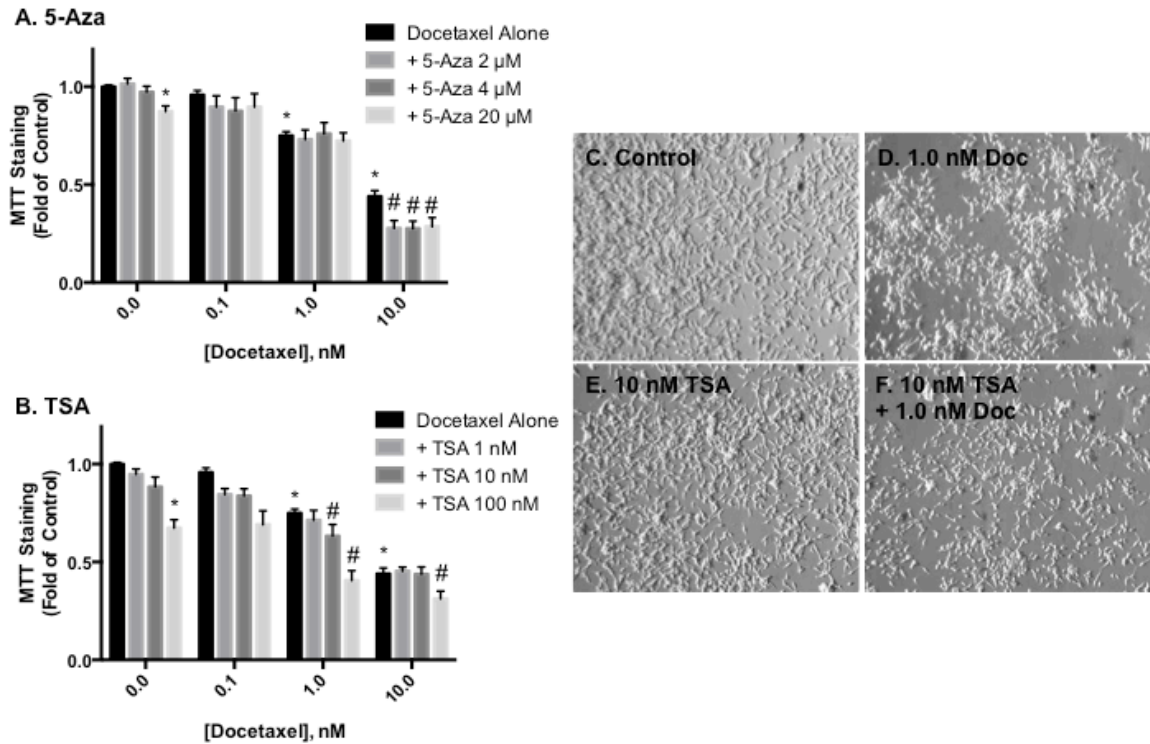


Figure 2. Effect of epigenetic inhibitors on docetaxel-induced cytotoxicity in LNCaP cells. Cells were pretreated with 5-Aza or TSA for 30 min prior to 48 hr exposure to docetaxel and cytotoxicity was measured via changes in MTT staining (**A**, **B**) and phase contrast microscopy (**C-F**). Data in **A** and **B** are indicative of results from at least 3 separate passages per cell line ($n=3$) and are expressed as mean $\pm$ SEM. (* $p < 0.05$ compared to control, # $p < 0.05$ compared to respective docetaxel treatment alone by One-way ANOVA followed by Bonferonni post-hoc test).

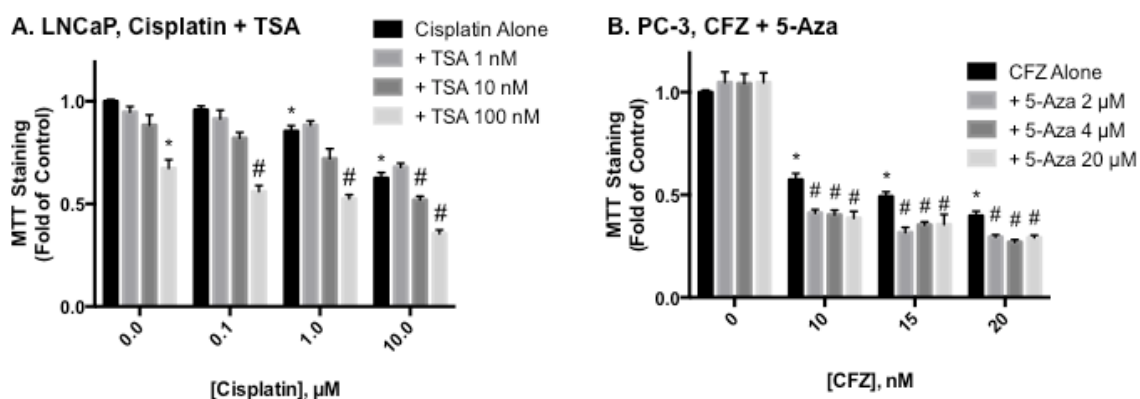


Figure 3. Effect of epigenetic inhibitors on cisplatin- and CFZ-induced cytotoxicity in prostate cancer cells. LNCaP (**A**) cells were combinatorial treated with TSA and cisplatin and PC-3 (**B**) cells with 5-Aza and CFZ. The effect on cytotoxicity was measured using MTT staining and data are indicative of results from at least 3 separate passages per cell line ($n=3$) and are expressed as mean $\pm$ SEM. (* $p < 0.05$ compared to control, # $p < 0.05$ compared to respective chemotherapeutic treatment alone by One-way ANOVA followed by Bonferonni post-hoc test).

Table 1. Effect of epigenetic inhibitors on chemotherapeutic-induced toxicity in prostate cancer cells^a

	+ 5-Aza ^b		+ CBZ		+ TSA	
	LNCa	PC-3	LNCa	PC-3	LNCaP	PC-3
Docetax	✓ ^c	✓	+	-	-	-
Cisplatin	✓	✓	-	-	-	-
CFZ	-	✓	-	+	-	-

^aCells were exposed to docetaxel, cisplatin or carfilzomib (CFZ) for 48 hr, after which toxicity was assessed using MTT staining.

^bCells were pretreated for 30 min with 5-Aza = 5-azacytidine, CBZ = carbamezapine or TSA = trichostatin A prior to chemotherapeutic exposure.

^c✓ indicates that the given chemotherapeutic was toxic, while - indicates no significant effect and + indicates increased toxicity in the presence of the specified epigenetic inhibitor.

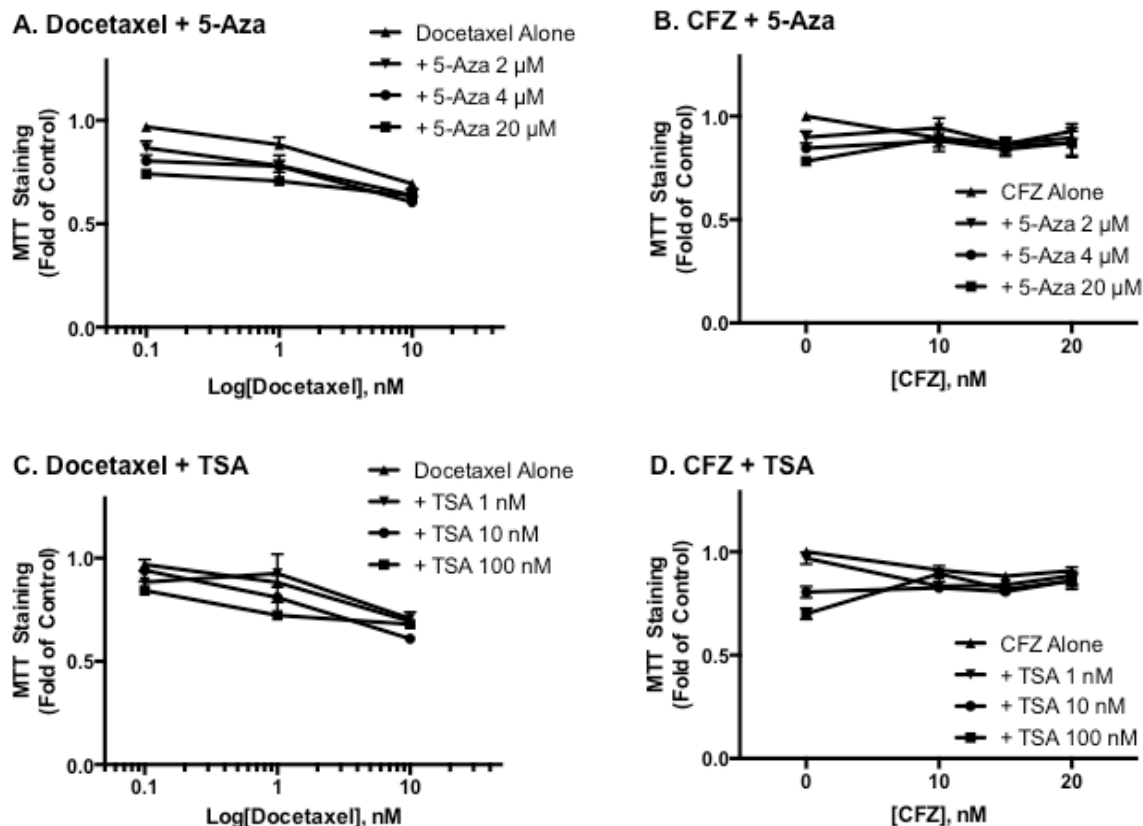


Figure 4. Effect of epigenetic inhibitors on docetaxel and CFZ-induced toxicity in HEK293 cells. Cells were exposed to the same treatments as prostate cancer cells and the effects of epigenetic inhibitors on docetaxel- (**A**, **C**) and CFZ-induced (**B**, **D**) toxicity were examined using MTT staining. Data are indicative of results from at least 3 separate passages per cell line (n=3) and are expressed as mean $\pm$ SEM.

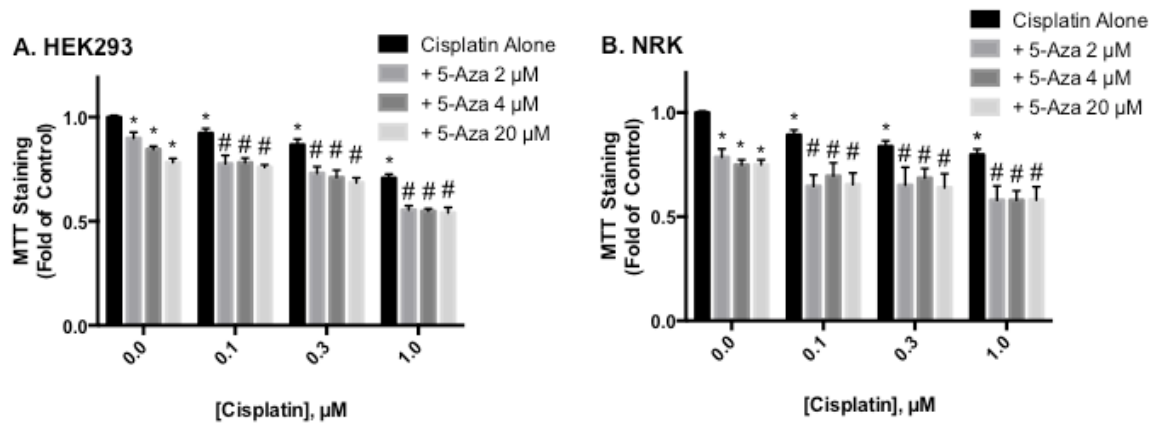


Figure 5. Effect of 5-Aza pretreatment on cisplatin-induced toxicity in kidney cells. HEK293 (**A**) and NRK (**B**) cells were pretreated with 5-Aza prior to 48 hr exposure to cisplatin and analysis via MTT staining. Data are indicative of results from at least 3 separate passages per cell line ($n=3$) and are expressed as mean $\pm$ SEM. (* $p < 0.05$ compared to control, # $p < 0.05$ compared to respective chemotherapeutic treatment alone by One-way ANOVA followed by Bonferonni post-hoc test).

Discussion

Multiple studies have shown that epigenetic inhibitors, including DNA methyltransferase and histone deacetylase inhibitors, induce nephrotoxicity (Nadasz et al. 2009; Peterson et al. 1981). Even so, these compounds are continuously being investigated as potential chemotherapeutics. In support of this research, data in this study showed that pretreatment of prostate cancer cells with these inhibitors enhanced the toxicity of several diverse chemotherapeutics. Unfortunately, data in this study also showed that epigenetic inhibitors alone were more toxic to kidney cells than to prostate cancer cells. Further, combinatorial treatment of kidney cells with certain epigenetic inhibitors increased the toxicity of select chemotherapeutics. Fortunately, these increases were only seen for the combination of 5-Aza and cisplatin.

While data in this study showed that epigenetic inhibitors in general did not increase chemotherapeutic-induced nephrotoxicity, they also demonstrated that these inhibitors did not protect against this toxicity. This is in contrast to data from Tikoo et al. (2009) who reported that 5-Aza decreased the nephrotoxicity of cisplatin *in vivo* while enhancing its anti-neoplastic activity against colon and breast cancer. It should be noted, however, that the concentrations of 5-Aza used in the aforementioned study did not induce nephrotoxicity alone, unlike those used for our study. Thus, these differences could be due in part to the different concentrations used. In support of this hypothesis, Dong et al. (2010) observed that TSA also lessened cisplatin-induced nephrotoxicity; however, concentrations of TSA shown in a previous study by the same group to be

nontoxic alone (Dong et al. 2008) were used for this study. TSA undergoes extensive hepatic metabolism (Sanderson et al. 2004), so while we observe toxicity *in vitro*, this effect may be limited *in vivo*. Conversely, it is also possible that differences between *in vitro* and *in vivo* studies may be related to differences in kinetics. For example, renal excretion is the primary route of 5-Aza clearance (Troetel et al. 1972), which in combination with the data presented here, suggests the need for a DNA methyltransferase inhibitor with alternate pharmacokinetic properties for use as a therapeutic treatment.

The finding that kidney cells were more susceptible to epigenetic inhibitor-induced toxicity when exposed at confluence compared to during log phase growth was initially surprising. In general, chemotherapeutics target dividing cells, more of which are present during log phase growth. The mechanism of action of these epigenetic inhibitors, however, is different from that of chemotherapeutics, and these differences may explain our findings. 5-Aza incorporates into DNA where it irreversibly binds to DNMTs rendering them inactive resulting in depletion of cellular DNMT levels (Christman 2002; Stresemann and Lyko 2008). Increased DNMT expression and activity, such as that present in actively dividing cells, could decrease the effectiveness of 5-Aza. In support of this hypothesis, DNMT activity has been shown to be downregulated during the G₀ phase of the cell cycle (Hayes et al. 2005; Robertson et al. 2000). Therefore, less 5-Aza could be necessary to saturate DNMT activity in confluent cells than in cells at log phase growth, shifting the

dose response. Similarly, TSA binds directly to HDACs, so the same principal could be behind the toxicity pattern observed with this compound.

In general, epigenetic inhibitors had little to no effect on chemotherapeutic-induced toxicity in kidney cells; however, pretreatment with epigenetic inhibitors did enhance chemotherapeutic-induced toxicity in prostate cancer cells in a cell- and compound-specific manner, as outlined in **Table 1**. This is an exciting and potentially clinically beneficial finding, but the mechanisms behind these enhancements remain unknown. Furthermore, it should be mentioned that while epigenetic events are integral to cancer formation and progression, studies suggest that these combinatorial effects may actually be independent of epigenetic alterations. For example, studies by Abbruzzese and Frost (1992) indicate that DNA methyltransferase inhibitor incorporation into DNA, not the resulting hypomethylation, may increase cisplatin binding, resulting in synergistic cytotoxicity. Therefore, further studies are needed to discern the mechanisms mediating alteration in toxicity induced by combinatorial treatment of epigenetic inhibitors and chemotherapeutics.

The innovative design of this study investigated the anti-cancer activity of combinatorial treatments with various epigenetic inhibitors and chemotherapeutics in tandem with non-target organ toxicity. The data presented here corroborate the potential therapeutic use of epigenetic inhibitors, as indicated by increased chemotherapeutic-induced toxicity in prostate cancer cells with select co-treatments. However, the DNA methyltransferase and histone deacetylase inhibitors used proved more toxic to kidney cells than prostate

cancer cells. Further, we report the novel finding that epigenetic-inhibitor induced toxicity in kidney cells is dependent on the stage of cell growth at the time of exposure, with confluent cells being more susceptible than those in log phase growth. Taken together, these data further validate the need to thoroughly examine non-target toxicity when developing potential chemotherapeutic treatments.

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