Cocaine-mediated Induction and Gene Targets of Hippocampal *FosB*

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Abstract
Relapse to drug use is driven by hippocampus-mediated associations between the drug and the environmental context in which drugs are taken. The synaptic connections within the hippocampus play an essential role in learning and memory, and chronic exposure to drugs of abuse evokes changes in synaptic strength within the hippocampus. However, the changes in gene expression that underlie drug-induced alterations in hippocampal synaptic function remain unknown. One mechanism by which these changes could occur and be maintained is through the action of stable, long-lasting transcription factors, such as ΔFosB. ΔFosB has been extensively studied in the reward pathway - however, its role in the hippocampus is unknown. Therefore, I will investigate whether ΔFosB regulates transcription of genes involved in hippocampal synaptic function to control formation of drug-environment associations. To accomplish this, I will elucidate the mechanism through which cocaine mediates the induction of ΔFosB and determine whether ΔFosB is necessary for changes in gene expression caused by chronic cocaine exposure. Understanding the molecular mechanisms behind drug addiction is a key step for developing treatments that prevent the development of, or relapse to, addiction.

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Specific Aims

The health and financial burden of substance abuse will continue to rise unless specific facets of addiction, such as relapse, are better treated. Relapse to drug use after prolonged abstinence requires hippocampus-mediated associations between the drug and environmental context. Repeated exposure to drugs of abuse leads to changes in transcriptional activity throughout the brain, including the hippocampus[5]. These changes may occur via the action of stable transcription factors, such as ΔFosB. ΔFosB plays a critical role in the reward pathway - however, its role in hippocampus has not yet been investigated. Our preliminary data demonstrate that hippocampal ΔFosB is necessary for both general learning and formation of drug-environment associations. *Therefore, my central hypothesis is that ΔFosB regulates transcription of genes involved in hippocampal synaptic function to control formation of drug-environment associations.* We will determine histone modifications associated with the *FosB* gene during cocaine-mediated induction, manipulate these modifications using novel viral tools, and determine the transcriptional targets of ΔFosB in the hippocampus. To accomplish this, I propose the following aims:

Aim 1: Characterize induction of the *FosB* gene in hippocampus in response to chronic cocaine

Expression of *FosB* gene products is increased throughout the brain after drug exposure, and this correlates with changes in histone modifications at the *FosB* gene in nucleus accumbens (NAc)[6, 7]. In this aim, we will use immunohistochemistry (IHC) and chromatin immunoprecipitation (ChIP) to quantify induction of *FosB* gene products in hippocampus and determine whether histone modifications associated with the *FosB* gene in hippocampus are regulated in response to cocaine.

1a: Characterize the induction of *FosB* gene products in hippocampal subregions in response to chronic cocaine

1b: Determine epigenetic changes at the *FosB* gene in response to chronic cocaine

Aim 2: Determine whether *FosB* induction in hippocampus is mediated by histone modification writers or erasers

Our preliminary data indicate that cocaine induces *FosB* gene products in hippocampus, and they are critical for forming drug-environment associations. Here, we will determine whether histone modification writers or erasers have an impact on the induction of *FosB* in hippocampus using direct injection of pharmacological inhibitors or viral vectors to overexpress these enzymes. In addition, to determine whether dimethylation of lysine 9 on histone H3 (H3K9me2) specifically at the *FosB* promoter regulates cocaine-mediated induction of *FosB* in hippocampus, we will utilize novel zinc-finger fusion protein viral vectors.

2a: Determine whether specific histone modification writers or erasers drive cocaine-mediated *FosB* induction

2b: Determine whether epigenetic modification of the *FosB* gene regulates cocaine-mediated induction in hippocampus

Aim 3: Identify FosB gene targets in the hippocampus

The downstream mechanisms by which FosB regulates hippocampal function are unknown. To identify gene targets of ΔFosB in hippocampus, we will conduct ChIP followed by qPCR for candidate genes known from NAc, as well as ChIPseq to determine novel targets. To elucidate the role of *FosB* in cocaine-mediated changes in gene expression, we will use *floxed FosB* mice in combination with a novel ribosomal trapping technology to determine global FosB-dependent cocaine-mediated gene transcription in hippocampus

3a: Determine FosB gene targets in hippocampus

3b: Determine hippocampal FosB gene targets regulated by cocaine

Drugs of Abuse and Addiction

Drug addiction may be defined as the continued seeking and taking of drugs despite adverse consequences. These negative consequences include significant physical, criminal, and mental health problems for the individual and their families, as well as considerable societal costs. For example, the National Drug Intelligence Center reported that illicit drugs cost the United States more than $193 billion in 2011 alone[8]. These expenses resulted from costs associated with putting individuals through the criminal justice system, providing health care, and from productivity lost due to drug abuse and drug-related incarceration. Current treatments for addiction have limited effectiveness, with virtually no pharmacotherapies for some forms of drug addiction. While there are addiction treatment centers available, not enough individuals are getting the help they need. In 2013, the Substance Abuse and Mental Health Services Administration (SAMHSA) reported that 22.7 million people in the US needed treatment for an illicit substance or alcohol-use problem, though only 10.9% of them received treatment at a specialized facility (hospital inpatient, rehabilitation or mental health center)[9]. Therefore, new and more effective targeted therapeutics are necessary to stem the rapidly growing costs of drug addiction.

Cocaine is the second most abused illegal substance in the US, behind marijuana[10]. It is a highly addictive stimulant that has the potential to quickly progress from experimentation to abuse and dependence. Cocaine produces its pleasurable effects by increasing dopamine signaling in the nucleus accumbens (NAc), a brain region that modulates feelings of pleasure and reward. This pathway is normally activated in response to pleasurable activities such as eating, sex, or social interaction, but drugs of abuse hijack this reward circuitry and have the ability to make all other normally rewarding activities seem less pleasurable. Although the mechanism by which cocaine acts is well established, efficacious pharmacological treatments for cocaine addiction remain elusive. More importantly, even if an individual maintains decades of abstinence after abuse, the daily threat of relapse remains. Presently, there is no FDA-approved pharmacological treatment for cocaine addiction. However, within this thesis I propose targeting a key feature of cocaine addiction, context-induced relapse to cocaine-seeking, which could ultimately lead to the development of more effective pharmacotherapies. Before such therapies can be developed, we require a better understanding of the functional role of the central nervous system in this particular characteristic of cocaine addiction.

Cocaine and the Central Nervous System

Cocaine exerts complex effects on multiple regions of the central nervous system. Primarily it leads to activation of the reward pathway, which begins with dopamine release in the NAc from ventral tegmental area (VTA) projections. As an inhibitor of the dopamine transporter, cocaine prevents reuptake of dopamine, leading to a buildup of it in the synaptic cleft and increased activation of dopamine receptors in the NAc (Figure 1). The reward pathway is also modulated by

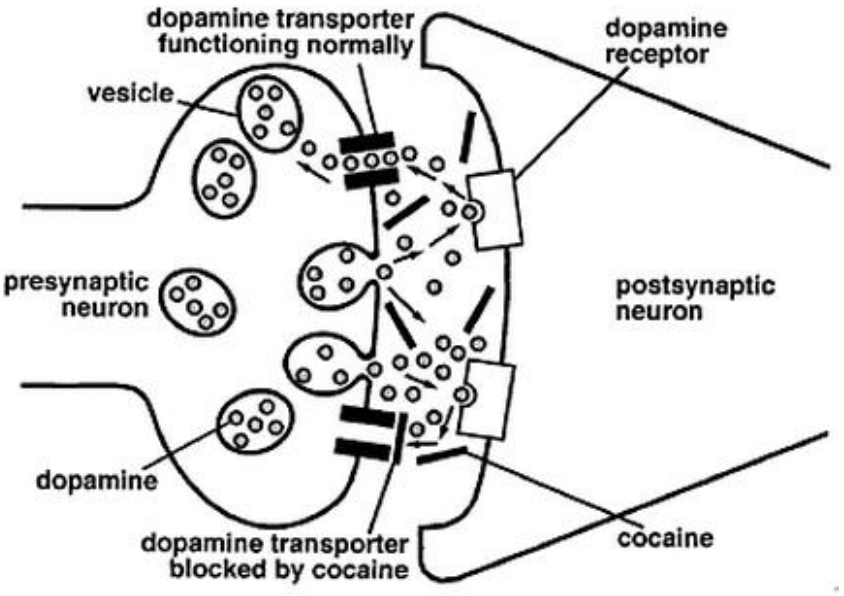


Figure 1. Cocaine mechanism of action at the synapse (BSCS and Videodiscovery, Inc.[2])

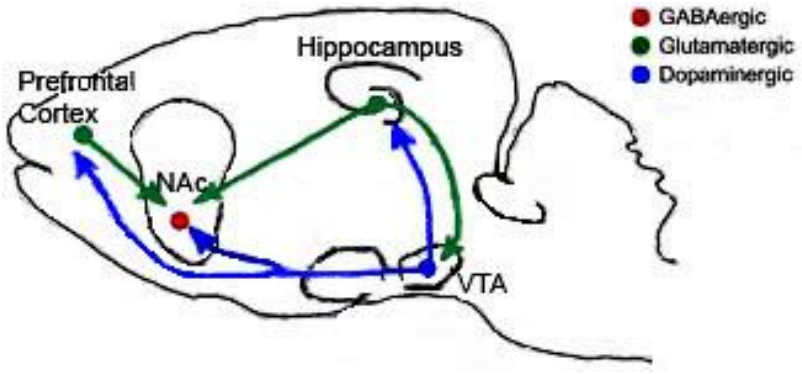


Figure 2. Brain circuitry showing the connections between the reward pathway and the hippocampus (modified from Nestler and Carlezon[3])

glutamatergic inputs from a variety of brain regions (Figure 2), including the hippocampus, a specialized limbic structure essential for the consolidation of spatial, contextual, and declarative memories for storage in the cortex. Stimulation of the hippocampus causes increased firing of VTA dopamine neurons and relapse to cocaine-seeking behavior in rats[11]. Furthermore, in an experiment where animals learned to self-administer cocaine in the presence of a specific contextual environment, inactivation of the ventral hippocampus significantly decreased the relapse to context-induced cocaine-seeking after withdrawal[12]. Contextual memories are strongly implicated in addiction. For example, the pleasurable feelings from using cocaine are strongly associated with the house, people, or paraphernalia involved in acquiring and consuming the drug. Modeled in the rodent, cocaine conditioned place preference (CPP) pairs an injection of cocaine or saline with a contextually distinct chamber and allows us to measure the strength of the associations between the drug and the environment. After lesion of the dorsal hippocampus, rats were unable to display cocaine CPP, suggesting that the hippocampus plays a critical role in drug-environmental association[13]. These spatial and contextual cues have the potential to trigger craving for the drug during abstinence because these strong associations continue to exist even after successful treatment[14]. The development of these associations is in part due to how cocaine changes hippocampal synaptic plasticity, for instance through the enhancement of long-term potentiation, a mechanism proposed to underlie memory formation [15, 16]. However, the molecular mechanism behind how these long-term effects are maintained is not well understood. It has been hypothesized that long-term exposure to drugs causes stable changes in gene expression in the brain resulting in aberrant behaviors that characterize addiction[5], but the transcriptional and epigenetic changes responsible for this remain unclear. One target candidate in addiction is ΔFosB, a member of the Fos family of transcription factors, which robustly accumulates with chronic cocaine exposure in multiple brain regions (including NAc and hippocampus), and is stable for weeks after the last cocaine injection[17-20]. The investigation of certain stable transcription factors, such as ΔFosB, may elucidate a role for specific gene expression changes that underlie addiction behavior.

FosB Gene and Drug Exposure

The Fos family of transcription factors dimerize with Jun family proteins to form AP1 complexes, which regulate transcription of many genes, acting as either a transcriptional activators or repressors. *FosB* is an immediate early gene (IEG) that is rapidly induced in response to a variety of stimuli. Most IEG proteins undergo a quick turnover rate, lasting only a few hours in the cell, which is true of the full-length FosB protein. However, the ΔFosB isoform, a splice variant which incorporates an early stop codon truncating ~13 kDa from the c-terminus of the protein (Figure 3), remains stable in the cell for many days because it lacks two degron domains found in full-length FosB[1]. Overexpression of ΔFosB in NAc and dorsal striatum in bitransgeneic mice enhances the locomotor response to cocaine and cocaine reward[21], and these mice self-administer more cocaine than wild type mice[22]. Conversely, inhibition of ΔFosB in NAc reduces the locomotor sensitization to cocaine, a behavioral phenomenon dependent on plasticity changes in response to psychostimulants[23]. These findings suggest that normal accumulation of

ΔFosB in the brain after chronic cocaine exposure may underlie the development of addictive behaviors. FosB isoforms are also functionally relevant in the hippocampus. Our preliminary data show that a dominant negative version of FosB (ΔJunD) injected into the hippocampus using a viral vector impairs cocaine CPP (Figure 4), suggesting that FosB is mediating the formation of drug-environment associations. ΔJunD is a truncated version of JunD and when it dimerizes with FosB gene products, the heterodimers are unable to bind to AP1 sites[23].

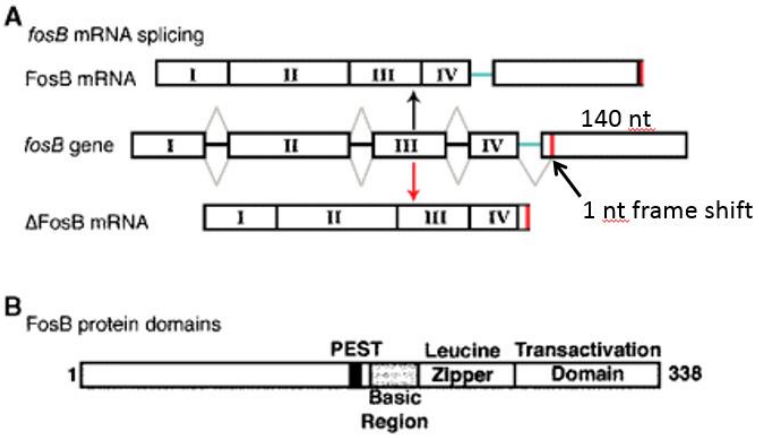


Figure 3. A. Alternative splice variants obtained from the FosB gene. B. Specific protein domains found in all FosB proteins (modified from Carle et al[1])

Furthermore, recent evidence indicates that the induction of ΔFosB may be regulated by epigenetic changes at the *FosB* gene promoter. After chronic cocaine exposure, rats were found to have decreased histone H3 dimethylation at lysine 9 (H3K9me2) at the *FosB* promoter in the NAc[6]. This decrease in a modification associated with transcriptional silencing corresponds to the induction of FosB isoforms that is seen after cocaine exposure. These findings suggest that epigenetic modifications by chronic cocaine are an intriguing target for further study.

<u>Epigenetic Effects of Chronic Stimuli</u>

Epigenetic modifications are an especially elegant adaptation that allow for changes in gene expression without altering DNA sequences, which could have potentially lethal effects to the cell. Epigenetic modifications can act to either activate or repress activity through enzymatic modification of DNA, modification of nucleosomes, or the actions of non-coding RNAs. For example, DNA methyltransferases add a methyl group to cytosines and these regions are typically associated with less transcriptionally active chromatin, perhaps through recruitment of silencing machinery[24]. Another epigenetic mechanism involves microRNA – small, non-coding RNAs that act within a RNA-induced silencing complex (RISC) to target specific mRNAs for degradation[25]. Lastly, post-translational modifications of histones occur at specific residues on their N-terminal tails, which project out of the nucleosome and are easily accessible to modifying enzymes. The close interaction between histones and DNA allows these modifications to influence the wrapping of DNA and the recruitment of transcriptional activation or silencing factors[26, 27]. The modifications include acetylation, phosphorylation, methylation, ubiquitination and sumoylation at a variety of residues on the tails of multiple histones.

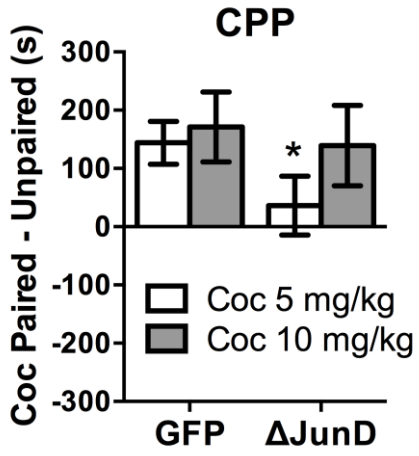


*Figure 4. Cocaine conditioned place preference reveals a reduced drug-environment association with low-dose cocaine (5mg/kg) in mice expressing AAV-ΔJunD in Hippocampus. * p<0.05 compared to GFP.*

Recent evidence suggests that histone modifications may have a role in regulating hippocampal function and physiology. Upon overexpression of a histone deacetylase (HDAC) throughout the brain, mice showed decreased hippocampal spine density, synapse number, synaptic

plasticity and memory formation. Conversely, when the HDAC was knocked-down, there was increased hippocampal synapse number and memory formation[28]. Additionally, when an HDAC inhibitor (HDACi) was infused into the hippocampus, *ex vivo* slice electrophysiology showed an increase in the induction of long-term potentiation. *In vivo* treatment of HDACi prior to contextual fear conditioning, a behavioral test which measures the memory trace of an association between an aversive stimulus with a contextually distinct environment, led to enhanced long-term fear memory in mice[29]. These findings demonstrate that histone acetylation in the hippocampus influences and controls normal memory consolidation.

Furthermore, evidence implicates histone modifications as the epigenetic cause for changes seen in gene expression after cocaine exposure, specifically in the NAc. Acute and chronic cocaine exposures are known to separately induce expression of different subsets of genes[30, 31] as well as specific histone modifications[32]. For instance, genes such as *BDNF* and *Cdk5* are only induced after chronic cocaine exposure and their promoters show histone H3 hyperacetylation after chronic but not acute cocaine. In addition, these modifications continued to be present seven days after the final administration of cocaine[7]. These findings indicate that epigenetic changes, such as histone post-translational modifications, can lead to differential gene expression observed after cocaine use, and also have the potential to encode long-lasting changes in transcriptional activity. Chronic cocaine also decreases global levels of H3K9me2 in the NAc[33]. This modification is associated with transcriptional silencing through its ability to recruit certain repressive machinery, such as heterochromatin protein 1[27]. The reduction in H3K9me2 was found to be due to repression of the histone methyltransferase, G9a, which is regulated by ΔFosB[33]. Therefore, chronic cocaine exposure leads to an induction of ΔFosB, which represses *G9a* transcription, which in turn lowers global levels of H3K9me2, a finding corroborated by the decrease in H3K9me2 found at the *FosB* promoter after chronic cocaine exposure[6]. However, whether there are similar cocaine-induced epigenetic changes in the hippocampus remains to be seen. Thus, we propose to address whether cocaine induces epigenetic changes in gene expression in the hippocampus, including at the *FosB* gene, which may be responsible for the formation of strong memories associating cocaine and environment.

Approach

Aim 1: Characterize induction of the *FosB* gene in hippocampus in response to chronic cocaine

Prior studies demonstrate that chronic cocaine increases ΔFosB levels throughout the brain, including the hippocampus and all of its subregions[19]. However, these studies were qualitative, and therefore the exact changes in expression remain to be determined. Here we show preliminary data that this induction is due in part to histone post-translational modifications occurring at the *FosB* gene promoter in the hippocampus. The specific histone modification, H3K9me2, is associated with transcriptional repression through recruitment of silencing machinery such as heterochromatin protein 1[26, 27]. Therefore the decrease in H3K9me2 that we find at the *FosB* gene promoter is indicative of an increase in gene expression. We plan to validate and quantitate the cocaine-mediated induction of FosB isoforms in hippocampal subregions by conducting immunohistochemistry (IHC), as well as further explore and validate the epigenetic changes by investigating other histone modifications at the *FosB* promoter.

1a: Characterize the induction of *FosB* gene products in hippocampal subregions in response to chronic cocaine

Given that previous data show chronic cocaine exposure leads to an increase in ΔFosB expression in the whole hippocampus[17], our goal in this subaim is to determine the quantitative subregion-specific changes of FosB isoform protein expression. The subregions will be defined as the dentate gyrus (DG), CA3 and CA1 of both the dorsal and ventral hippocampus (Figure 5). To determine whether there is differential expression of *FosB* gene products, we will administer intraperitoneal (i.p.) injections of 20mg/kg cocaine or a 0.9% saline solution to male C57BL6 mice for 10 days. We have previously determined that this cocaine concentration produces a significant induction of ΔFosB after only three days of injections (Figure 6), and maximal expression by 10 days[23]. The mice will be sacrificed 24 hours after the last injection to enrich for the ΔFosB isoform. This will occur via chloral hydrate overdose and perfusion with formalin to fix brain tissue. The brains will be extracted and prepared for IHC. Slices containing either the dorsal or ventral hippocampus will be identified and probed for FosB using the previously validated antibody from Santa Cruz[34].

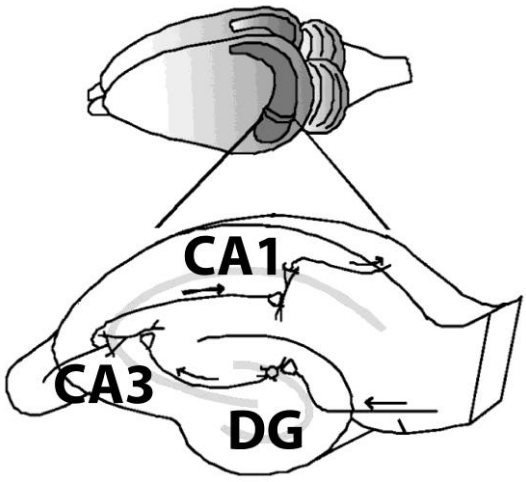


Figure 5. Depiction of the mouse hippocampus and hippocampal subregions (modified from Nguyen[4])

1b: Determine epigenetic changes at the *FosB* gene in response to chronic cocaine

Since it has been demonstrated in rats that the *FosB* gene promoter undergoes epigenetic changes in the NAc in response to cocaine exposure[6], in this subaim we will determine if similar alterations are found in hippocampus. We have put mice through the same regimen of cocaine or saline treatment as proposed in subaim 1a, they were sacrificed one hour after the last injection and the whole hippocampus was micro dissected for analysis. Preliminary data from ChIP (protocol adapted from Barski et al[35]) for H3K9me2 indicate a cocaine-mediated decrease in the histone modification around 900 base pairs upstream of the *FosB* transcription start site (TSS) (Figure 7). We will now determine whether there are changes to histone modifications associated with transcriptionally active chromatin, such as H3K9ac and H3K4ac, as well as further validate our findings for H3K9me2.

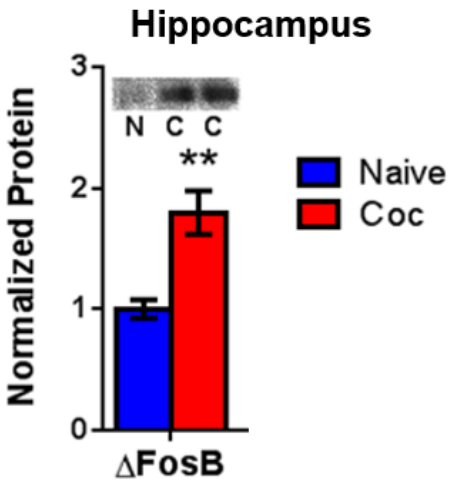


*Figure 6. Western blot reveals significant induction of ΔFosB in response to 20 mg/kg cocaine for 3 days. Mice were sacrificed one hour after the last injection **p<0.001*

Expected Outcomes: Preliminary data from our lab indicate that another stimulus, chronic exposure to the antidepressant fluoxetine, produces an induction of the FosB isoforms similar to cocaine, and that there are no differences between dorsal and ventral hippocampus. Also, based on qualitative data, there appears to be induction of ΔFosB in all of the hippocampal subregions after exposure to many types of drugs, including cocaine[19]. Thus, we predict we will find cocaine-mediated induction of FosB isoforms throughout all subregions in both dorsal and ventral hippocampus.

Preliminary ChIP data for H3K9me2 show a reduction at the *FosB* gene promoter, around 900 base pairs upstream of the TSS. This is a promising preliminary result because of similar findings in the NAc of rats chronically treated with cocaine[6]. In the same rats, there were no changes in the amount of H3K9ac, another histone modification we would like to investigate. Therefore, we are expecting to find either no change or an increase in histone acetylation in this region, which may suggest another mechanism for the increase in gene expression in response to cocaine.

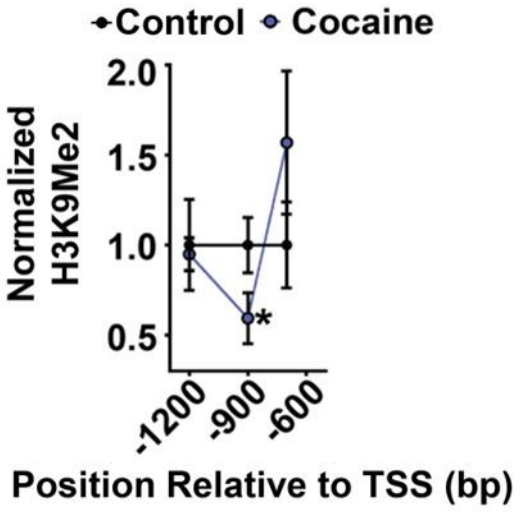


*Figure 7. Epigenetic regulation of the FosB promoter. Mice were exposed to 10 days of 20mg/kg i.p. cocaine in home cage. Hippocampus was removed and ChIP assay reveals a reduction in enrichment for the repressive H3K9Me2 mark centering at a region 900 base pairs upstream of the FosB TSS relative to control animals. Data are normalized to control animals at each site. Error bars represent mean +/- SEM, * indicates p<0.05 compared to control; n=7 immunoprecipitations from 28 total mice per group.*

Alternative Approaches: Although our lab is proficient in conducting IHC, if there are problems with this technique, we could quantify FosB proteins in hippocampal neurons using immunofluorescence. This would also allow us to do automated quantification of signal intensity by hippocampal subregion. Similarly, as shown in Figure 7, we have conducted successful histone ChIP experiments in our lab. However, we may discover problems with specificity of primer pairs and therefore would need to utilize different primers for qPCR, in which case we would shift our primer locations across the *FosB* promoter by ~50 bp in either direction. If we find that we do not have sufficient chromatin for all of the ChIP assays we propose, it may prove more efficient to micro dissect the whole hippocampus instead of punching it - a technique common in proteomic studies[36].

Aim 2: Determine whether *FosB* induction in hippocampus is mediated by histone modification writers or erasers

Drug-mediated binding of histone modification writers and erasers to the *FosB* promoter has been found in the NAc[33, 37]. Since preliminary data indicate similarities in histone modifications between NAc and hippocampus, we want to investigate whether there are similar drug-induced changes to histone modifiers that effect the induction of *FosB* in the hippocampus. Therefore, the objective of this aim is to test whether changes in histone modification writers or erasers are involved in *FosB* gene product induction in the hippocampus in response to chronic cocaine. We will use a combined pharmacological and viral approach to alter levels or activity of histone methyltransferase, G9a, or HDACs in the hippocampus and then evaluate changes in FosB isoform levels in response to cocaine.

2a: Determine whether specific histone modification writers or erasers drive cocaine-mediated *FosB* induction

A ΔFosB-dependent decrease in G9a was found in NAc in response to chronic cocaine[33]. G9a catalyzes the dimethylation of H3K9, and therefore this result coincides with the cocaine-mediated decrease in NAc H3K9me2 both globally and at the *FosB* gene promoter[6, 33]. Therefore, we will determine whether the G9a inhibitor, BIX01294 hydrochloride (BIX), or the HDACi, MS-275, effect the *FosB* induction seen in hippocampus in response to cocaine. These inhibitors have been shown to effectively and selectively inhibit these histone modifiers in the mouse brain[38, 39]. Mice will undergo stereotaxic surgery for bilateral hippocampal cannula placement and then recover for 1 week[40]. After surgery, mice will be singly housed to eliminate any contact and disruption of

the cannula. Either BIX, MS-275, or vehicle, will be injected into the cannula an hour before each cocaine exposure. We will maintain within-animal controls such that one side of the brain will receive the pharmacological inhibitor and the other side will receive the vehicle. The mice will be given either 10 mg/kg i.p. injections of cocaine or a 0.9% saline solution for 5 days. This submaximal dose of cocaine will allow us to see changes in ΔFosB induction in response to the inhibitors, and ΔFosB levels at this dose are still significantly increased[23]. After day 5, the mice will be sacrificed one hour after the last injection and the hippocampus will be micro dissected and processed for Western blotting. We will probe for FosB isoforms and the control samples will also be used to determine cocaine-mediated changes in hippocampal G9a and multiple HDACs and HATs.

In addition, we will determine whether an increase in hippocampal G9a could mediate the decrease in cocaine-dependent induction of *FosB* gene products. We will utilize HSV vectors to express either GFP or G9a+GFP in hippocampus via stereotaxic surgery (HSV-GFP on one side and HSV-G9a+GFP on the other side for within-animal controls). The mice will recover for 24 hours and be given 10 mg/kg cocaine or 0.9% saline i.p. injections for 5 days, which coincides with the length of expression for these HSVs[41]. The mice will be sacrificed 24 hours after the last injection and perfused. GFP signal will be used to validate hippocampal targeting, and immunofluorescence will be used to quantify FosB proteins in GFP-positive cells, allowing us to determine the effect of G9a overexpression on cocaine-mediated *FosB* induction in the hippocampus.

2b: Determine whether epigenetic modification of the *FosB* gene regulates cocaine-mediated induction in hippocampus

In order to determine whether specific epigenetic changes to the *FosB* gene promoter play a role in the cocaine-mediated induction, we will manipulate histone modifications at a single promoter in a single brain region. To do this, we will perform stereotaxic surgery to bilaterally inject HSV vectors into the hippocampus that contain a GFP reporter and a zinc-finger protein fused to G9a, HSV-*FosB*-ZFP-G9a[42]. The zinc-finger protein contains a DNA binding domain specific to the *FosB* gene promoter, and fusion to G9a allows the histone methyltransferase to act only at the *FosB* promoter to enhance H3K9me2. The control HSVs contain a non-functional domain (NFD) fused to the same zinc finger protein, HSV-*FosB*-ZFP-NFD. We will maintain within-animal controls so that each mouse is injected with the NFD fusion on one side and the G9a fusion on the other. The mice will recover for 24 hours and then be subjected to i.p. injections of 10 mg/kg cocaine or 0.9% saline for 5 days. Mice will be sacrificed by perfusion 24 hours after the last injection. GFP signal will be used to validate hippocampal targeting, and immunofluorescence will be used to quantify FosB proteins in GFP-positive cells, allowing us to determine the effect of specific H3K9 dimethylation of histones at the *FosB* promoter on cocaine-mediated induction in hippocampus.

Expected Outcomes: The G9a inhibitor, BIX, will decrease the amount of dimethylation occurring at H3K9, and therefore we expect it will increase the cocaine-mediated induction of hippocampal ΔFosB. Similarly we anticipate that MS-275 administration will increase the induction of hippocampal ΔFosB, suggesting that acetylation of histones at the *FosB* promoter is important for this process. In contrast, viral-mediated overexpression of G9a in hippocampus should decrease the induction of FosB isoforms, presumably by increasing H3K9me2 at the *FosB* gene promoter. Given our preliminary data showing a decrease in H3K9me2 at the *FosB* gene promoter in hippocampus in response to cocaine, and the effects of H3K9me2 on *FosB* induction in NAc[42], we expect that inducing H3K9me2 at the *FosB* promoter in hippocampus will decrease cocaine-mediated induction.

Alternative Approaches: It is possible that the proposed histone modification inhibitors will have nonspecific effects on other molecules. An alternative method would be to utilize known siRNA duplexes that inhibit either G9a or a specific HDAC in the mouse brain[43]. This would have the added benefit of allowing us to specifically examine the effects of individual HDACs, since MS-275 inhibits HDAC1 preferentially[44].
It is possible that our NFD fusion zinc finger protein may itself lead to changes in expression of the *FosB* gene because it still binds to the *FosB* DNA sequence and may sterically interfere with the binding of other elements critical for induction. Therefore, an alternative control would be an HSV containing a zinc finger fusion protein that binds to a different sequence of DNA, such as HSV-*VEGF*-ZFP-G9a[42], which is also available to our lab.

Aim 3: Identify FosB gene targets in the hippocampus
Previous data collected from the NAc indicate that ΔFosB has many target genes and that AP1 binding can lead to either activation or repression depending on the gene and conditions[21, 23, 45-47]. Known ΔFosB target genes, such as *CaMKIIα*, *GluA2*, and *Cdk5* play essential roles in hippocampal synaptic plasticity[48-50]. CaMKII is a serine/threonine kinase that is essential to the process of long-term potentiation in the CA1 region of hippocampus[48], GluA2 is a subunit of the AMPA receptor that regulates the conductance and calcium permeability of the channel[49], and Cdk5 is a neuronal kinase implicated in memory formation[51]. However, it is not known whether ΔFosB is the major regulator of their expression in the hippocampus. Thus, the objective of this aim is to determine whether ΔFosB mediates expression of these and/or other genes in response to cocaine, perhaps suggesting a mechanism for the extreme strength of drug-associated memories. We will conduct FosB ChIP and ChIPseq from hippocampus of mice exposed to either cocaine or saline. We will test for binding to candidate genes as well as look for novel gene targets of FosB isoforms. In a parallel study, we will use our *floxed FosB* mouse to identify genes whose induction by cocaine in the hippocampus depends on *FosB* gene products. Together these approaches will allow us to identify specific changes in both candidate and novel gene expression that are due to hippocampal FosB protein induction in response to chronic cocaine.

3a: Determine FosB gene targets in hippocampus
Cocaine-mediated changes in gene expression have been extensively studied in regions of the reward pathway such as the NAc. In D1-type medium spiny neurons of the NAc shell, ΔFosB binds to the *CaMKIIα* promoter in response to cocaine, but this does not occur in the NAc core[23], demonstrating the cell- and region-specific nature of ΔFosB activity. In addition, ΔFosB overexpression increases *GluA2* transcription by over 50% in the NAc[21], and it is enriched at the *GluA2* promoter in the NAc in response to stress[46]. Furthermore, using cDNA array analysis, *Cdk5* has been found to be a target of ΔFosB in both NAc and hippocampus[52, 53]. Additionally, Hevin (or SPARC-like 1) is a transmembrane protein that is known to be important for synaptic plasticity and ΔFosB binds its promoter in NAc in response to antidepressant treatment and can increase *Hevin* expression when overexpressed in NAc[46, 54]. These are all promising and functionally significant candidates for FosB isoform binding in the hippocampus due to their roles in synaptic strength and plasticity as well as learning and memory. In order to determine whether ΔFosB regulates the transcription of these genes in hippocampus, we will conduct ChIP using the validated Cell Signaling FosB antibody[23] with four mice per group in 16 groups (64 mice total, 8 groups of cocaine and 8 groups of saline). After 10 days of saline or 20 mg/kg cocaine exposure, we will sacrifice 24 hours after the last injection and micro dissect the hippocampus. We will then conduct qPCR with predetermined primers for candidate genes to determine whether FosB proteins bind the promoter regions of any of these genes, and whether binding is regulated by cocaine:

Gene	Forward Primer	Reverse Primer	Citation
CaMKII	ACTGACTCAGGAAGAGGGATA	TGTGCTCCTCAGAATCCACAA	Robison et al[23]
GluA2	TTTGGGAGTTGTCCCTTCAG	GGAAGCCGAACTGCTAATTG	Vialou et al[46]
Hevin	CGGTGCCATCATAGGCTTAC	CGTAAGCAGCTCTGGAAAATG	Robison et al[23]
Cdk5	N/A	N/A	Pending Design

In addition, 10% of the total prepared chromatin will be reserved for ChIPseq[55] (sequencing to be done by the Genomics Core Facility of Mount Sinai School of Medicine, New York), which will allow us to find novel transcriptional targets of the FosB isoforms. We have previously performed this technique in NAc tissue, and the results show enrichment of FosB binding to genes involved in synaptic function (Figure 8), indicating that FosB may also regulate the expression of proteins important for hippocampal synaptic plasticity. Validation of differential expression of all candidate and any novel proteins in response to chronic cocaine will utilize a new cohort of mice under the same cocaine regimen with Western blotting conducted at the end.

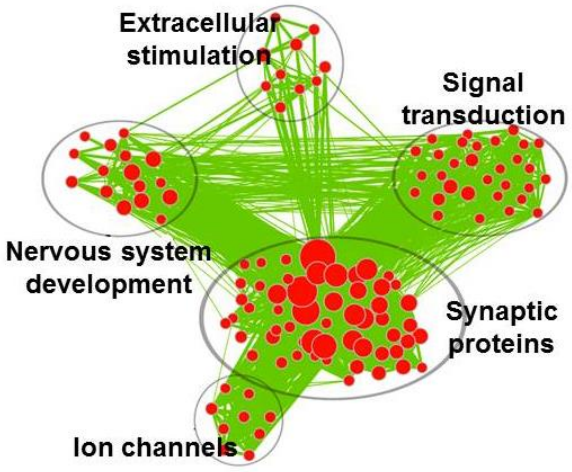


Figure 8. FosB ChIPseq results from NAc demonstrate that the majority of genes bound by FosB are synaptic proteins.

3b: Determine hippocampal FosB gene targets regulated by cocaine

FosB null mice[56] display impaired neurogenesis in the adult hippocampus as well as spontaneous epilepsy[57]. However, mice that retain expression of ΔFosB but lack full-length FosB do not have these phenotypes[57]. This indicates that ΔFosB may be sufficient for the proper development and function of the hippocampus. However, it is unknown whether the phenotype of the *FosB* null mouse is due to lack of FosB function specifically in hippocampus, or lack of *FosB* expression in other regions or during early development that leads to hippocampal dysfunction. Therefore, we are breeding *floxed FosB* mice with mice expressing Cre using the Neurotensin Receptor 2 (NtsR2) promoter, which has enriched expression in the DG and CA3 (Figure 9), allowing for a more region-specific knockout of *FosB* expression (there is some Cre expression in the hypothalamus of these mice). These mice have been bred with an L10-GFP reporter line, which expresses GFP fused to a ribosomal protein. Since antibodies specific to GFP are widely used and validated, this will allow us to conduct novel ribosomal trapping by immunoprecipitating ribosomes and their associated mRNAs only from NtsR2-positive cells[58]. When bred with *floxed FosB* mice and then treated with chronic cocaine or saline (Figure 10), we can determine genes whose cocaine-mediated expression in hippocampus is mediated by FosB proteins. A pilot study will be conducted to determine how many mice per treatment group will be needed in order to obtain a sufficient yield of mRNA. Once enough F1 offspring are bred, we will conduct an initial

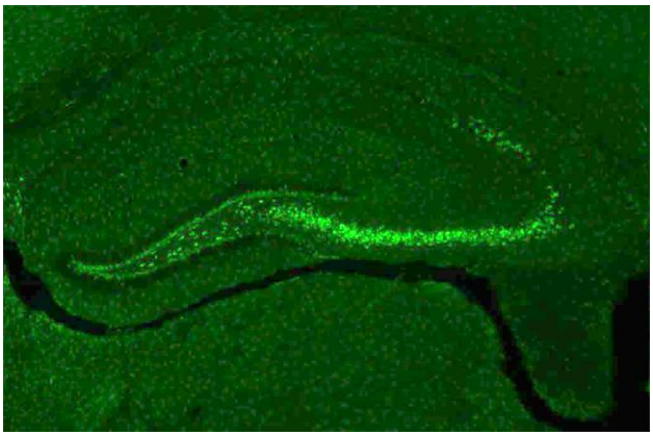


Figure 9. GFP expression is enriched in hippocampal subregions DG and CA3 in NtsR2 L10-Cre reporter mice.

ribosomal trapping test with at least 3 mice. If this number allows us to yield sufficient mRNA (validated by nanodrop) then we will continue with this cohort size or adjust it as needed. Therefore, we propose that 12 mice of each genotype will be given saline or 20 mg/kg cocaine for 10 days. This will allow us to pool three mice per pull-down with four replicates. The mice will be sacrificed 24 hours after the last injection and the hippocampus will be micro dissected and prepared for ribosomal trapping as described by Sanz et al[58], and resulting mRNAs will be reverse transcribed and sequenced as for ChIPseq above.

Expected Outcomes: Given previous work that shows *CaMKII*α, *GluA2*, *Cdk5* and *Hevin* are all gene targets for FosB isoforms in the NAc, we expect to see enrichment of FosB at some or all of these targets in the hippocampus when mice are exposed to chronic cocaine. However, increased ΔFosB expression does not always lead to increased ΔFosB binding at AP1 sites[54], and it is possible that cocaine could cause decreased ΔFosB binding to some of these promoters. We also expect to obtain novel targets of the FosB isoforms through ChIPseq in order to better characterize the cocaine-mediated transcriptional changes that occur in the hippocampus. Peak analysis for both ChIPseq and RNA will be done by Dr. Li Shen at Mount Sinai School of Medicine. Ribosomal trapping will allow us to precipitate out mRNA that is actively being translated, allowing us to find *FosB*-dependent cocaine-driven changes in hippocampal gene expression. At the moment we have parental breeding pairs set up and have obtained F_1 offspring. We expect to have enough F_1 to breed for the F_2 generation by the summer of 2015. Therefore, the creation of enough F_2 male mice to conduct ribosomal trapping should occur between three and six months after F_1 breeding starts. We predict that many of the genes uncovered by ChIPseq and ribosomal trapping will be linked to hippocampal synaptic function and plasticity, leading to future experiments in which we manipulate the expression of these target genes in hippocampus and determine the physiological and behavioral consequences.

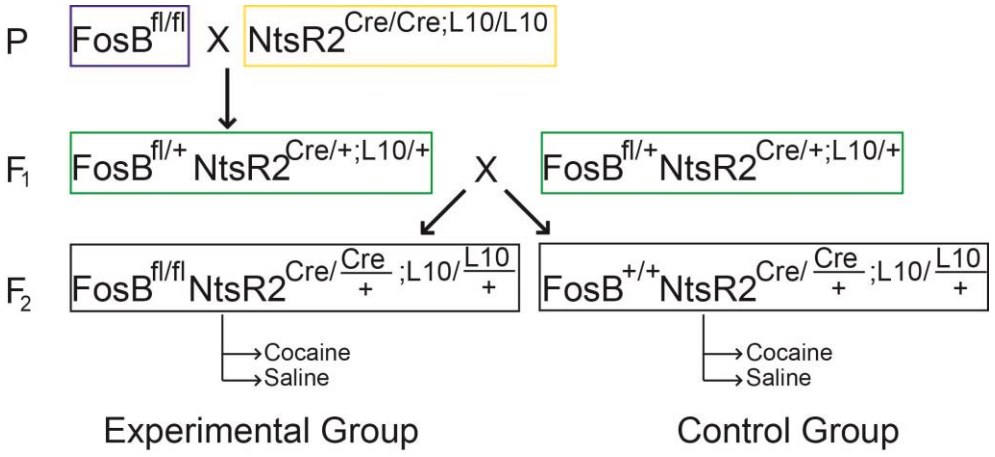
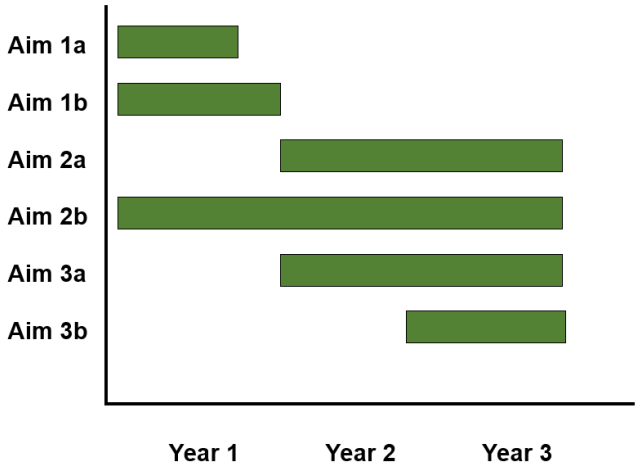


Figure 10. Breeding strategy for floxed FosB and NtsR2-Cre mice. F_2 will be the offspring used in the proposed ribosomal trapping experiments. P: parental generation, F_1: first generation hybrids, F_2: second generation offspring.

Alternative Approaches: One caveat of FosB ChIP is that we may not be able to precipitate out enough of our protein of interest. Previous problems have been noted with conducting ChIP for transcription factors, since their expression levels are low compared to histones, and the FosB antibody is not 100% efficient. In this case, we may need to utilize a knock-in mouse line that

expresses a FosB-HA fusion protein, available to us through the Nestler lab at Mount Sinai School of Medicine. This would allow us to precipitate with an efficient and validated antibody for HA instead of FosB. Also, after novel targets have been found using ChIPseq, we may not find effective antibodies for Western blot validation of protein expression. In this case, we could conduct qPCR to look for differences in mRNA expression instead of protein.
The proposed ribosomal trapping approach has not been previously used in our lab. Although this technique has been used by our collaborators[58] and we are confident it can be adapted for our purposes, we may have to instead observe differences in gene expression in our *floxed FosB* mice using RNAseq. Also, in order to obtain a sufficient yield of mRNA we may need to increase our sample size. Therefore, the previously mentioned pilot study will allow us to determine whether our initial sample size is large enough or if it will need to be increased.

Tentative Timetable: Many of the proposed studies are currently underway, and we have made significant strides in examining *FosB* gene induction in hippocampus and in studying the epigenetic mechanisms underlying *FosB* induction in response to cocaine. The following depicts the estimated start and end time for each of the subaims:



Potential Funding Resources: This work is relevant to NIDA's high priority interest in research utilizing next-generation sequencing and exploring the transcriptional and epigenetic effects of drug exposure, and will uncover crucial new gene pathways underlying drug/environment associations that may be exploited in the search for novel therapeutic interventions in the addiction process.

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