HIPPOCAMPAL EPIGENETIC REGULATION OF THE FOSB GENE IN LEARNING AND ADDICTION

By

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PUBLIC ABSTRACT

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The health and financial burden of substance abuse will continue to rise unless specific facets of addiction, particularly relapse, are better treated. Relapse to drug use after prolonged abstinence requires strong associations between the drug and the environment in which it is experienced, and it is essential that we understand the molecular basis of these strong associations if we are to successfully prevent or treat addiction. These associations are mediated by the hippocampus, a brain region critical for memory formation which can drive craving and relapse. The formation of these memories requires long-term changes in hippocampal gene expression, which may occur via the action of stable transcription factors, such as ΔFosB. ΔFosB, a truncated stable product of the *FosB* gene, is uniquely positioned to affect the expression of many genes that play a role in hippocampal synaptic function and plasticity, as it is induced in the hippocampus by many drugs of abuse and is necessary for the formation of drug-environment associations. My data reveal changes in the regulation of the *FosB* gene in the hippocampus after chronic cocaine exposure via an epigenetic mechanism, altered histone methylation. Using novel, locus-specific viral tools to modify histones, I have elucidated how the epigenetic state of the *FosB* promoter drives gene expression and cocaine-related behaviors and memories. These findings identify novel molecular changes in the hippocampus due to cocaine exposure which furthers our understanding of drug-associated memory formation.

Additionally, my work also examined the translation of our research to a clinical setting. Although we have a great deal of evidence that *FosB* gene expression regulates behavior in mouse models of addiction, much less is known about the expression and function of *FosB* in the human brain. To this end, I obtained postmortem samples of human brains from cocaine addicts, depression

patients, and matched controls. I found dysfunction in the regulation of *FosB* gene products in the human hippocampus in response to cocaine and depression, and observed a decrease in ΔFosB protein levels, suggesting hippocampal dysfunction. Taken together, this suggests that ΔFosB in the hippocampus is important for drug-related memories, that cocaine changes how this gene is epigenetically regulated, and that regulation is also disrupted in the hippocampus of cocaine-addicted individuals. Future work will investigate the specific gene targets of ΔFosB, which may reveal new inroads for therapeutic intervention in addiction.

ABSTRACT

HIPPOCAMPAL EPIGENETIC REGULATION OF THE FOSB GENE IN LEARNING AND ADDICTION

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Drug addiction results in part from maladaptive learning, including the formation of strong associations between the drug, the environment, and circumstances of its use. However, the patterns of gene regulation critical for this learning remain unknown. Consolidation of explicit memories occurs through synaptic plasticity in the hippocampus, and some of the molecular mechanisms of this process are well characterized, but epigenetic regulation underlying changes in hippocampal gene expression and the distribution of altered gene expression is poorly understood. The transcription factor ΔFosB is an important arbitrator of activity-dependent gene expression, and its expression in hippocampus is critical for learning. Previous studies demonstrate that drugs of abuse strongly upregulate ΔFosB in rodent hippocampus, but the mechanism of its induction by cocaine and its role in hippocampus-dependent cocaine responses is unknown. I demonstrate here that ΔFosB induction occurs exclusively within the CA1 subregion of the hippocampus and is facilitated by cocaine-mediated decreases in a repressive histone modification, H3K9me2. Subsequent locus-specific increase of this histone mark in hippocampus is sufficient to impair general learning and memory and cocaine-environment associations. Furthermore, human hippocampus post-mortem samples reveal a decrease in multiple ΔFosB isoforms and some ΔFosB target genes in cocaine-addicted individuals, as well as in depressed individuals, indicating a potential role for this gene in hippocampal pathologies associated with human addiction. These findings collectively suggest that salient stimuli, such as formation of drug-environment associations, induce epigenetic changes in the hippocampal *FosB* gene promoter that regulate ΔFosB induction, which in turn may control the transcription of genes that underlie hippocampal cell function and cocaine-related learning. Moreover, dysregulation of the

FosB gene may contribute to the effects of chronic drug exposure and may underlie cognitive deficits that accompany drug addiction and depression.

To my parents, Arthur and Marcy Gajewski, whose encouragement has lifted me to new heights and whose unconditional love and support continuously keeps me moving forward. I love you both so much.

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COCAINE ADDICTION

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General Overview:

Drug addiction is a chronic brain disease that is characterized by the loss of control over drug use and the continued seeking and taking of drugs despite adverse consequences (Robison and Nestler, 2011). These negative consequences include significant physical and mental health and quality of life problems for the individuals 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 (NDIC, 2011), primarily due to costs associated with the criminal justice system, health care, and from productivity lost due to drug abuse and drug-related incarceration. Thus, it is critical to develop a better understanding of the etiology of addiction to individual illicit substances in order to develop novel methods to prevent or treat addiction.

The most recent World Drug Report notes that cocaine remains the second most popular illicit drug (after cannabis), and is consumed by 17 million people worldwide, with the most problematic use concentrating in the Americas (Crime, 2015). Cocaine is a Schedule II, highly addictive stimulant that has the potential to quickly progress from experimentation to abuse and dependence (Ciccocioppo et al., 2004). The effects of cocaine are dependent on the dose, with a small dose leading to feelings of euphoria and mental alertness in the user, as well as increased energy, talking, sensitivity to sight, sound, and touch (NIDA, 2016). Higher doses of cocaine can

lead to adverse psychological or physiological effects, such as auditory hallucinations or cardiac arrest. Abuse of cocaine can also damage the gastrointestinal tract and cardiovascular system and lead to increased risk for stroke, seizures, and other neurological problems (NIDA, 2016). Currently, there are no medications approved by the FDA to treat cocaine addiction, and the use of behavioral therapies requires long-term participation by the individual which is not always completed, again indicating that the need for novel therapies is pressing.

Mechanism of action:

Cocaine produces its rewarding effects by binding to and blocking transporters of dopamine (DAT), serotonin (SERT), and norepinephrine (NET) (**Figure 1)**. At synapses, neurotransmitters are released from one neuron to activate receptors on the receiving neuron. Many neurotransmitters are then taken back up by the presynaptic neuron via transporters and repackaged into vesicles to await future transmission. The blockade of these transporters leads to an increase in action of their respective neurotransmitters, and the resulting over-activation of their receptors can have varying effects depending on the brain region and neurotransmitter involved.

Many investigations into brain regions affected by cocaine have led to interrogation of regions rich in dopamine (DA) receptors. These regions include the amygdala, hippocampus, septum, nucleus accumbens (NAc), and prefrontal cortex (PFC) and are all modulated by DA, and thus impacted by cocaine. Together, these regions are important for emotional processing, learning, memory, and reward and motivation. The NAc is a key structure in the processing of reward and motivated behaviors and receives input from multiple regions which modulates its function **(Figure 2)**, and is where the effects of drugs of abuse have been intensely investigated. The NAc, as well as the above-mentioned regions, receives DA input from the ventral tegmental area (VTA). It responds to DA via two different types of receptors, D1- and D2-type, which are G Protein-Coupled

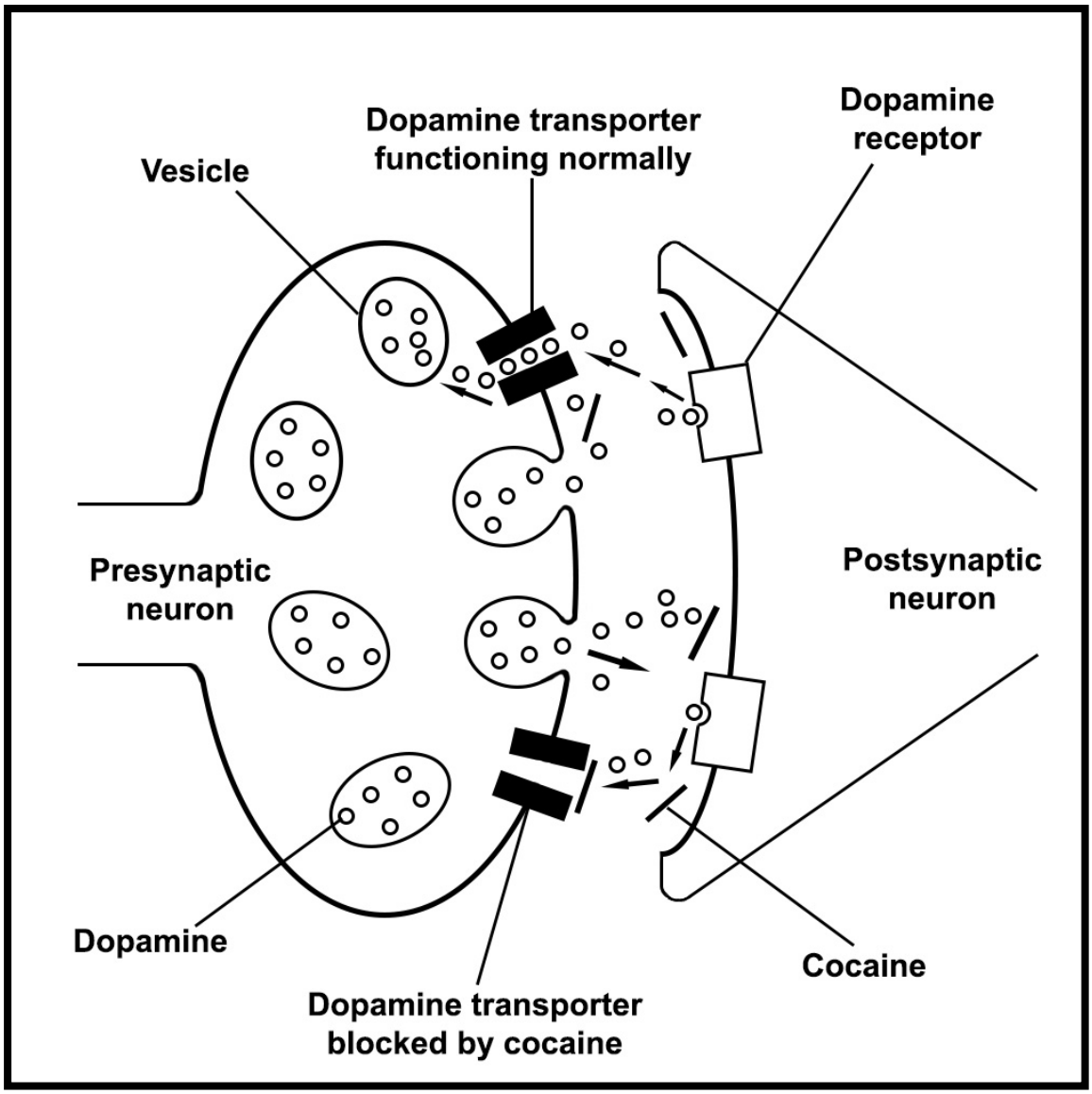

Figure 1. Cocaine mechanism of action. Vesicles fuse with the plasma membrane of the presynaptic neuron after an action potential is reached in order to release dopamine. Dopamine binds to and activates dopamine receptors on the postsynaptic neuron and is taken back up into the presynaptic terminal via the action of dopamine transporters. This allows the dopamine to be repackaged into vesicles for later synaptic transmission and limits the amount of time and concentration of dopamine available to the receptors. When cocaine is present, it selectively binds to and blocks the action of the dopamine transporters, leading to an increase in dopamine in the synaptic cleft that will continue to bind and activate dopamine receptors. Adapted from: BSCS and Videodiscovery, Inc. The Brain: Understanding Neurobiology, Lesson 3 – Drugs Change the Way Neurons Communicate (2010b).

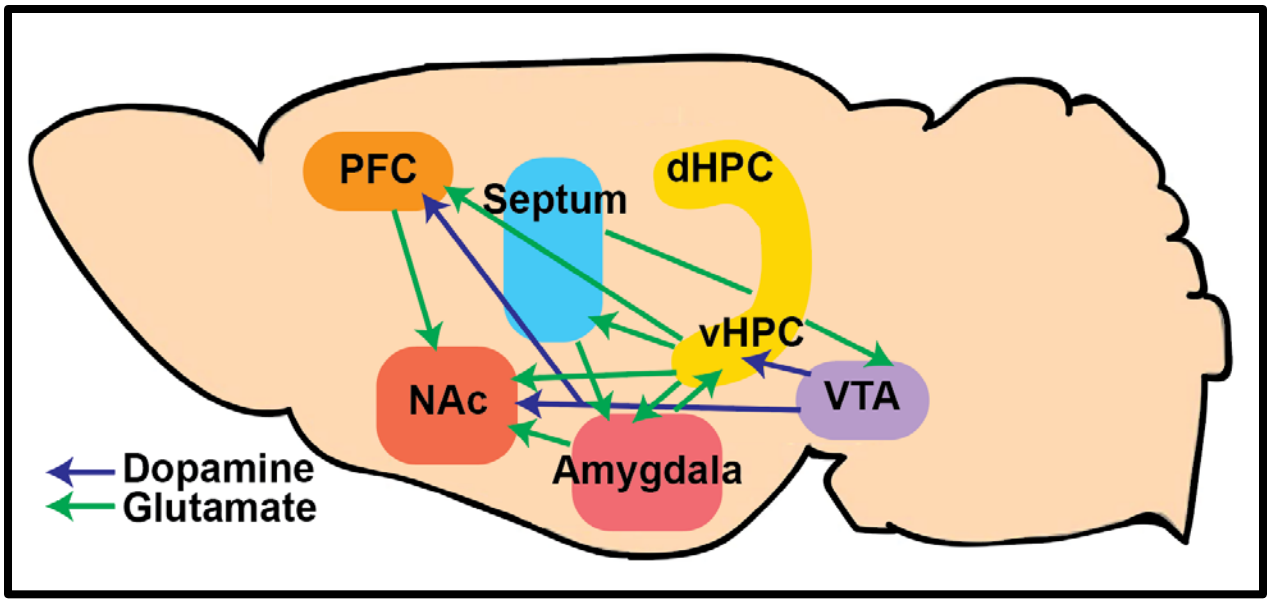

Figure 2. Reward circuitry inputs. The nucleus accumbens (NAc) integrates information from many other brain regions encoding emotion, motivation, and memory into the rewarding aspects of an event. The NAc, as well as ventral hippocampus (vHPC) and prefrontal cortex (PFC), receive dopamine (blue arrow) from the ventral tegmental area (VTA). Glutamatergic inputs (green arrows) from other regions, like the vHPC, amygdala, and PFC, regulate NAc excitability and reward processing This figure is modified from: (Gajewski, 2017).

Receptors (GPCRs), each expressed on separate subtypes of medium spiny neurons (MSNs), the main neuron population in the NAc. D1-type DA receptors (D1DRs) are G_s-coupled GPCRs, and thus activate adenylyl cyclase in response to DA, while D2DRs are G_i-coupled and have the opposite effect. The resulting elevation or suppression of cyclic adenosine monophosphate (cAMP) signaling regulates the function and expression of a variety of ion channels and their effectors, and ultimately increases (D1) or decreases (D2) excitatory responses to glutamate (Self and Nestler, 1995). NAc MSNs are GABAergic (they release the inhibitory neurotransmitter, gamma-Aminobutyric acid (GABA)), and their projection targets differ by subtype. D1 neurons synapse onto and inhibit the globus pallidus interna (GPi) and substantia nigra pars reticulata (SNr), which typically inhibit the thalamus. Thus, inhibition of the GPi/SNr allows for activation of the thalamus and its downstream target, the cortex. This path is referred to as the direct pathway

and ultimately increases thalamo-cortical drive. On the other hand, D2-type MSNs synapse onto the globus pallidus externa (GPe), which inhibits the GPi/SNr. Activation of this path will keep the GPi/SNr inhibition in place and is referred to as the indirect pathway. Thus, DA release in the NAc both stimulates the direct pathway and inhibits the indirect pathway, ultimately increasing cortical drive and reinforcing motivated behaviors (Floresco, 2015) . The balance between these two pathways likely relies on regulated glutamatergic and dopaminergic inputs as described above and depicted in Figure 2.

After cocaine exposure, the enhanced DA activity in the NAc modulates the neurons which also receive excitatory glutamatergic inputs from other regions, such as cortical, amygdalar and hippocampal regions. These glutamatergic inputs serve to control the output of the NAc in many respects, both on a synaptic level and behaviorally. Input from the prefrontal cortex (PFC), which is the seat of executive function and behavioral inhibition, provides information on planning. Input from the amygdala modulates conditioned associations and emotions, and inputs from the ventral hippocampus (vHPC) integrate contextual, spatial, and emotion-related experiences. The inputs from the vHPC have considerable importance in addiction-related behaviors, as contextual and environmental cues associated with the drug play a large role in future drug craving and relapse to drug seeking (Vorel et al., 2001).

THE HIPPOCAMPUS AND ADDICTION

Hippocampal circuitry:

Experiences generate sensory information that is processed in the cortex then transmitted through the entorhinal cortex (EC) into the hippocampus, where memories of these experiences are formed, consolidated, and modified. These include explicit and spatial information and associations between different environmental stimuli, cues, and environments. The hippocampus

has multiple subregions and cell types that play distinct roles in various aspects of memory formation and consolidation.

The establishment of explicit long-term memories requires excitatory glutamatergic inputs into EC from both cortex and limbic regions such as amygdala. The hippocampus can be divided into three main subregions: dentate gyrus (DG), CA3 (Cornu Ammonis 3) and CA1, which form the tri-synaptic loop **(Figure 3)**. Connectivity between these subregions is mostly unidirectional and excitatory (glutamatergic). Information primarily enters this circuit via EC projections onto DG, known as the perforant pathway, though EC also projects to CA3, CA1, and subiculum. Glutamatergic DG granule cells project to pyramidal neurons in CA3 (the mossy fiber pathway), which send glutamatergic Schaffer collateral projections to pyramidal neurons in CA1. Virtually all synapses in these pathways display enduring forms of activity-dependent synaptic plasticity known as long-term potentiation (LTP) or depression (LTD). From CA1, consolidated memories can be passed through subiculum and back to EC, where they are distributed throughout cortex for long-term storage. Plasticity of the tri-synaptic loop of DG-CA3-CA1 underlies nearly all forms of explicit memory consolidation, making the hippocampus central to cocaine-related memories (Gajewski, 2017).

During drug use, many associations are formed between the drug and environmental cues, like location, paraphernalia, social interactions, etc. Addicts will often abstain from drugs for months or years, but upon re-exposure to a drug-associated cue, like a neighborhood or a friend, they experience strong urges to use drugs and will often relapse to addiction. These associations that drive relapse are formed and maintained, in part, by the hippocampus. Importantly, the hippocampus can be functionally divided into dorsal (dHPC) and ventral (vHPC) regions which are known to distinctly regulate different aspects of memory. Episodic memories, mainly comprising spatial and contextual memories, are consolidated by dHPC, and these include associations between cocaine and the environment and context of its use. vHPC regulates

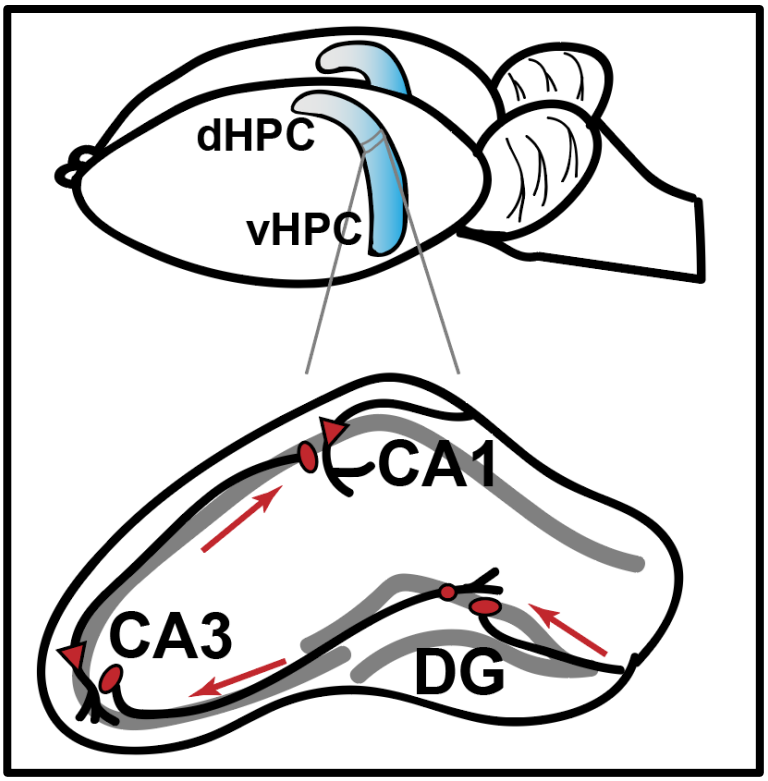

Figure 3. Hippocampal structure in rodent brain. The hippocampus can be separated into dorsal and ventral regions (dHPC, dorsal; vHPC, ventral), and also into specific subregions. The subregions are the dentate gyrus (DG), cornu ammonis 1 (CA1), and CA3, and can be differentiated by specific cell types, but work as a unidirectional tri-synaptic loop for the hippocampus to function. Information comes into the hippocampus primarily at the granule neurons of the DG, which begins the loop, and leads to activation of CA3 pyramidal neurons, which in turn activate pyramidal CA1 neurons. This loop allows for a specific subset of neurons to be activated and involved in the formation of one memory with others being involved in a different memory.

emotional memory, including drug-related motivational behaviors. Unlike dHPC, vHPC extends monosynaptic glutamatergic afferents to regions associated with reward and emotion, such as amygdala, lateral septum, and NAc **(Figure 2)**. This "top-down" control of the reward and emotional circuitry by the vHPC contributes directly to drug responses and drug-seeking behavior (Gajewski, 2017).

Cocaine's effects on hippocampal activity:

Hippocampal function appears to be particularly important in drug-environment associations. This

is highlighted by increased craving for drugs of abuse induced by exposure to environmental stimuli previously co-experienced with the drug. Cues have been associated with alterations in hippocampal activity of drug abusers in imaging studies using functional magnetic resonance imaging (fMRI) and positron emission tomography (PET). For example, environmental cues paired with cocaine use, such as those associated with drug preparation, trigger drug craving that is correlated with increases in glucose metabolism in portions of the hippocampus as measured by PET scans, and cocaine cue presentation increases hippocampal activation in cocaine-dependent subjects measured by fMRI (Grant et al., 1996; Prisciandaro et al., 2014). Furthermore, subjects watching cocaine-cue videos have also shown changes in downstream hippocampal circuits, such as increased activation of the amygdala and cingulate cortex as measured by fMRI (Breiter et al., 1997; Gajewski, 2017).

Additionally, assessment of neuronal activity via blood oxygen level dependent (BOLD) fMRI in awake rats showed a significant increase in hippocampal activation after initial exposure to cocaine, and activation similar to vehicle injection after repeated exposure (Febo et al., 2005). These pre-clinical imaging findings suggest that hippocampal activation is important for the initial consolidation of a memory, e.g. acute experience, but that activation may have been attenuated in these cells after chronic exposure (Guzowski, 2002). These findings suggest that neural sensitization, e.g. changes in synapses and circuits of the hippocampus, may underlie behavioral phenomena associated with the drug (Gajewski, 2017).

Functional divergence within hippocampus and hippocampal circuitry:

dHPC and vHPC mediate distinct cocaine-dependent behaviors. These regions have divergent projections which may influence various aspects of drug-environment related memories and behavioral outputs. Dorsal CA1 sends excitatory projections to the dorsal subiculum (dSub) which in turn projects to cortical regions involved in memory processing and spatial navigation

(Fanselow and Dong, 2010). This dHPC-dSub-cortex pathway is particularly important for contextual associations, e.g. associating a distinct environment with the events that occur in the context of that environment (i.e., a context in which cocaine is experienced and the drug effects). General dHPC function can be assessed using spatial learning paradigms, such as the Morris Water Maze in which a rodent is trained to escape a pool of water by using spatial cues around the room to find the location of a submerged platform. In contrast, vHPC appears to regulate emotional memory. vHPC output to regions of amygdala, including basolateral and central amygdala, regulates anxiety and fear learning. The vHPC forms a series of direct and indirect connections with the lateral septum and medial and central amygdalar nuclei, key regions known to regulate feeding, sex, and defensive behaviors, all of which have strong emotional components (Fanselow and Dong, 2010). Additionally, there are direct projections from vHPC CA1 and ventral subiculum to the NAc, which plays a critical role in reward processing, and stimulation of this circuit is intrinsically rewarding (Britt et al., 2012; Gajewski, 2017).

To further identify which regions of the hippocampus are important for drug associations, craving, and relapse, studies have focused on a few different behavioral paradigms, such as cocaine conditioned place preference (cocaine CPP) and cocaine self-administration **(Figure 4)**. In CPP, an animal is placed into a multi-chamber box in which each chamber is contextually distinct from the other (e.g. wall coloring and patterns, floor textural differences, etc), and is given free access to each chamber to establish whether a preference for one of the chambers exists, this is called the pre-test **(Figure 4A)**. The next day begins a conditioning phase in which the animal is exposed to an experimenter-administered vehicle/saline injection and is confined to one chamber in the morning, then in the afternoon is given a cocaine injection and placed in the other chamber. The animal thus forms an association between a unique environmental context and the euphoria induced by cocaine. This association can be measured by giving the animal free access to all chambers in a drug-free state and measuring the time spent in each chamber, with greater time spent in the drug-paired chamber indicating a stronger association between the rewarding effects

of the drug and the environmental context. After conditioning using the CPP paradigm, re-exposure to the cocaine-paired side results in an increase in c-Fos immunoreactivity, a proxy marker for neuronal activation, in the DG of both the dHPC and vHPC (Barr and Unterwald, 2015). However, lesions to dHPC, but not vHPC, impair cocaine CPP, suggesting that dHPC plays a role in the formation of drug-environment associations, highlighting its well-known role in spatial and contextual memory (Meyers et al., 2003). Importantly, there remain subregions of the vHPC that are not affected by this lesioning technique, specifically the ventral CA1 which contains neurons that project to the NAc and which we now know may facilitate the reward processing and related memories. However, LTD induced at these ventral CA1-NAc projections using optogenetic stimulation demonstrated the importance of this circuit, as it reversed the cocaine-mediated plasticity changes and mice subsequently displayed a decreased motivation to self-administer cocaine (Pascoli et al., 2014).

Animal models of cocaine self-administration, particularly those using a reinstatement model of cocaine-seeking behavior, are translationally relevant because they replicate relapse in addicts. In this paradigm, animals are trained to self-administer cocaine (typically through a jugular catheter) using an operant apparatus until stable responding for cocaine is acquired **(Figure 4B)**. They then go through an extinction process where responding no longer causes cocaine injection, thus gradually depressing responding for cocaine. Once responses are extinguished, cocaine seeking can be reinstated using a variety of stimuli including cocaine, cue, and context. vHPC activity is increased in context-induced reinstatement and may recruit specific neuronal ensembles in the NAc (Cruz et al., 2014; Bossert et al., 2016). Electrical stimulation of vHPC, which activates VTA dopamine output to NAc, can produce reinstatement of cocaine seeking (Vorel et al., 2001). Stimulation of vHPC enhances VTA dopamine neuron firing which drives an increase in DA release in the NAc (Legault et al., 2000). This is mediated by vHPC outputs to the lateral septum which, in turn, project to VTA, as chemical disconnection of any of these circuits attenuates the evoked responses of VTA neurons and context-induced reinstatement of cocaine

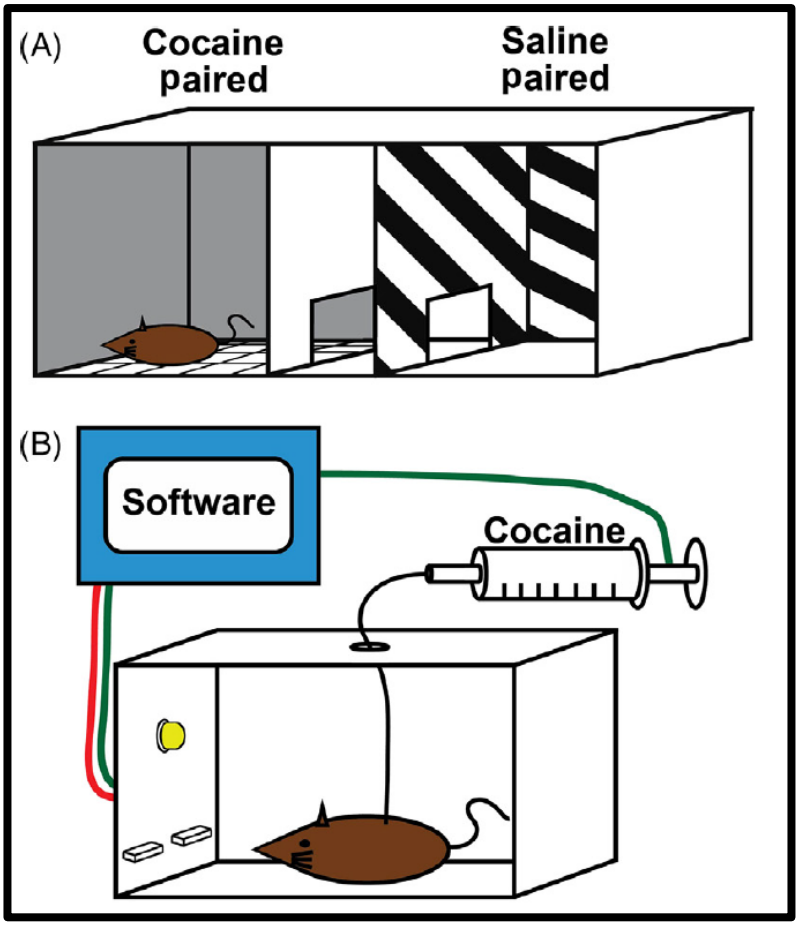

Figure 4. Behavioral paradigms used to assay drug reward. A. Cocaine conditioned place preference (CPP) is an experimenter-administered drug approach that pairs an injection of saline or cocaine with a contextually distinct chamber within a three-chamber box. After two days of pairing the animal will develop a preference for the chamber paired with the cocaine and this can be measured by giving the animal access to all chambers and tracking the amount of time that is spent in each. A wild type rodent will spend most of its time in the chamber paired with cocaine. **B.** Cocaine self-administration is an approach that allows an animal to work toward an infusion of cocaine by either pressing levers or nose poking a hole (not shown). The animal will be placed in the operant chamber after undergoing surgery to insert a jugular catheter. For a few hours each day they have the opportunity to press the lever the appropriate number of times which will allow it to receive a specific infusion volume of cocaine as the levers are synced up to software and an infusion pump. This figure is adapted from: (Gajewski, 2017).

seeking (Luo et al., 2011). Inactivation of both dHPC and vHPC block context-induced reinstatement, indicating that both dHPC integration of context and vHPC regulation of reward circuitry are important for this behavior (Fuchs et al., 2005; Lasseter et al., 2010). However, it appears that the vHPC, but not dHPC, is important for cue- and cocaine-induced reinstatement, which may suggest vHPC's role in reinstatement is not specific to general contextual memory that is known to be regulated by dHPC (Fuchs et al., 2005; Rogers and See, 2007; Lasseter et al., 2010). Finally, vHPC has direct afferents to the NAc, allowing for direct monosynaptic regulation of cocaine-dependent behaviors (Ciocchi et al., 2015). vHPC inputs to NAc are potentiated by cocaine, mediate cocaine-induced locomotion, and are capable of supporting self-stimulation (Britt et al., 2012). In addition, vHPC afferents drive neuroplasticity, e.g. increased synaptic strength, in synapses in the NAc and this vHPC-NAc-driven plasticity is directly related to cocaine seeking (Pascoli et al., 2014). Along with the CPP findings, these data collectively highlight the distinct roles of hippocampal subregions in cocaine-related memory formation and reward.

MEMORY FORMATION

General Overview:

The hippocampus is essential for the formation of new memories, in particular, declarative memories, which are facts or events that can be consciously recalled. Input from other brain regions allows the hippocampus to integrate and consolidate multiple aspects of an experience into a memory like sensory, motor, and emotional information (Deadwyler et al., 1987). It is then necessary to be able to temporally distinguish between events, and the hippocampus likely accomplishes this through the specific activation of ensembles of neurons (Rubin et al., 2015). Neuronal ensembles consist of a specific subset of cells that are activated during a given event, inducing a persistent change in the synaptic connections between them (Sjulson et al., 2017).

Synaptic plasticity is a change in the strength of connections between neurons, typically by altering the number of glutamate receptors on the postsynaptic cell. Along with active changes in gene expression, hippocampal synaptic plasticity is necessary for the formation of memories. After an experience, learning facilitates gene transcription and subsequent protein synthesis critical for the changes in synaptic connectivity that generate a stable ensemble encoding the memory (Nguyen et al., 1994; Blum and Abbott, 1996). There are many forms of synaptic plasticity. One form of synaptic strengthening is termed Long-Term Potentiation (LTP) and is the enhancement of synaptic transmission ranging from hours to days after an initial stimulus, often a rapid, repetitive, high-frequency stimulation **(Figure 5)**. Conversely, Long-Term Depression (LTD) can occur after low-frequency stimulation and leads to a reduction in synaptic strength.

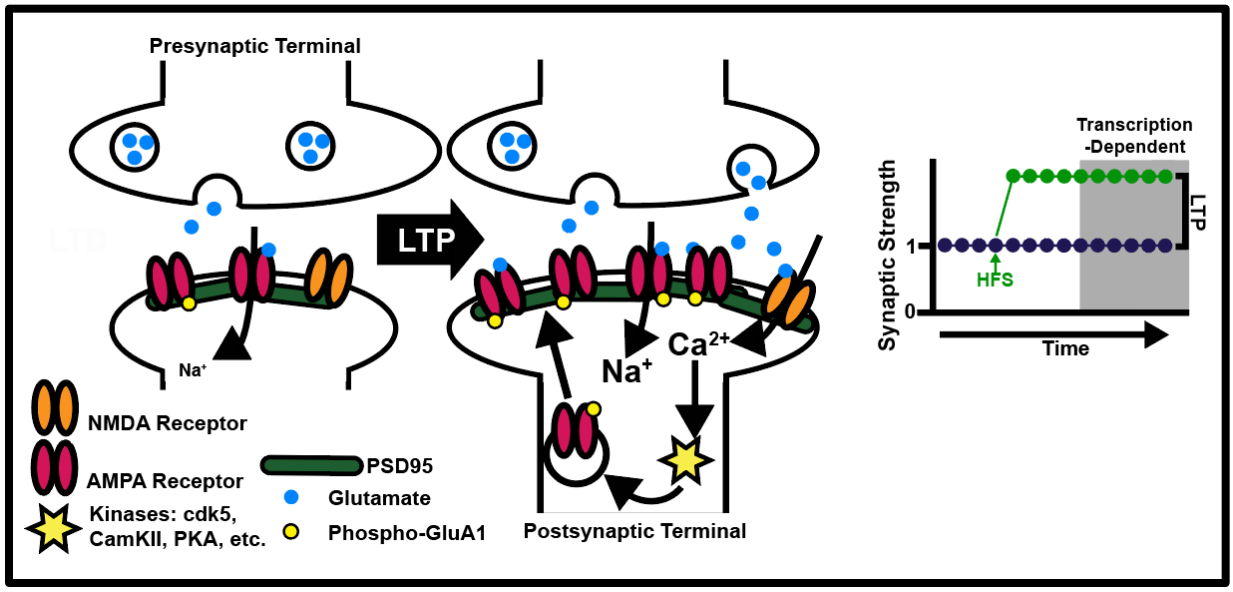

Figure 5. Long-Term Potentiation at hippocampal synapses. At basal conditions, glutamate (blue) is released from the presynaptic terminal, binds to receptors on the postsynaptic neuron, and the AMPA glutamate receptors (pink) let in amounts of Na^+ in which depolarizes the neuron while the NMDA glutamate receptors (orange) remain inactive. After LTP, more AMPA receptors become activated by glutamate, which depolarizes the neuron enough to release the Mg^{2+} ion blocking the NMDA receptor. This causes an influx of Ca^{2+} via the NMDA receptors which activates CaMKII and downstream signaling cascades, ultimately leading to an increase in AMPA receptors being added to the postsynaptic terminal and increase in phosphorylation of the GluA1 subunit. The graph to the right is a representation of LTP induced by high frequency stimulation (HFS). The gray region indicates long-lasting change in synaptic strength that is dependent on active changes in transcription. This figure is modified from: (Eagle et al., 2016).

Synaptic plasticity occurs throughout the brain, but much of the research has probed LTP in the synapses between CA1 axons and CA3 dendrites, often referred to as Schaffer collateral fibers. LTP in the CA1 region of the hippocampus requires activation of Ca^{2+}-permeable glutamate receptors, called N-methyl-D-aspartate (NMDA) receptors. Prior to activation, the NMDA receptor contains a Mg^{2+} ion blockade in its channel pore that cannot be released until depolarization of the neuron. Depolarization can occur once glutamate is released from the presynaptic neuron and binds to α-amino-3-hydroxy-5-methyl-4-isoxzolepropionic (AMPA) receptors, another type of glutamate receptor. This activation results in an influx of Na^{+} and subsequent depolarization of the postsynaptic membrane, releasing the Mg^{2+} blockade in the NMDA receptor and allowing for the influx of Ca^{2+}, which is essential for LTP (Lynch et al., 1983; Malenka et al., 1988) **(Figure 5)**. Once inside the neuron, Ca^{2+} can activate a variety of signaling mechanisms, including calmodulin-dependent kinases and adenylyl cyclase, ultimately increasing phosphorylation and insertion of AMPA receptors into the postsynaptic membrane. Long-term maintenance of these effects is dependent on transcription and translation (Banke et al., 2000; Matamales and Girault, 2011).

Gene expression and memory:

Since the late 1960's, researchers have been investigating the role of gene transcription in hippocampal-dependent memory formation (Nakajima, 1969). Transcription occurs when RNA polymerase II transcribes a sequence of DNA into corresponding messenger RNA (mRNA). The process requires an orchestra of molecular machinery including the promoter regions that direct transcription, transcription factors that facilitate RNA polymerase binding, coregulators and cofactors which mediate the interactions with transcription factors, and chromatin regulators that can modulate the remodeling of the chromatin structure to enhance or suppress transcription factor binding to DNA (Eagle et al., 2016). Pharmacological tools inhibiting RNA synthesis, such

as actinomycin D, have been utilized to identify the role of gene transcription in learning and memory tasks. After actinomycin injection into dHPC, rats showed deficits in both learning and relearning when put through position discrimination using a T maze, and in a brightness discrimination task (Nakajima, 1969; Wetzel et al., 1976). When added directly onto an *ex vivo* brain slice, actinomycin impaired only the late phase, or maintenance, of LTP, as the early phase utilizes already available proteins and is transcription-independent (Nguyen et al., 1994). After learning takes place, neurons display depressed LTP, suggesting that the process of learning and memory formation potentiates the synapses, occluding further strengthening (Barnes et al., 1994). The changes in gene expression necessary for LTP ultimately lead to changes in the density of ion channels and receptors, increasing the depolarizing responses to subsequent glutamate release (Appleby et al., 2011).

Many of the early studies into transcription-dependent memory formation, including the ones stated above, utilized actinomycin D, which was ultimately determined to have highly toxic effects as it irreversibly blocks transcription by all three classes of RNA polymerases (Neale et al., 1973). More recent studies investigating the role of transcription in hippocampal-dependent memory formation have utilized RNA polymerase II inhibitors which are less toxic and allow investigators to determine with temporal specificity when transcription is necessary. Injection of either 5,6-dichloro-1-beta-d-ribofuranosylbenzimidazole or alpha-amanitin into the dorsal CA1 region during the time of training or 3-6 hours later impaired long-term memory (Igaz et al., 2002). These two time points have been hypothesized to represent the expression of different genes, with the early phase expressing transcription factors and immediate early genes (IEGs) and the later phase expressing structural genes that will be important for synaptic remodeling. These two phases of active gene transcription critical for hippocampal-dependent memory formation can also be blocked with a protein kinase A (PKA) inhibitor, suggesting that the cAMP signaling pathway regulates transcription required for memory formation (Bourtchouladze et al., 1998). More detailed information regarding the PKA signaling pathway is discussed below in the "*Cocaine-related*

transcriptional effects" section.

Changes in chromatin structure may be a central mechanism in the regulation of hippocampal gene expression. Investigation into epigenetic changes, such as DNA methylation and histone post-translational modifications, has revealed an important role for epigenetics in memory formation (Zovkic et al., 2013). The addition of chemical groups to DNA or histone tails regulates gene transcription in response to environmental changes, including neuronal activation, via regulation of DNA compaction directly or indirectly through the recruitment of suppressor or activator molecules (Miller et al., 2008; Kramer et al., 2011).

Epigenetic mechanisms:

Epigenetic modifications change gene expression without altering DNA sequences, avoiding potentially lethal long-term effects of DNA mutation. The majority of modifications allow for the manipulation of the chromatin structure. Chromatin is the collection of DNA wrapped around histone cores, the basic structural unit of which is called a nucleosome. The compaction of DNA in this manner is essential to not only control gene expression but also for packaging the entire genome into the nucleus. Approximately 146 base pairs of DNA fit around an octamer of histone proteins, which contains two of each core histone, H2A, H2B, H3, and H4 **(Figure 6)**. Connecting each of the nucleosomes is the linker histone, H1, and together this structure is called chromatin. Chromatin can exist in two different states – highly compact, called heterochromatin, or open and available for active transcription, called euchromatin (Arney and Fisher, 2004). Epigenetic modifications can act to either activate or repress transcriptional activity through enzymatic modification of DNA or histones. 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 (Dantas Machado et al., 2014). Post-translational modification of histone proteins occurs 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 activating or silencing factors (Bannister et al., 2001; Blackledge and Klose, 2010). The modifications include acetylation, phosphorylation, methylation, ubiquitination, and sumoylation at a variety of residues on the tails of multiple

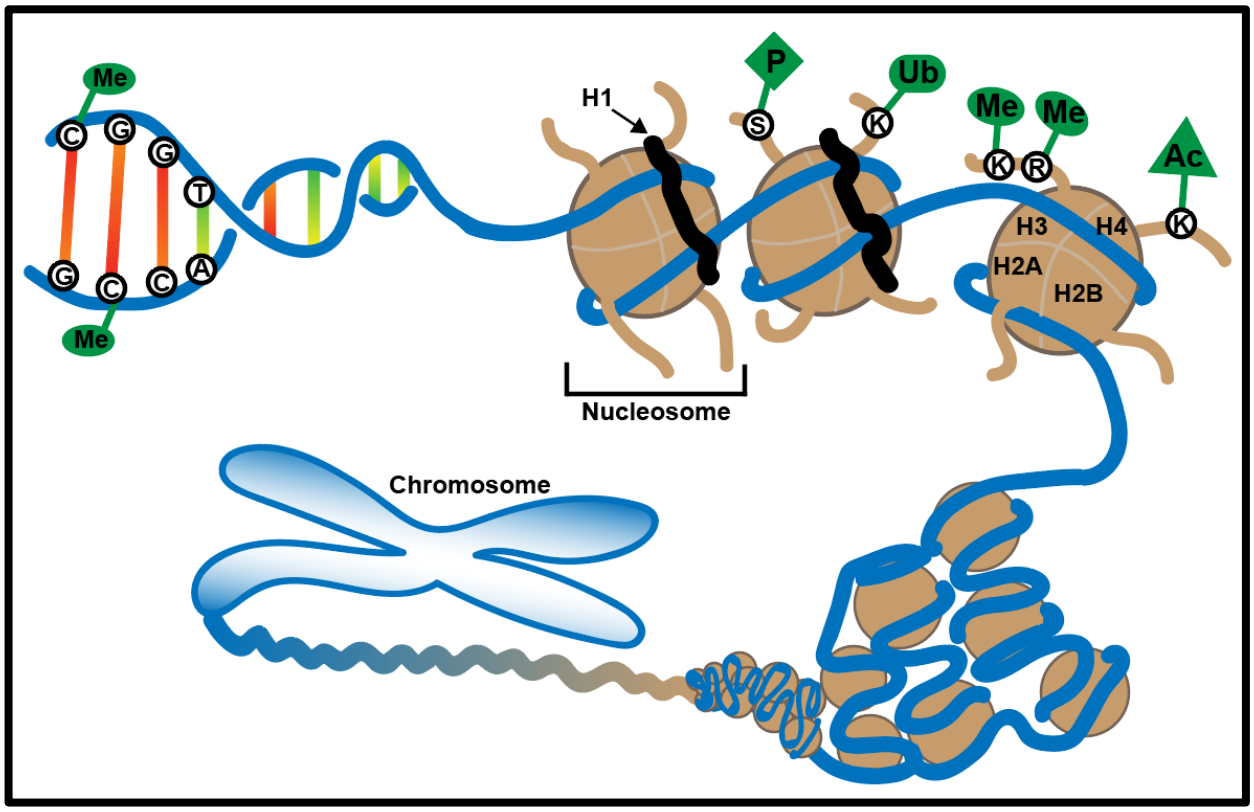

Figure 6. Epigenetic modifications made to chromatin. Cytosines (red) in the DNA sequence may become methylated (Me) by DNA methyltransferases. This modification is most typically associated with a decrease in transcription (Other nucleotides Guanine: orange; Alanine: yellow; Thymine: green). DNA first compacted into nucleosomes after it is wrapped around a core set of histone (composed on two copies of each H2A, H2B, H3, and H4) and held together by histone H1. Each of the core histones has a tail that sticks out from the core and freely interacts with DNA as well as enzymes that can add or remove chemical groups. Many histone tail residues are targets for modifications, which ultimately influence the compaction of the surrounding DNA either by direct interactions or through the recruitment of activator/repressor factors (amino acid residues: S: serine, K: lysine, R: arginine; modifications: P: phosphorylation, Ub: ubiquitination, Me: methylation, Ac: acetylation). Nucleosomes are further compacted regardless of the various histone modifications in order to condense the chromatin into a state in which it fits neatly in the nucleus with the rest of the DNA. The state of compaction in which we typically visualize DNA is at the chromosomal level.

histones **(Figure 5)**. Additionally, another epigenetic mechanism involves microRNA – small, non-coding RNAs that act within a RNA-induced silencing complex (RISC) to target specific mRNAs for degradation (Varela et al., 2013). For the purpose of this text I will focus on changes that influence the chromatin structure and subsequent transcription, such as DNA methylation and histone modifications

Epigenetic mechanisms in memory formation:

Recent evidence suggests that histone modifications may have a role in regulating hippocampal function and physiology. Specifically, the acetylation of lysine residues has been investigated for its role in transcriptional activation after learning. Lysine is a positively charged amino acid, and those found on histone tails interact in close proximity with DNA, which has a backbone of negatively charged phosphates. This interaction, without any modification to the lysine residue, creates a tight covalent bond between the positively charged lysine and the negatively charged DNA backbone, which has the effect of more tightly wrapping the DNA around the histone core and inhibiting the binding of transcriptional machinery. Upon acetylation of the lysine residue, the positive charge is neutralized, leading to more loosely wrapped DNA, opening it up for transcription factor and RNA polymerase binding to activate transcription (Rosato and Grant, 2005). This type of histone modification, one that increases the likely-hood of transcription, is called permissive. Upon overexpression of a histone deacetylase (HDAC) throughout the brain, mice showed decreased hippocampal spine density, synapse number, synaptic plasticity, and memory formation (Guan et al., 2009). Conversely, when the HDAC was knocked-down, this resulted in an increased number of hippocampal synapses and increased LTP (Guan et al., 2009). Treatment with HDAC inhibitors prior to the hippocampal-dependent learning paradigm, contextual fear conditioning, led to enhanced long-term fear memory in mice (Levenson et al., 2004). These findings demonstrate that histone acetylation in the hippocampus controls normal

memory consolidation.

The methylation of various lysine residues on histone tails has also been implicated in dynamic regulation of memory processing (Gupta et al., 2010). Histone methylation can occur via the addition of one, two, or three methyl groups onto a lysine residue, or up to two methyl groups on arginine residues, and depending which residue is affected, the modification can be permissive or repressive. For example, mono-, di-, and trimethylated H3 lysine 4 (H3K4) and monomethylation of H3K9 is permissive, while di- and trimethylation of H3K9 is repressive. Methylation does not directly impact the bond between residues on the histone tails and DNA, but instead recruits transcriptional modulators which have specific domains that recognize and bind to the modification and influence transcription. During the retrieval of a fear memory, the CA1 subregion of the hippocampus, and not the basoloteral amygdala, of rats showed increases in the trimethylation of lysine 4 on histone H3 (H3K4me3). Furthermore, knockdown of the histone methyltransferase, Mll1, in the CA1 impaired fear memory (Webb et al., 2017). H3K4me3 has also been examined during memory consolidation, and was found to be increased at the promoter of two genes, *Zif268* and *bdnf*, both of which play a role in memory consolidation (Bramham, 2007; Poirier et al., 2007; Gupta et al., 2010). The increase in H3K4me3 has been associated with an increase in DNA methylation at the *Zif268* gene, indicating that regulation of histone modifications can also influence the action of DNA methyltransferases (Gupta et al., 2010). Thus, the regulation of these histone methylation marks is essential for normal cognitive processes including hippocampal-dependent memory formation.

Cocaine-related transcriptional effects:

After cocaine exposure, DA receptor activation induces many downstream signaling cascades. In the case of D1-type DA receptors, stimulation activates adenylyl cyclase activity which activates the kinase, PKA. Activated PKA then phosphorylates multiple nuclear proteins involved in gene

transcription, such as CREB (cAMP response element binding protein, **Figure 7**) (Self and Nestler, 1995; Shaywitz and Greenberg, 1999; Matamales and Girault, 2011). In addition to the known changes occurring via the DA receptor signaling pathway, transcriptional profiles have been investigated in multiple brain regions in response to cocaine. Evidence from the NAc of human addicts reveals that heroin and cocaine addiction, while both ultimately increase DA signaling, lead to distinct gene expression profiles with only around 25 out of 39,000 transcripts being altered in both cohorts (Albertson et al., 2006). Another study examined the hippocampus of cocaine-addicted and alcoholic individuals and found extensive overlap in gene expression changes, with 29 out of 413 genes being differentially expressed in both cohorts, as well as significant overlap in the location of the histone modification, H3K4me2 (Zhou et al., 2011).

Animal studies have similarly given us insights into transcriptional changes in response to cocaine exposure, while also allowing for a more consistent timing between individuals and a more controlled behavioral regimen. Profiling of the PFC after various stages of cocaine withdrawal revealed time dependent decreases in energy and protein metabolism pathways, increases in plasticity related pathways, like LTP, and changes to the ERK pathway, which is activated in the mesolimbic reward system after cocaine exposure (Lu et al., 2006; Li et al., 2017). Lastly, the transcription factor CREB (**Figure 7**) is phosphorylated in response to cocaine and is critical in amygdala for stress-induced reinstatement to drug seeking (Kreibich and Blendy, 2004). CREB knockdown also impairs cocaine-mediated transcription and changes the expression profile of amygdalar stress- and immune-related genes (Ecke et al., 2011). Together, these studies indicate that cocaine exposure changes the transcriptional profiles of multiple brain regions and that there are many changes specific to cocaine addiction.

Transcription Factors:

The regulation of appropriate hippocampal function relies heavily on activity dependent changes in transcription. Activity-dependent transcription factors are induced by the stimulation of a

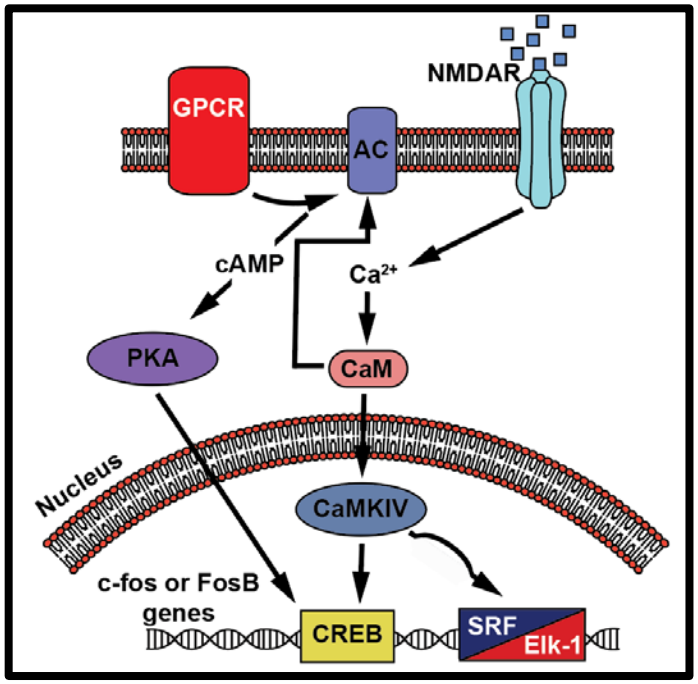

Figure 7. **Common signaling cascades leading to CREB activation.** Activation of G Protein-Coupled Receptors (GPCR) with a stimulatory subunit activates adenylyl cyclase (AC), resulting in the formation of cyclic AMP (cAMP) and activation of protein kinase A (PKA). PKA phosphorylates CREB, activating it, resulting in its binding to specific elements in promoter regions to regulate transcription of various immediate early genes, such as *c-fos* or *FosB*. Activation of NMDA receptors by glutamate also leads to CREB phosphorylation via a signaling cascade that follows an influx of Ca^{2+}. Calmodulin (CaM), Ca2+/calmodulin kinase IV (CaMKIV), Serum response factor (SRF). This figure is modified from (Eagle et al., 2016)

neuron, which leads to an action potential. This drives the expression of immediate early genes (IEGs) via signaling cascades, which encode many of the necessary transcription factors for normal hippocampal and general brain function. The identification of multiple transcription factors associated with hippocampal plasticity and learning has led to the dissection of their possible dysfunction in disease states. Egr1 (Zif268) and ATF4 are two such transcription factors that when knocked down, decrease LTP and impair long-term memory formation (Jones et al., 2001; Pasini et al., 2015). c-Fos also plays a role in hippocampal function, as brain-specific knockout impairs learning and memory and hippocampal plasticity (Sagar et al., 1988; Fleischmann et al., 2003). The silencing of another IEG in the hippocampus, FosB, leads to impairments in spatial learning

and general memory formation (Eagle et al., 2015). Additionally, overexpression of a uniquely stable splice variant of the *FosB* gene, called ΔFosB, similarly impairs learning and memory and induces immature spine formation in dHPC CA1 pyramidal neurons (Eagle et al., 2015). Transcription factors are master regulators of general and specific transcriptional changes that can be brain region- and cell type-specific, and thus may mediate the myriad effects of cocaine on gene transcription. Cocaine exposure changes the levels of IEGs such as c-Fos, c-jun, FosB, junB, and Zif268 in the NAc (**Table 1**) (Hope et al., 1992). Examination of some of these transcription factors revealed molecular- and circuit-specific changes in their levels after cocaine. For instance, CPP elicits an increase in c-Fos expression in cells projecting from the basolateral amygdala and not the prelimbic cortex, supporting the view that the amygdala specifically mediates cue-elicited drug seeking (Miller and Marshall, 2005). Additionally, acute and chronic cocaine exposure increases the expression levels of FosB and its splice variant, ΔFosB, in the NAc and striatum (Hope et al., 1992; Sato et al., 2011), and overexpression of ΔFosB in the NAc causes mice to display similar behavioral phenotypes to chronic drug exposure (Kelz et al., 1999a). However, much of what is known about the *FosB* isoforms and their role in drug addiction comes almost exclusively from the NAc, with few studies probing the effects in other brain regions

Table 1. Immediate Early Genes (IEGs) impacted by cocaine exposure

Gene	Cocaine-mediated changes	References
c-Fos	Induction throughout NAc, overexpression reduces sensitization and cocaine CPP extinction	(Zhang et al., 2006)
c-Jun	Induction in NAc, overexpression of dominant-negative form reduces cocaine CPP	(Kosofsky et al., 1996 Peakman et al., 2003)
FosB	Induction in D1-type NAc neurons, overexpression of ΔFosB increases locomotor sensitization, cocaine CPP, and cocaine self-administration	(Kelz et al., 1999b, Colby et al., 2003)
JunB	Induction in NAc	(Hope et al., 1994b)
Zif268	Induction in D1-type NAc neurons, overexpression in D1 neurons increases locomotor sensitization and cocaine CPP, overexpression in D2 neurons reduces locomotion and CPP	(Chandra et al., 2015)

essential for addiction-related behaviors, like the hippocampus (Perrotti et al., 2008).

ΔFOSB

Gene Structure:

FosB is a member of the Fos family of transcription factors, and is thus an IEG that is rapidly induced in response to a variety of stimuli, such as electroconvulsive seizures, drug exposure, spatial learning, and stress (Hope et al., 1994a; Hope et al., 1994b; Vialou et al., 2010; Eagle et al., 2015). As previously stated, multiple splice variants are produced from the four exons of the *FosB* gene **(Figure 8)**. Generally, three isoforms are recognized: FosB, ΔFosB, and Δ2ΔFosB. FosB includes all four exons, while ΔFosB and Δ2ΔFosB are truncated versions that are formed from alternative splicing. ΔFosB incorporates an early stop codon from a one nucleotide frame-shift caused by the alternative splice site, truncating 101 amino acids from the C-terminus of the protein. Δ2ΔFosB is translated from an alternative start codon located within the ΔFosB mRNA causing it to lack 78 amino acids from the N-terminus (Chen et al., 1997). The four exons encode for a DNA-binding basic leucine zipper domain, which allows for the formation of a heterodimer with a Jun family protein to form the activator protein 1 (AP-1) transcription factor complex, and a C-terminal transactivation domain (Morgan and Curran, 1995; Chinenov and Kerppola, 2001) **(Figure 8)**. The C-terminal truncation in ΔFosB and Δ2ΔFosB causes an exclusion of two degron domains encoded by the fourth exon which increases the stability of these isoforms as they are not targeted for the same proteasomal degradation as FosB (Carle et al., 2007). The truncation leading to the formation of ΔFosB also appears to influence the activation of different genetic profiles when compared with FosB, with each having potentially different effects at the same promoter (Chen et al., 1995; McClung and Nestler, 2003). Additionally, ΔFosB can act as a transcriptional repressor for some genes and a transcriptional activator for others (Dobrazanski

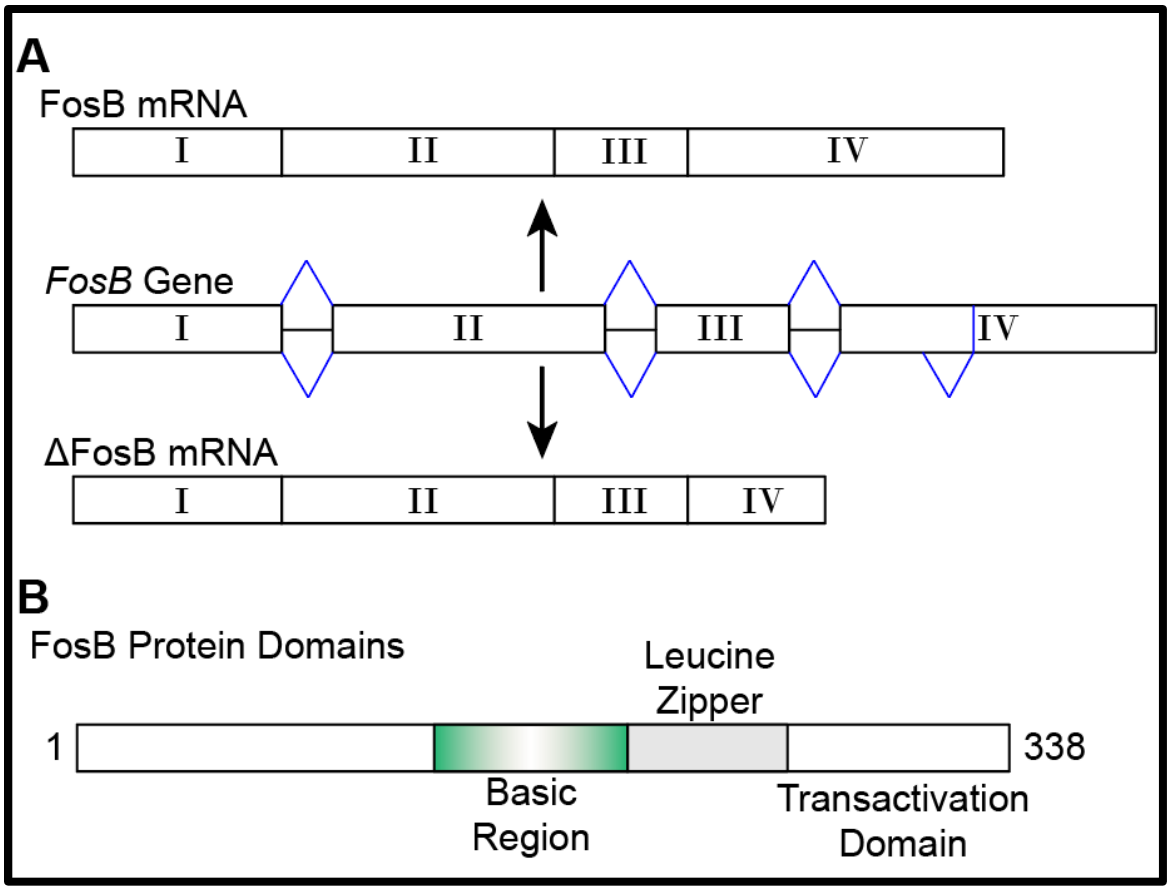

Figure 8. *FosB* mRNA splicing and protein domains. A. The *FosB* gene produces two splice variants, full length FosB and a truncated ΔFosB. ΔFosB mRNA results from a splicing event that occurs in exon 4 that causes a one nucleotide frame shift ending in a premature stop codon. This leads to the exclusion of two degron domains from exon 4 that are found in full length FosB, which contributes to the increase in ΔFosB's stability. **B.** Full length FosB and ΔFosB have similar protein structure for the most part. This includes a basic region upstream of the leucine zipper domain and a C-terminal transactivation domain. As ΔFosB is truncated at the C-terminal, the transactivation domains between FosB and ΔFosB are different. Full length FosB also contains degron domains at the C-terminal. This figure is modified from: (Carle et al., 2007).

et al., 1991; Nakabeppu and Nathans, 1991; McClung and Nestler, 2003; Carle et al., 2007). The *FosB* gene is found on chromosome 7 in mice, chromosome 1 in rats, and chromosome 19 in humans. The promoter region contains binding motifs for transcription factors CREB and serum response factor (SRF) which are known to regulate its induction in the brain (Vialou et al., 2012). Sequence analysis in a mouse embryonic fibroblast cell line revealed AP-1 like sequences around

900 and 400 base pairs upstream of the transcriptional start site, indicating that Fos proteins may regulate *FosB* transcription. However, though overexpression of FosB or c-Fos has the ability to repress *FosB* transcription, this occurs even in the presence of a mutated promoter containing only the serum response element (Lazo et al., 1992), indicating that the repression may be indirect.

Stability of ΔFosB:

Most IEG proteins undergo quick turnover, lasting only a few hours in the cell, which is true of the full-length FosB protein. However, ΔFosB remains stable in the cell for many days due to its lack of degron domains and to specific phosphorylation occurring at serine 27 (S27) (Ulery et al., 2006; Carle et al., 2007; Ulery-Reynolds et al., 2009). Phosphorylation of S27 has been shown to be mediated by casein kinase-2 (CK2) *in vitro* and Calcium/calmodulin-dependent protein kinase II (CaMKII) *in vivo*, and while there are additional sites of phosphorylation on ΔFosB, this one is particularly important for protecting the protein from proteasomal degradation (Ulery et al., 2006; Robison et al., 2013b). ΔFosB accumulates gradually during chronic stimulation and then persists in the brain for weeks, even after the mRNA has degraded (Chen et al., 1997; Alibhai et al., 2007). The native form of ΔFosB induced by acute treatments has a molecular weight of 33 kilodaltons (kDa), while after chronic stimulation the 35-57 kDa isoforms arise, likely due to posttranslational modifications related to its stability (Chen et al., 1997). With controlled regulation of ΔFosB, short-term and long-term expression had the opposite effects on expression pattern – over half of the genes that were upregulated in the short-term were later downregulated after weeks of overexpression, indicating that ΔFosB's stability plays a role in its activity and ability to regulate gene expression (McClung and Nestler, 2003), or that other homeostatic factors may regulate target gene expression over time.

Target Genes:

ΔFosB binds JunD with high affinity, although it has the ability to form a heterodimer with other Jun family proteins (Hiroi et al., 1998). Together, they form the AP-1 complex which recognizes the consensus sequence, TGAC/GTCA, in promoter regions of genes. ΔFosB dynamically regulates transcription as both an activator and a repressor. Genes targeted by ΔFosB have been determined using a candidate approach as well as with microarrays. The *GRIA2* promoter contains an AP-1 site which is bound by ΔFosB and is upregulated after ΔFosB overexpression in the NAc (Kelz et al., 1999a; Peakman et al., 2003). The *GRIA2* gene product, GluR2, is an AMPA receptor subunit and is involved in the assembly and trafficking of the receptor to the membrane (Isaac et al., 2007). Additionally, GluR2-lacking receptors are permeable to Ca^{2+} and thus play a role in LTP, as an NMDA receptor-independent form of plasticity (Mahanty and Sah, 1998). ΔFosB also regulates the NMDA receptor subunit, NMDAR1, in the cerebral cortex. Chronic seizures upregulate NMDAR1 expression, as well as ΔFosB expression, and in *FosB* knockout mice NMDAR1 induction does not take place (Hiroi et al., 1998). Cyclin-dependent kinase-5 (*cdk5*) was found to be activated by ΔFosB in the hippocampus after examining electroconvulsive seizures as an antidepressant treatment (Chen et al., 2000). Cdk5 has been directly associated with the phosphorylation states of multiple synaptic proteins implicated in memory formation (Guan et al., 2011). Activation of nuclear factor-kappaB (*NFκB*) subunits by ΔFosB in the NAc has also been shown, and induction of NFκB leads to an increase in dendritic spine number in NAc medium spiny neurons and sensitization to drug responses (Russo et al., 2009). CaMKIIα, while also a regulator of ΔFosB's stability, appears to be a gene target for ΔFosB in NAc, causing a feedforward loop between the two proteins, with CaMKIIα phosphorylating and stabilizing ΔFosB which can then bind the *CaMKIIα* promoter and increase transcription (Robison et al., 2013b). In addition to being a stabilizing agent for ΔFosB, CaMKII plays an important role in LTP and hippocampal-dependent learning, as it also phosphorylates the GluR1 subunit of the

AMPA receptor, which increases its conductance, and drives its transport to the synapse (**Figure 5**) (Barria et al., 1997; Hayashi et al., 2000; Malenka, 2003). Dynorphin, the endogenous opioid peptide, also contains AP-1-like sites in its promoter region, and when ΔFosB is selectively overexpressed in dynorphin-positive neurons in the striatum, there is a subsequent decrease in dynorphin mRNA (Zachariou et al., 2006). Lastly, upon overexpression of hippocampal ΔFosB, or after electroconvulsive seizures (which also increase hippocampal ΔFosB), CCAAT-enhancing binding protein-beta (C/EBPβ) is downregulated and was investigated as a molecular mechanism by which electroconvulsive seizures may be mediating their antidepressant effects (Chen et al., 2004). C/EBPβ has been previously examined in the context of long-term memory in Aplysia, and later in mammals as having a role in the consolidation of long-term memory (Alberini et al., 1994; Taubenfeld et al., 2001).

These target genes do not encompass all the transcriptional effects of ΔFosB, and not all of the above genes have been validated as target genes in the hippocampus. However, as many of the currently known target genes play a known role in synaptic plasticity throughout the brain, ΔFosB is a candidate for mediating the hippocampal synaptic changes seen after chronic cocaine exposure.

The role of ΔFosB in cocaine addiction:

ΔFosB robustly accumulates with chronic cocaine exposure in multiple brain regions including NAc and hippocampus, and is stable for weeks after the last cocaine injection (Hope et al., 1994b; Nestler et al., 2001; Nestler, 2008; Perrotti et al., 2008). To control the temporal and spatial expression of ΔFosB, a system was developed using bitransgenic mice in which ΔFosB could be specifically overexpressed in the NAc in a doxycycline-dependent manner (Kelz et al., 1999a). Overexpression of NAc ΔFosB during drug exposure enhanced the locomotor response to cocaine and cocaine reward, as the mice responded to approximately one-third the dose required

to produce a response in wild-type mice (Kelz et al., 1999a). Additionally, these mice self-administered more cocaine than wild type and displayed an increased motivation to receive an infusion of cocaine (Colby et al., 2003). Conversely, inhibition of NAc ΔFosB reduced locomotor sensitization to cocaine, a behavioral phenomenon dependent on plasticity changes in response to psychostimulants in which an animal experiences a progressive and long-lasting increase in their locomotor activity (Robison et al., 2013b). In rodents, this sensitization correlates with an increased predisposition to self-administer psychostimulants, including cocaine, and reinstatement of previously extinguished self-administration (De Vries et al., 1998; Schenk and Partridge, 2000; Vezina et al., 2002). These findings suggest that normal accumulation of ΔFosB in the brain after chronic cocaine exposure may underlie the development of addictive behaviors. One way ΔFosB is changing the synaptic connections in NAc after cocaine exposure is by altering the number of silent synapses in a specific subtype of MSNs, the D1 dopamine receptor expressing neurons (Grueter et al., 2013). Silent synapses contain NMDA receptors without expression of any AMPA receptors, so they have a reduced depolarization in response to glutamate, as the NMDA receptors will remain blocked by the Mg^{2+} ion. The insertion of NMDA receptors indicates a sort of priming for the synapse, as the addition of AMPA receptors may still occur after some time and will immediately allow the synapse to respond to a glutamate signal via membrane depolarization (Morita et al., 2014).

Overexpression of ΔFosB in D1-type neurons led to a decrease in AMPA receptor-mediated synaptic transmissions due to an increase in the proportion of silent synapses. In contrast, ΔFosB overexpression in D2-type neurons caused an increase in AMPA receptor-mediated synaptic transmission (Grueter et al., 2013). Not surprisingly, these synaptic changes elicited behavioral changes as well, with short-term overexpression in D1, but not D2 neurons causing an increased locomotor sensitization and cocaine CPP to a subthreshold dose of cocaine. The generation of silent synapses was also observed in the NAc after short-term withdrawal from cocaine and was rescued with a dominant negative form of CREB, but also induced by the expression of a

constitutively active form of CREB, which also induces silent synapses in the hippocampus (Marie et al., 2005; Huang et al., 2009; Brown et al., 2011; Grueter et al., 2013).

NAc ΔFosB appears to play a role in mediating some susceptibility to cocaine addiction, as it is not only induced throughout the NAc in response to chronic cocaine exposure, but overexpression of it allows rodents to react in the same manner to low doses of cocaine as wild type rodents react to high doses. The mechanism by which cocaine is leading to an increase in ΔFosB is thus of great interest and has been the focus of study for many researchers.

Regulation of cocaine-mediated ΔFosB induction:

Synaptic plasticity requires active changes in chromatin structure and gene transcription, and exposure to cocaine leads to the differential expression of hundreds of genes and specific histone post-translational modifications (Walton et al., 1999; Miller and Marshall, 2005; Rodriguez-Espinosa and Fernandez-Espejo, 2015; Sadri-Vakili, 2015; Kennedy et al., 2016; McEwen, 2016). Above sections detailed the role of intracellular signaling cascades after cocaine exposure, including activation of PKA, which leads to the phosphorylation of several transcription factors, such as SRF and CREB (Matamales and Girault, 2011). Activation of these two transcription factors is essential in the cocaine-mediated induction of ΔFosB, as genetic deletion of each one alone from NAc did not affect cocaine-mediated induction of ΔFosB, but deletion of both blocks ΔFosB induction and impairs cocaine CPP (Vialou et al., 2012).

Additionally, chronic cocaine decreases global levels of H3K9me2 in the NAc, a modification associated with transcriptional silencing through its ability to recruit certain repressive machinery, such as heterochromatin protein 1 (Renthal et al., 2009; Blackledge and Klose, 2010; Maze et al., 2010). The reduction in H3K9me2 was found to be due to repression of the histone methyltransferase, G9a, which is regulated by ΔFosB (Maze et al., 2010). 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 (Damez-Werno et al., 2012).

Hippocampal ΔFosB:

Many of ΔFosB's gene targets are critical to hippocampal synaptic plasticity and morphology, but until recently, a specific hippocampal role for ΔFosB had not been thoroughly investigated. In a whole body developmental knockout of the *FosB* gene, there are hippocampal malformations and impairments to spatial memory (Solecki et al., 2008; Yutsudo et al., 2013). Previous evidence also implicates the transcription factors SRF and CREB in hippocampal function, as they are induced by spatial learning (Mizuno et al., 2002; Porte et al., 2008; Alberini and Kandel, 2014). These pieces of evidence all pointed to ΔFosB playing an important role in hippocampal function. Our lab used Morris water maze (MWM) to assess the role of hippocampal ΔFosB in spatial learning and observed an increase in FosB-positive cells specifically in the dorsal CA1 subregion (Eagle et al., 2015). Moreover, silencing ΔFosB's transcriptional activity by overexpressing a dominant negative form of JunD, known as ΔJunD, impaired MWM and other forms of hippocampal-dependent learning and memory (Eagle et al., 2015). In addition to impairing learning, silencing ΔFosB activity produced a decrease in the total number of dendritic spines in CA1 neurons. General overexpression of ΔFosB in the dHPC also impaired the same learning and memory tasks, but increased the number of immature, thin and stubby, spines. This suggests that cell-specific activation of ΔFosB, as opposed to region-wide expression, may be necessary for memory formation (Eagle et al., 2016). A memory is formed by relatively few hippocampal neurons (i.e. the engram), and their stimulation causes an accumulation of *FosB* gene products, affecting their synaptic and excitability properties (**Figure 9**). The non-specific overexpression of ΔFosB throughout the entire hippocampus may obscure the function of specific ΔFosB induction, and its effects in the engram neurons may be ultimately lost in this "noise" (Eagle et al., 2015).

Dorsal CA1 and DG subregions display especially large inductions of ΔFosB in response to treatment with the antidepressant fluoxetine (Vialou et al., 2015). Antidepressant treatment increases adult neurogenesis in the DG subregion of the hippocampus, and genetic deletion of *FosB* impairs adult neurogenesis, creating a molecular link between the antidepressant-mediated increase in adult neurogenesis (Santarelli et al., 2003; Yutsudo et al., 2013; Vialou et al., 2015). Brief investigation into drug-mediated induction of ΔFosB examined qualitative induction in

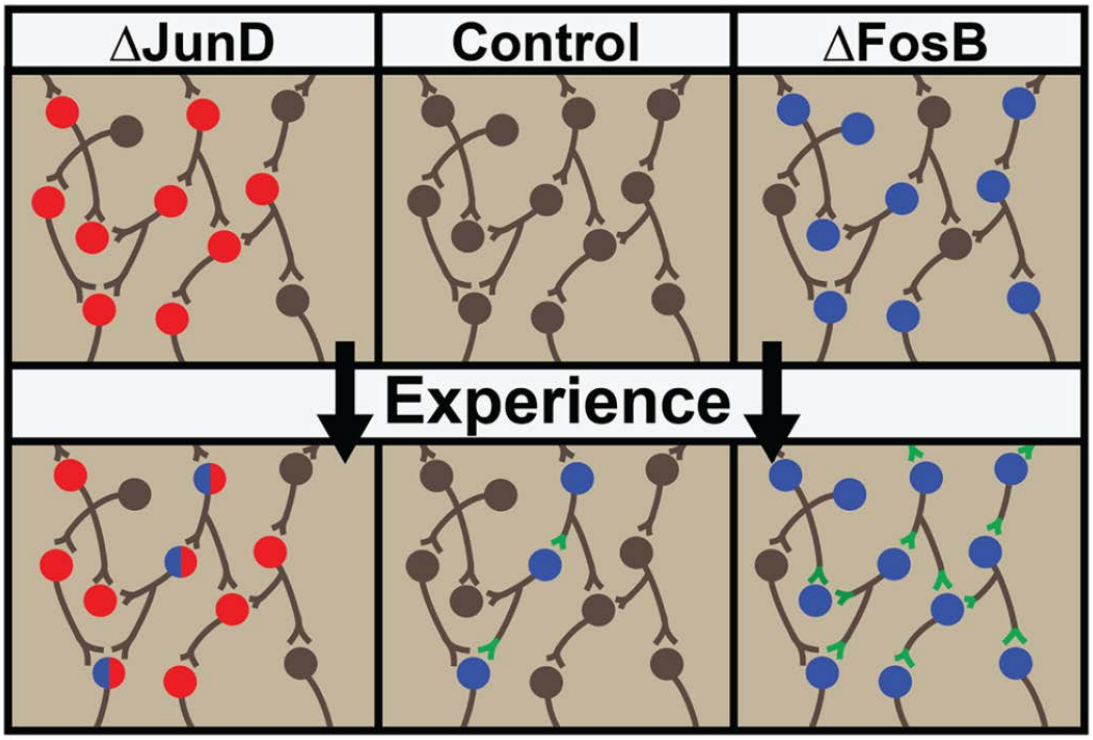

Figure 9. Model depicting experience-dependent changes in hippocampal cells under various conditions. Under control conditions an experience elicits the activation of a subset of neurons, which induces ΔFosB (blue) expression. Green indicates changes in synaptic structure/function resulting from ΔFosB expression, which may underlie learning. Viral overexpression of ΔJunD (red), causes the subset of neurons activated by an experience/learning to silence ΔFosB, and thus changes in synaptic function do not occur. Viral overexpression of ΔFosB leads to structural/function changes in a non-specific manner, increasing the "noise," and losing the signal of the specific subset of neurons activated. This figure was adapted from (Eagle et al., 2015)

various brain regions, including the dHPC, in response to various drugs of abuse such as morphine, ethanol, and cocaine (Perrotti et al., 2008). There appeared to be more FosB-positive neurons in all subregions of the hippocampus after chronic exposure to morphine or cocaine when compared with saline controls. Importantly, the rats in the study were given the drugs in their home cage, so the induction of FosB was discussed as possibly having a role in cognitive performance, as many addicts display a cognitive decline after chronic drug use (Wood et al., 2014). However, as evidence demonstrates that the drug-environment associations, such as those formed during cocaine CPP, rely on hippocampal activation, investigation into the role of hippocampal ΔFosB in this context remains an outstanding question. Furthermore, the mechanism by which cocaine drives ΔFosB induction requires further elucidation. Insights into the mechanism from NAc reveal an interesting role for epigenetics.

Epigenetic Regulation of the FosB gene:

The mechanism of ΔFosB induction in NAc has recently focused on changes in epigenetic factors, specifically histone 3 lysine 9 dimethylation (H3K9me2) by the histone methyltransferase G9a, as mentioned briefly above (Nunez et al., 2010; Damez-Werno et al., 2012). Examination of a variety of histone post-translational modifications revealed that only H3K9me2 was altered at the *FosB* promoter after chronic cocaine exposure – there was a significant decrease in the repressive histone modification at approximately 900 base pairs upstream of the transcriptional start site (Damez-Werno et al., 2012). The decrease was observed after a 28-day withdrawal period either with or without the presence of a challenging dose of cocaine, indicating that the decrease in H3K9me2 may in fact be priming the *FosB* gene for transcription once exposed to cocaine in the future.

One of the more recent goals has been to further elucidate the role of histone modifications in behavioral outcomes. Therefore, after observing the decrease in H3K9me2 at the *FosB* promoter,

it was hypothesized that interrupting the change in H3K9me2 might influence NAc-related behaviors. The regulation of histone modifications has generally been investigated with use of inhibitors of the histone modifying enzymes themselves. However, this technique does not allow for enough specificity to determine whether the outcome is due to changes in expression of a certain gene, or even subset of genes, as the entire activity of the enzyme is being altered. Therefore, a more precise approach was established by our collaborator Dr. Elizabeth Heller with the use of specific zinc-finger proteins (ZFP), which are DNA binding domains (Heller et al., 2014b). By fusing histone modifying enzymes to specifically engineered ZFPs, histone modifications can be artificially regulated at specific genes. The DNA encoding these ZFP-fusion proteins can then be packaged into viral vectors to obtain temporal and spatial specificity of expression within the adult animal brain. The ZFP that Heller synthesized recognizes a sequence in the *FosB* promoter approximately 300 base pairs upstream of the transcriptional start site (**Figure 10**). Fused to the ZFP was either the transcriptional activator, p65, which promotes histone acetylation via the recruitment of p300/CBP (CREB-binding protein), or the catalytic domain of the histone methytransferase, G9a (Heller et al., 2014b). Any cells infected with the virus would express the ZFP-fusion protein which would bind to the *FosB* promoter, where the attached enzyme domain would either increase histone acetylation, in the case of p65, or increase H3K9me2, in the case of the G9a catalytic domain. Validation of the ZFP binding found no changes in expression of any other genes with sequences similar to the *FosB* promoter (Heller et al., 2014b). Thus, it could be used to change the histone modifications specifically at the *FosB* gene and determine if it was enough to influence behavioral outcomes. They found that the ZFP-G9a fusion protein did indeed impair the cocaine-mediated *FosB* induction in the NAc, and blocked cocaine locomotor sensitization, while ZFP-p65 was sufficient to enhance the locomotor sensitization effects of cocaine given at a subthreshold dose (Heller et al., 2014b). As previous evidence has implicated NAc ΔFosB in depressive-like behaviors as well, they also examined stress-evoked behaviors and found that mice that received ZFP-G9a in the NAc displayed pro-

depressive behaviors when compared with controls. This is in line with the finding that increased FosB/ΔFosB expression in NAc protects animals from the depressive-like phenotype seen after chronic stress (Vialou et al., 2010).

Taken together, these findings demonstrate that changes in H3K9me2 are sufficient to decrease expression of the *FosB* gene and control behavioral effects of cocaine and stress exposure. These data imply that changes in histone modifications directly cause changes in gene expression. However, not all brain regions are similar in their chromatin state, and thus do not all express the same genes. Therefore, ΔFosB's role in cocaine-related hippocampal-dependent behaviors requires further analysis.

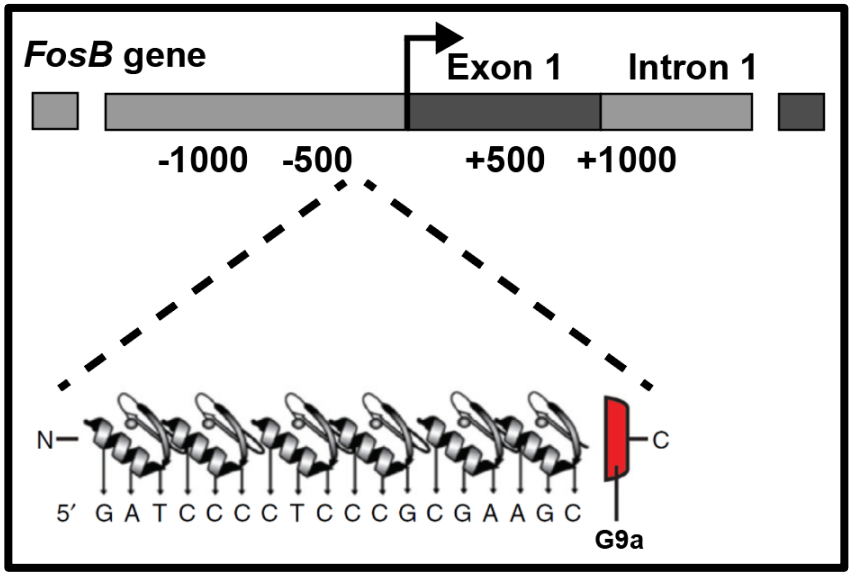

Figure 10. Diagram showing binding of zinc finger protein fused to histone methyltransferase G9a. The zinc finger protein (ZFP) is composed of multiple domains that will bind DNA at a specific sequence at the *FosB* promoter, approximately 300 base pairs upstream of the transcriptional start site (arrow). The ZFP is fused to the catalytic domain of the histone methyltransferase, G9a, which will dimethylate lysine 9 on the surrounding H3 histones. This figure was modified from (Heller et al., 2014b)

HYPOTHESIS AND SPECIFIC AIMS:

Hypothesis: Active epigenetic mechanisms engaged by cocaine are critical for induction of ΔFosB in hippocampus and drug-associated learning.

Aim I: To characterize the induction of the FosB gene in hippocampus in response to chronic cocaine.

Aim II: To determine whether FosB induction in hippocampus is mediated by histone modifying enzymes.

Aim III: To determine the effect of cocaine or antidepressant treatment on human post-mortem brain samples.

MATERIALS AND METHODS

CHROMATIN IMMUNIPRECIPITATION (CHIP)

Adult (10-11 weeks) male mice were sacrificed by cervical dislocation, forebrains were removed, and whole hippocampus was microdissected and immediately frozen on dry ice. H3K9me2 ChIP was performed essentially as described (Maze et al., 2015). Briefly, tissue (bilateral hippocampus from two mice were pooled per sample) was crosslinked with 1% formaldehyde for 12 minutes at room temperature and then quenched for five minutes with 2M glycine. Tissue was pelleted and washed 5 times with phosphate buffered saline (PBS) containing protease inhibitors (Roche, Complete-mini EDTA free, 11-836-170-001). Tissue was homogenized by passing through a 1ml syringe with a 22-gauge needle 10 times, then centrifuged at 4ºC at 1230rcf for 5 minutes. Pelleted cells/nuclei were resuspended and rotated for 15 minutes in 1ml LB1 buffer [50 mM Hepes-KOH, pH 7.5, 140mM NaCl, 1mM EDTA, 10% glycerol, 0.5% NP-40, 0.25% Triton-X 100, plus EDTA-free protease inhibitors] at 4ºC. Following pelleting, cells were then resuspended in 1ml LB2 buffer [10mM Tris-HCL, pH 8.0, 200mM NaCl, 1mM EDTA, 0.5mM EGTA, plus EDTA-free protease inhibitors] and rotated for 10 minutes at 4ºC. Pelleted cells/nuclei were then resuspended in 300µl LB3 buffer [10mM Tris-HCl, pH 8.0, 100mM NaCl, 1mM EDTA, 0.5mM EGTA, 0.1% Na-Deoxycholate, 0.5% N-lauroylsarcosine, plus EDTA-free protease inhibitors], passed through a 27-gauge needle 3X and were then put through sonication using a Bioruptor (Diagenode) for 60 minutes (30 second on/30 seconds off for 60 cycles; setting = high) at 4ºC. All samples were sonicated to approximately 150-300 base pairs prior to further processing. Following sonication, 1/10 volume of 10% Triton-X 100 was added and samples were centrifuged at 20,000 x g for 10 minutes at 4ºC to crack nuclei. Supernatants were then collected and incubated with antibody (10µg/sample, H3K9me2 antibody, abcam, ab1220) bound to sheep anti-mouse IgG M-280 Dynabeads (Invitrogen, 11201D) on a rotator at 4ºC overnight. The following day,

immunoprecipitates were washed once with 1ml Low Salt Wash Buffer [0.1% SDS, 1% Triton-X 100, 2mM EDTA, 150mM NaCl, 20mM Tris-HCl, pH 8.0], then washed once with 1ml High Salt Wash Buffer [0.1% SDS, 1% Triton-X 100, 2mM EDTA, 500mM NaCl, 20mM Tris-HCl, pH 8.0], then washed once with 1ml LiCl Wash Buffer [150mM LiCl, 1% NP-40, 1% Na-Deoxycholate, 1mM EDTA, 10mM Tris-HCl, pH 8.0] all at 4ºC. Samples were then washed 8 times with 1ml RIPA buffer [50mM Hepes-KOH, pH 7.6, 500 mM LiCl, 1mM EDTA, 1% NP-40, 0.7% Na-Deoxycholate], rinsed once with 1ml Tris-EDTA (TE) + 50 mM NaCl buffer [5mM NaCl, 1M Tris-HCl, pH 8, 0.5M EDTA]. Supernatant was removed and samples were eluted at 65ºC for 30 minutes (shaking at 1000 rpm) in 210µl elution buffer [1% SDS, 50mM Tris-HCl, pH 8.0, 10mM EDTA]. After centrifugation, samples and respective inputs (10%) were reversed crosslinked in elution buffer at 65ºC overnight. RNA and protein were digested with RNAse (one hour at 37ºC, Roche, 11119915001) and proteinase K (two hours at 55ºC, Roche, 03115887001), respectively, prior to DNA fragment purification using a Qiagen PCR purification kit.

COCAINE CONDITIONED PLACE PREFERENCE

Cocaine conditioned place preference (CPP) consisted of a 4-day protocol as previously described (Iniguez et al., 2015). Briefly, mice were placed into a three-compartment apparatus (San Diego Instruments, Place Preference) on the preconditioning day and allowed to freely explore all three chambers for 15 min to obtain baseline preference. The preconditioning trial took place 1-2 days prior to any stereotaxic surgery to eliminate any stress-evoked biases. Conditioning trials (30 min, two per day) were given on two consecutive days and began 48 hours after stereotaxic surgery. During the conditional trials, mice received an intraperitoneal (ip) injection of saline in the morning and were confined to one chamber. After 4 h, mice received an ip injection of cocaine (5 mg/kg) and were confined to the opposite chamber. 24 hours later, on test day, mice were allowed to freely explore all three chambers for 15 minutes and the amount

of time spent in each chamber was recorded and used to assess preference. Data was captured by recording movements with a 4 X 6 photobeam array and analyzed using PAS Software and the Place Preference Reporter.

CONTEXTUAL FEAR CONDITIONING

Contextual fear conditioning (CFC) consisted of a 2-day procedure as described previously (Eagle et al., 2015). For conditioning, mice were placed into an operant chamber for a total of 6 minutes. After 3 minutes in the chamber, three mild electric foot-shocks (0.8 mA, 1 second duration), each separated by a 60 second interval, were administered, and the mouse was removed from the chamber after the 6^{th} minute. 24 hours after conditioning, mice were placed back into the conditioning chamber for 8 minutes and behavior was video recorded. The percentage of time spent freezing was video scored by a blind, independent observer. Freezing was defined as the lack of skeletal movement for periods >1 second.

HUMAN SAMPLES

Post-mortem human brain tissues were obtained from the Douglas Bell-Canada Brain Bank (Douglas Mental Health University Institute, Montreal, Quebec, Canada). Substance use information regarding human cocaine addicts, depression patients, and matched controls can be found in **Table 2** The preservation of tissue proceeded essentially as described (Quirion et al., 1987). Briefly, once extracted, the brain is placed on wet ice in a Styrofoam box and rushed to the Douglas Bell-Canada Brain Bank facilities. Hemispheres are immediately separated by a sagittal cut in the middle of the brain, brain stem, and cerebellum. Blood vessels, pineal gland, choroid plexus, half cerebellum, and half brain stem are typically dissected from the left hemisphere which is then cut coronally into 1 cm-thick slices before freezing. The latter half

Table 2. Substance dependence, toxicology, and use of antidepressant medication in human cocaine addicts, depression patients, and matched control groups

Group (number of subjects)	Additional Substance Dependence	Toxicology at Death (Drugs of Abuse)	Psychiatric Medication (Previous 3 months)
Control (18)	• Alcohol (1/18)	Total = 2/18 • Opioid (1/18) • Ethanol (1/18)	None
Cocaine Dependent (19)	Total = 15/19 • Alcohol (11/19) • Cannabis (3/19) • Opioid (1/19) • Sedative (1/19)	Total = 14/19 • Cocaine (11/19) • Opioid (2/19) • Ethanol (8/19)	Total = 6/19 • SSRI/SNRI (3/19) • Benzodiazepine (5/19) • Classic Antidepressant (2/19) • Antipsychotic (2/19)
Control (11)	None	Ethanol (1/11)	None
Depressed Non-Medicated (14)	• Alcohol (5/14)	Total = 4/14 • Opioid (1/14) • Ethanol (4/14)	• Benzodiazepine (2/14)
Depressed Medicated (13)	• Alcohol (1/13)	Total = 2/13 • Opioid (1/13) • Ethanol (1/13)	Total = 13/13 • SSRI/SNRI (9/13) • Benzodiazepine (6/13) • Classic Antidepressant (5/13) • Tricyclic Antidepressant (1/13) • Antipsychotic (3/13)

Parentheses show number of patients with given condition out of number of total patients in the group. Note that some patients abused multiple additional substances or used multiple medications, thus the total number in a group with an additional condition is given as "Total." SSRI: selective serotonin reuptake inhibitor, SSNI: selective norepinephrine reuptake inhibitor.

cerebellum is cut sagittally into 1cm-thick slices before freezing. Tissues are flash frozen in 2-methylbutane at -40°C for ~60 sec. All frozen tissues are kept separately in plastic bags at -80°C for long-term storage. Specific brain regions are dissected from frozen coronal slices on a stainless-steel plate with dry ice all around. PFC samples come from Brodmann area 8/9, and HPC samples are taken from center mass of the hippocampal formation (**Figure 11**).

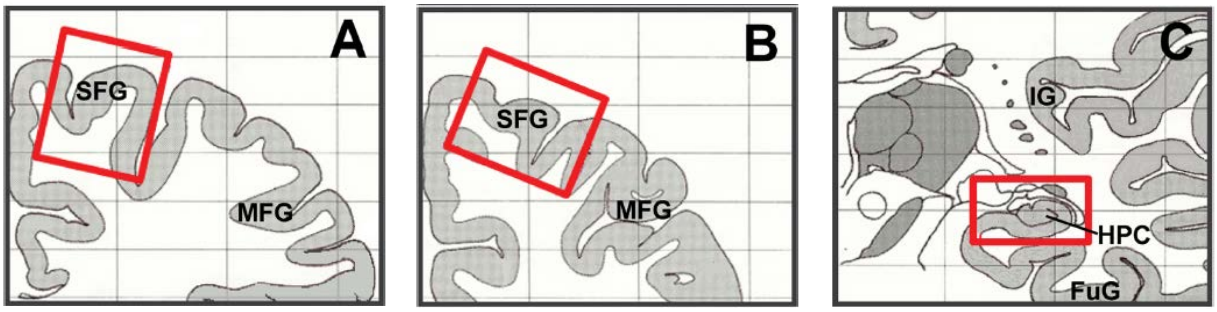

Figure 11. Diagram of dissection regions for human brain samples. Drawings represent anterior (A) and posterior (B) coronal sections of human brain used for dissection of prefrontal cortex samples, and (C) hippocampus (HPC) samples. Red boxes highlight areas of dissection. SFH: superior frontal gyrus; MFG: middle frontal gyrus; IG: insular gyrus; FuG: fusiform gyrus. (Gajewski et al., 2016)

IMMUNOFLUORESCENCE

Mice were perfused transcardially with ice-cold PBS followed by 10% formalin. Brains were post-fixed for 24 hours in 10% formalin, and cryopreserved in 30% sucrose overnight, and then sliced into 35µm sections. Immunofluorescence was performed using anti-FosB primary antibody (FosB 5G4, 1:1000, #2251S, Cell Signaling Technologies) and corresponding secondary antibody (Donkey anti-rabbit Cy5, 1:200, 711-175-152, Jackson Immunoresearch). Fluorescent images were visualized on an Olympus FluoView 1000 filter-based laser scanning confocal microscope. Immunofluorescence intensity per cell was assessed using NIH ImageJ software.

IMMUNOHISTOCHEMISTRY

Mice were perfused and brains sliced as above. Immunohistochemistry was performed using anti-FosB primary antibody (FosB, 1:1000, abcam, ab11959), and secondary (biotinylated donkey anti-mouse, 1:1000, BA-1000, Vector Laboratories) and visualized by 3,3'-diaminobenzidine (DAB) staining (Vector Laboratories). Cells were counted by a blind, independent observer, and also assayed using the Nikon Nis Element Imaging Software version 4.60 – cell counts were assayed using a threshold of 6000.

MICE AND TREATMENT

Male 7-week old C57BL/6J mice were group housed 4-5 per cage in a 12 h light/dark cycle and were provided food and water *ad libitum*. All experiments conducted were approved by the Institutional Animal Care and Use Committee at Michigan State University. Mice were habituated in our facility for at least 5 days before experimentation, and all tests were conducted during the light cycle. For ChIP, Immunohistochemistry, and Western blot experiments, mice received daily ip injections of either saline or cocaine (20 mg per kg) and placed into a novel environment

(cocaine CPP chambers were used for novel environments) for 30 minutes for 10 consecutive days, and were sacrificed 1 or 24 hours after the last injection (ChIP, Western blot: 1 hour; Immunohistochemistry: 24 hours).

NOVEL OBJECT RECOGNITION

Novel object recognition (NOR) was assessed using a 3-day paradigm as described previously (Eagle et al., 2015). Mice were first habituated to the open-field (OF) apparatus, which consisted of a custom-made, square white foamed polyvinylchloride box (38 X 38 X 35 cm), for 1 hour, and OF activity was recorded at this time (see below for full method). If the mice had undergone stereotaxic surgery for viral expression, the habituation occurred 48 hours after the surgery. Twenty-four hours later, two similar objects were placed at adjacent corners of the square apparatus, and animals were allowed to explore the apparatus for 30 min. Object pairs consisted of metal and plastic objects, including Lego blocks, miniature action figures, spark plugs, and metal knobs. Mice did not show consistent biases for any one object over another. The following day, mice were tested for NOR. One object was removed and replaced with another dissimilar object, and mice were allowed to freely explore the apparatus for 5 min. Behavior was video recorded, and time spent in the 7 cm square corners around the objects was assessed. Time spent investigating an object was measured by a blind, independent observer. Investigation of an object was defined as the nose of the mouse pointing toward the object, no less than 2 cm away from the object, with active sniffing (Leger et al., 2013). If the mouse displayed climbing behavior on the object, but was not actively pointing its nose towards it, this was not counted as investigative behavior.

OPEN FIELD

Open field analysis was performed on the first day of the NOR protocol. Briefly, animals were

placed in an open box as described above, and time spent within the center of the box (the center starts approximately 9.5cm from the edge of the wall), distance moved, and velocity were measured for 1 hour. Activity was video recorded with a digital CCD camera connected to a computer running automated video tracking software package (Clever Sys).

STEREOTAXIC SURGERY

Stereotaxic surgery was performed to inject viral vectors into the dorsal or ventral hippocampus of adult male mice. Thirty-gauge needles (Hamilton Company) were bilaterally placed at the following coordinates for dorsal hippocampus: 10º angle, -2.2 mm anteroposterior (AP), ±2.5 mm mediolateral, virus was infused (0.6µl) separately (0.3µl/infusion) over 3 min periods at two sites: -2.1 mm dorsoventral (DV) and -1.9 mm DV; coordinates for ventral hippocampus: 3º angle, -3.2 mm AP, ± 3.4 mm mediolateral, virus was infused (0.5µl) over 5 min period at one site: -4.8 DV. The needles remained at the injection site for 5 min after infusion to allow for diffusion of the viral particles. Previously validated (Robison et al., 2013b; Heller et al., 2014a) viral vectors included the following: herpes simplex virus (HSV) expressing GFP alone (HSV-GFP) or HSV expressing GFP and *FosB*-ZFP-G9a (HSV-*FosB*-ZFP-G9a). HSV-GFP and HSV-*FosB*-ZFP-G9a were obtained from the Massachusetts Institute of Technology Viral Core Facility.

WESTERN BLOTTING

Brains were extracted rapidly on ice and then sliced into 1 mm sections, and the dorsal hippocampus, ventral hippocampus, and ventral CA1 projection area were removed with a 12-gauge punch and frozen immediately on dry ice. Samples were then processed for SDS-PAGE and transferred to PVDF membranes for Western blotting with chemiluminescence. Blots were

probed for FosB (5G4; 1:500; Cell Signaling Technology), H3K9me2 (1:1,000, Millipore), and total H3 (1:6000, abcam) and assayed for total protein using Swift Membrane Stain (G Biosciences). Band intensity was quantified using NIH ImageJ software.

EPIGENETIC MECHANISMS OF COCAINE-MEDIATED HIPPOCAMPAL ΔFOSB INDUCTION

INTRODUCTION

The hippocampus is essential for the formation of drug-related memories, specifically drug-environment associations, and the establishment of these associations requires differential activation of the dorsal and ventral regions of the hippocampus (Robbins et al., 2008). The distinct functions of these regions appear to underlie multiple aspects of addiction, like relapse and reward, which can be modeled in rodents. Chronic exposure to cocaine alters the function of hippocampal cells, including plasticity of hippocampal synapses, a critical factor in learning (Thompson et al., 2002; Perez et al., 2010; Adinoff et al., 2015). This plasticity requires active changes in chromatin structure and gene transcription (Walton et al., 1999; Kennedy et al., 2016; McEwen, 2016), and exposure to cocaine leads to the differential expression of hundreds of genes in the hippocampus (Krasnova et al., 2008). Previous studies have focused on cocaine-mediated epigenetic changes in the nucleus accumbens (NAc), a component of the brain's reward circuity, and have identified numerous changes in expression and function of histone modifying enzymes, such as histone deacetylases and histone methyltransferases (Kalda and Zharkovsky, 2015; Walker et al., 2015). One in particular, a lysine methyltransferase called G9a, is decreased in NAc by repeated cocaine exposure leading to a decrease in the repressive histone modification, lysine nine dimethylation on histone H3 (H3K9me2), and this decrease is necessary for plasticity changes in the NAc and subsequent cocaine-related behavior (Maze et al., 2010; Benevento et al., 2015). Specific epigenetic modifications are also important for maintaining normal hippocampal function and plasticity, indicating a potential mechanism by which cocaine may cause changes in plasticity gene expression (Sweatt, 2013; Srivas and Thakur, 2016). Thus, in order to delineate the cocaine-dependent changes in hippocampal cell function that may underlie critical aspects of addiction, we must determine the hippocampal transcriptional and epigenetic

factors engaged by cocaine and their effects on drug responses.

Chronic exposure to drugs of abuse evokes long-term changes in synaptic morphology and function in the hippocampus (Keralapurath et al., 2014), implicating stable regulation of gene expression. ΔFosB is a stable transcription factor induced by chronic stimuli and studied extensively in the context of addiction (Robison and Nestler, 2011). ΔFosB is a splice variant originating from the *FosB* gene and is uniquely stable due to exclusion of two degron domains (Carle et al., 2007). ΔFosB is induced in many brain regions in response to chronic stimuli, including drugs of abuse (Perrotti et al., 2008), and study of the mechanism of its induction in the NAc has recently focused on changes in epigenetic factors, specifically H3K9me2 via the enzyme G9a (Nunez et al., 2010; Damez-Werno et al., 2012). Additionally, much of what we know about the target genes of ΔFosB comes from addiction and depression studies in the NAc, and many of those NAc target genes, such as *CaMKIIα*, *GRIA2*, and *Cdk5,* have a major role in regulating synaptic plasticity in the hippocampus (Kelz et al., 1999a; Chen et al., 2000; Robison et al., 2013b). Though it has long been known that ΔFosB is induced in dorsal hippocampus by chronic exposure to drugs or stress (Perrotti et al., 2004; Perrotti et al., 2008), its critical role in hippocampal synaptic structure and spatial learning were only recently reported (Eagle et al., 2015). Here, we investigate the epigenetic mechanism of ΔFosB induction in hippocampus by cocaine.

RESULTS

Induction of ΔFosB in hippocampal subregions:

Induction of ΔFosB has been observed in the dorsal hippocampus in response to drugs of abuse such as morphine and cocaine (Perrotti et al., 2008). However, there has been neither quantification of this induction nor comparison between dorsal and ventral hippocampus. Therefore, we exposed mice to a novel environment paired with saline or cocaine (20 mg/kg)

for 30 minutes/day for 10 days and compared the protein levels of FosB isoforms in dorsal and ventral hippocampus using Western blot. Additionally, in another cohort, we separated the ventral CA1 subregion from the rest of the ventral hippocampus, as this area has been identified as functionally relevant in drug-related behaviors. We thus enriched for hippocampal cells that project to other regions of the brain, such as the amygdala and NAc, projections critical for emotional learning and drug responses (Cooper et al., 2017). We found no significant changes in whole dorsal hippocampus FosB and ΔFosB protein levels between cocaine- and saline-treated mice (**Figure 12A**). Whole ventral hippocampus revealed a trend towards an increase in FosB and ΔFosB (**Figure 12B**). It was only when the ventral CA1 was enriched that there was a significant increase in FosB protein and a trend toward increase in ΔFosB (**Figure 12C,D**). While the rest of the ventral hippocampus, a punch containing mostly DG (vDG) revealed no significant changes in either FosB isoform (**Figure 12C**).

For the same samples I isolated mRNA to determine expression levels of each isoform and whether transcriptional regulation was changing in response to cocaine. There was significantly more FosB and ΔFosB mRNA found in dorsal hippocampus after chronic cocaine exposure (**Figure 13A**) However, no change in either isoform's mRNA was found in the ventral DG, while a trend towards an increase was found when the ventral CA1 was enriched for (**Figure 13B,C**).

In order to better assess changes in each of the specific subregions of the hippocampus, we completed the same protocol as above, but perfused the animals 24 hours after their last exposure to either saline or cocaine and took coronal slices to perform immunohistochemistry or immunofluorescence. As the antibody recognizes all FosB isoforms, we specifically quantified the stable ΔFosB isoform by sacrificing 24 hours after the last drug exposure. We found an increase in the number of ΔFosB-positive cells in the CA1 subregion of dorsal hippocampus after chronic

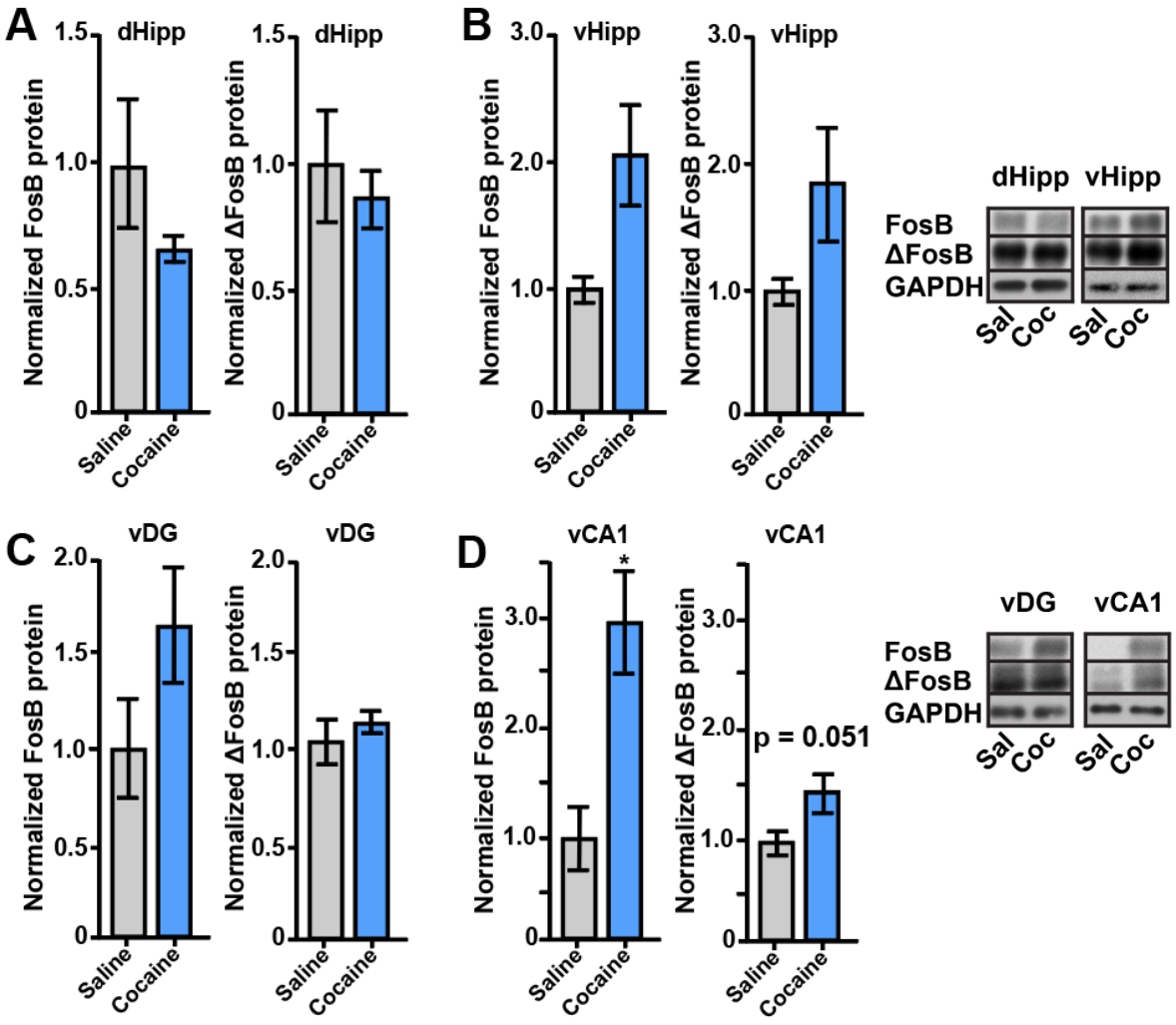

Figure 12. Only ventral CA1 displays a significant cocaine-mediated induction of FosB isoforms. A. Mice were sacrificed 1 hour after their last exposure to saline or cocaine paired with a novel environment. Whole dorsal hippocampus (dHipp) punches revealed no changes in either FosB or ΔFosB after Western blot analysis. **B.** Whole ventral hippocampus (vHipp) punches revealed a trend towards an increase in FosB and ΔFosB (p=0.072, p=0.089, respectively). **C-D.** In a separate cohort, vHipp was split into two separate punches, one to take the DG region, which did not show any significant changes in FosB isoform protein after cocaine exposure (**C**), and one to enrich for the ventral CA1, which is the source of the cocaine-mediated induction of FosB and ΔFosB (vCA1, **D**) (n=8/group). Right of the graphs are representative bands from Western blots for FosB, ΔFosB, and GAPDH.

cocaine exposure (**Figure 14A-B**), but no significant changes in the dorsal DG or CA3 (**Figure 14B**). In ventral hippocampus CA1 subregion there was also a significant increase in the number of ΔFosB-positive cells, with no change found in DG and CA3 (**Figure 14C-D**). We also examined the intensity of ΔFosB fluorescence signal per neuron in the dorsal CA1 and found an increase in fluorescence intensity per cell (**Figure 15**). This suggests that drugs of abuse selectively induce ΔFosB in specific hippocampal subregions in both the number of neurons expressing ΔFosB and the amount of ΔFosB being produced, which may contribute to the formation of the strong association between drugs and environment.

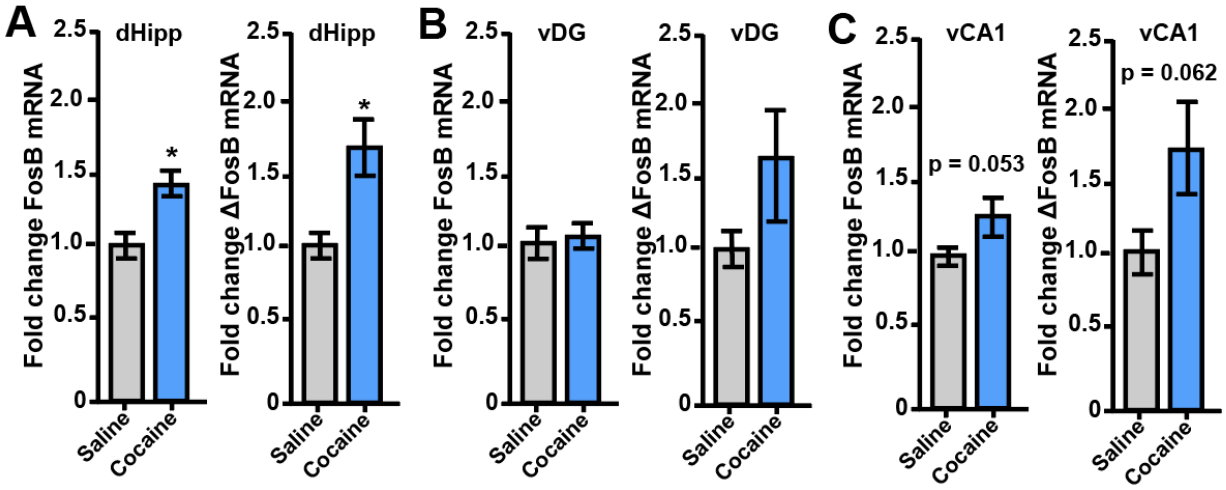

Figure 13. Only dorsal hippocampus displays increased expression levels of FosB isoform mRNA. A. Mice were sacrificed 1 hour after their last exposure to saline or cocaine paired with a novel environment. Whole dorsal hippocampus (dHipp) punches revealed significant increases in the transcript levels for FosB and ΔFosB after qPCR analysis. **B.** Ventral dentate gyrus (vDG) punches revealed no changes in full length FosB or ΔFosB mRNA. **C.** In punches specifically for the ventral CA1, there is a trend towards an increase in both FosB and ΔFosB mRNA (p=0.052, and p=0.062, respectively). (n=8/group)

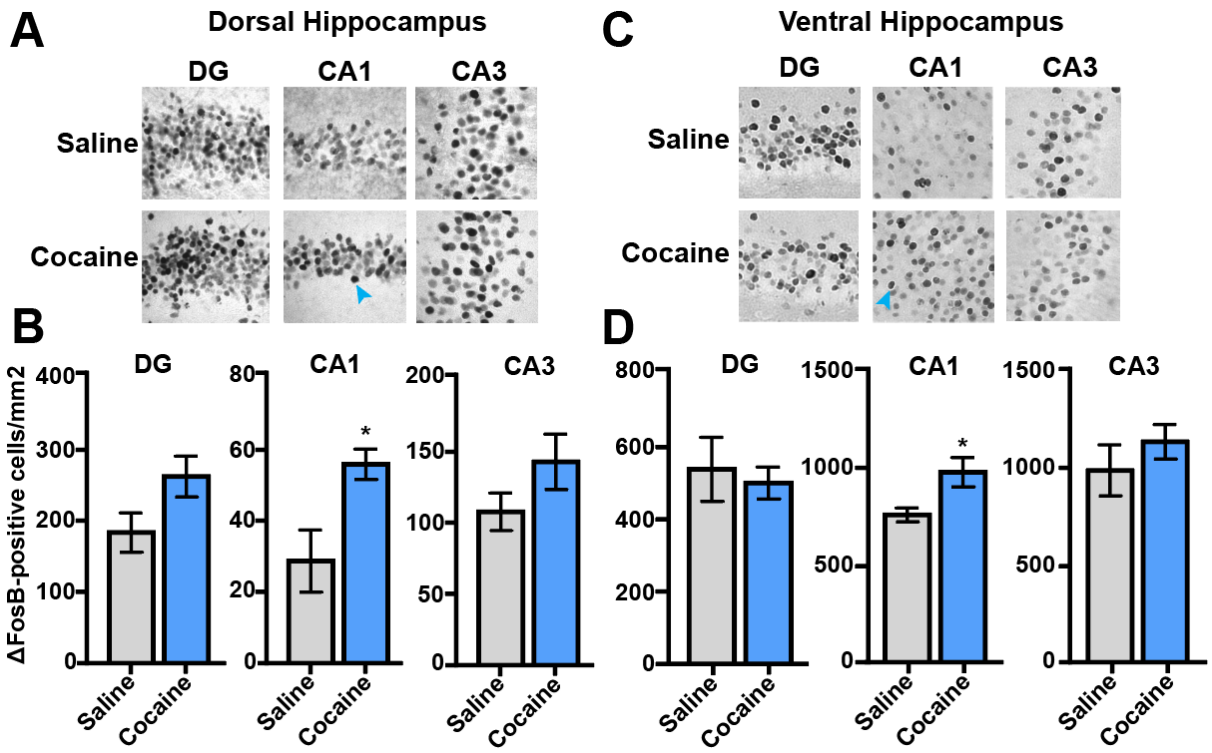

Figure 14. Induction of ΔFosB isoforms in subregions of the hippocampus by chronic cocaine exposure. A. Representative 40X images of coronal sections (dorsal hippocampus) stained for ΔFosB from mice given saline or cocaine in a novel environment. Arrowheads indicate ΔFosB+ cells. **B.** Quantitation of ΔFosB+ neurons in the dorsal DG (dentate gyrus), CA1, and CA3, with significant increase in number of ΔFosB+ cells found in CA1 after cocaine exposure (n=5, *p < 0.05). **C.** Representative 40X images of ventral hippocampus stained for ΔFosB from mice given saline or cocaine in a novel environment. **D.** Quantitation of ΔFosB+ neurons in the ventral DG, CA1, and CA3.

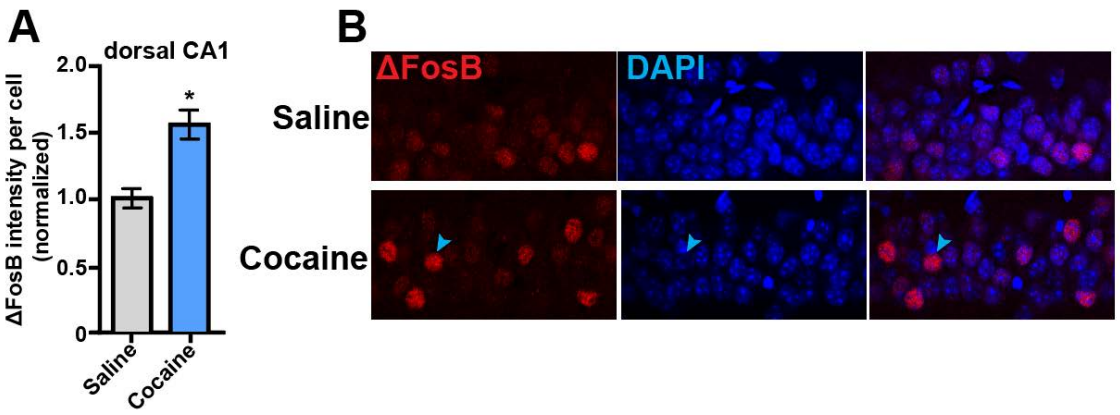

Figure 15. Cocaine-mediated induction of ΔFosB within the dorsal CA1. A. Quantified ΔFosB intensity per cell in dorsal hippocampus CA1 neurons via immunofluorescence after chronic cocaine exposure (n = 25-60, *p < 0.05). **B.** Representative images with ΔFosB in red and DAPI in blue. Arrowheads indicate one cell in which ΔFosB is induced at high levels following chronic cocaine exposure.

Epigenetic modifications at the FosB gene in response to chronic cocaine exposure:

Induction of ΔFosB in the NAc after cocaine exposure appears to be mediated in part by histone post-translational modifications at the *FosB* gene – specifically a decrease in the repressive histone modification H3K9me2 (Damez-Werno et al., 2012). Therefore, we assessed whether cocaine-mediated hippocampal ΔFosB induction was also associated with changes in histone modifications. We conducted chromatin immunoprecitipation (ChIP) for H3K9me2 and found a cocaine-mediated decrease in the histone mark at multiple points along the *FosB* promoter and gene in hippocampus (**Figure 16**), consistent with findings in the NAc. This suggests an epigenetic mechanism for cocaine-mediated hippocampal ΔFosB induction: the *FosB* gene is typically enriched with the repressive histone modification, H3K9me2, but after cocaine exposure, this mark is reduced. This could occur because the enzyme that catalyzes the modification, G9a, is no longer targeting the *FosB* gene, or because H3K9me2 is actively removed in response to cocaine.

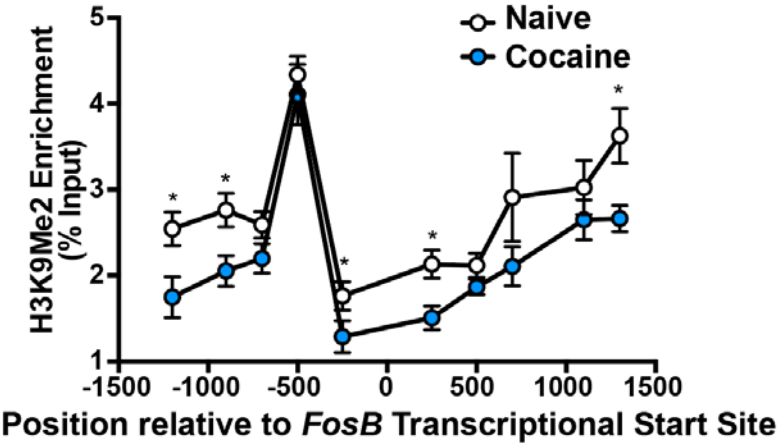

Figure 16. Histone post-translational modifications at the *FosB* gene influence cocaine-mediated ΔFosB induction. Mice were injected with cocaine (n = 8, 20 mg/kg, i.p.) or saline (n=8) and placed in a novel environment for 30 minutes/d for 10 d. Chromatin immunoprecipitation for H3K9me2 was conducted on microdissected dentate gyrus with primers spanning the *FosB* promoter and gene. Significant decreases in H3K9me2 enrichment were found at multiple sites along the *FosB* gene (*p < 0.05).

DISSCUSSION

This chapter describes the first quantitative evidence for a cocaine-mediated induction of FosB isoforms within specific subregions of the hippocampus and altered histone posttranslational modifications associated with this induction. The induction of full-length FosB and ΔFosB in the CA1 subregion of both dorsal and ventral hippocampus demonstrates the unique importance of this subregion in drug-related learning. The CA1 subregion is the primary output of the hippocampus, and also integrates information from the entorhinal cortex and the CA3 subregion (Barrientos and Tiznado, 2016). The dorsal CA1 is especially important for contextual encoding and contextual-dependent retrieval compared to the other subregions (Ji and Maren, 2008; Zhu et al., 2014). Ventral CA1 has also been identified as an important regulator of the NAc, a key structure in reward circuitry, suggesting that the CA1 may be uniquely positioned to influence drug-related behaviors (Zornoza et al., 2005). While dorsal and ventral hippocampus have distinct outputs and functions, it appears that drug-mediated learning induces expression of FosB isoforms in both regions.

Changes in the molecular profile of the hippocampus after cocaine exposure suggest that drugs of abuse engage the memory systems of the brain in addition to reward circuitry. The hippocampus is essential in the formation of associations concerning cues and events in a drug context (Fuchs et al., 2005). Furthermore, these drug-associated stimuli can elicit memory of the drug, which can induce craving and relapse even during abstinence (Gawin and Kleber, 1986). Memories of cocaine-associated stimuli are extremely persistent and resistant to extinction, thus cocaine addiction can be considered a learning and memory disease (Kutlu and Gould, 2016). Additionally, 30% of cocaine-addicted individuals display global cognitive impairment similar to that seen in diseases affecting hippocampal function, further implicating the hippocampus as a key region affected by cocaine use (Vonmoos et al., 2013; Vonmoos et al., 2014). Therefore, molecular and functional changes in the hippocampus that occur in response to cocaine exposure

may give us critical insight into the establishment of these drug-associated memories.

Our initial Western blots did not quite reveal the previously observed ΔFosB induction in dorsal hippocampus after cocaine exposure (Perrotti et al., 2008), but increases in both FosB and ΔFosB became robust when ventral CA1 was specifically targeting for dissection (**Figure 12**). To further investigate this potential subregion-specific induction, we chose to investigate each subregion individually using immunohistochemistry (Perrotti et al., 2008). However, Western blotting did allow us to interrogate the individual FosB isoforms by molecular weight. When mice were sacrificed one hour after their last drug exposure, we did indeed see an induction of full-length FosB and ΔFosB (to a lesser degree) in ventral hippocampus samples enriched for the CA1 subregion. Additionally, transcript levels of FosB and ΔFosB were increased in whole dorsal hippocampus without subsequent increases in proteins levels, suggesting that chronic cocaine exposure mediates active transcription of the *FosB* gene in this region, but that additional regulation at the translational or proteolytic level may prevent overall differences in protein. Further examination using immunohistochemistry revealed increases in ΔFosB-positive cells in the dorsal and ventral CA1. The dorsal CA1 subregion makes up a very small portion of the whole dorsal hippocampus, which explains the lack of induction of FosB/ΔFosB seen in the Western blot analysis. Increases in the number of ΔFosB+ cells in the dorsal CA1 has also been observed after mice go through spatial learning via the Morris Water Maze, and ΔFosB overexpression induces dendritic spine formation in the dorsal CA1 subregion (Eagle et al., 2015). This suggests that both general and drug-related learning may be mediated by similar mechanisms, which may lead to synaptic changes specifically in the CA1. Additionally, our control saline mice experienced a similar environment to the cocaine exposed mice, and being exposed to the same environment after even an injection of saline may allow for a mild amount of conditioned learning or spatial awareness, suggesting that the exposure to cocaine itself may cause an even larger number of cells to express ΔFosB when paired with a novel context.

On the other hand, induction of ΔFosB in the ventral CA1 subregion may be indicative of increased

activity of those neurons that project to the NAc. Exposure to repeated cocaine and subsequent stimulation of the ventral hippocampus enhances dopamine efflux in the NAc, suggesting a maladaptive response to cocaine exposure occurring in these projection specific neurons (Barr et al., 2014). Additionally, ventral hippocampal stimulation induces cocaine seeking in a self-administration paradigm even after extinction (Vorel et al., 2001). This, along with the cocaine-mediated increase in ΔFosB+ neurons in the ventral CA1 suggests that cocaine causes downstream synaptic function alterations of these neurons which may facilitate drug-related learning. Furthermore, the increase in ΔFosB was not only found in the number of cells expressing it, but also in the level of expression of ΔFosB per cell after cocaine exposure, as measured by immunofluorescence intensity. Together, the increases seen throughout the CA1 subregion suggest that those cells expressing ΔFosB are doing so at a higher level when mice are exposed to cocaine, which may be directly related to the cocaine-mediated changes in histone posttranslational modifications at the *FosB* gene.

It is possible that cocaine increases the transcription of FosB isoforms by decreasing the repressive histone modification, H3K9me2, a hypothesis I will test in the next chapter. This histone modification is written by the histone methyltransferase, G9a (**Figure 17**), and typically recognized by the silencing factor, heterochromatin protein 1 (HP1), via a chromodomain. HP1 also contains a chromo-shadow domain to bind other suppressors, like the histone methyltransferase, suppressor of variegation 3-9, which suggests that it may be a mechanism by which H3K9 methylation can spread once HP1 binds (Elgin and Reuter, 2013). HP1 can also recruit DNA methyltransferases (Honda et al., 2012), as well as form homodimers through its chromo-shadow domain, which can establish a crosslinking effect between adjacent nucleosomes, effectively blocking RNA polymerase II access, all of which appear to add to th repressive effects of HP1 binding (Canzio et al., 2011; Eissenberg and Elgin, 2014). Removal of the dimethyl mark is facilitated by lysine-specific demethylase 1A (LSD1), which can also remove H3K4me2 (Shi et

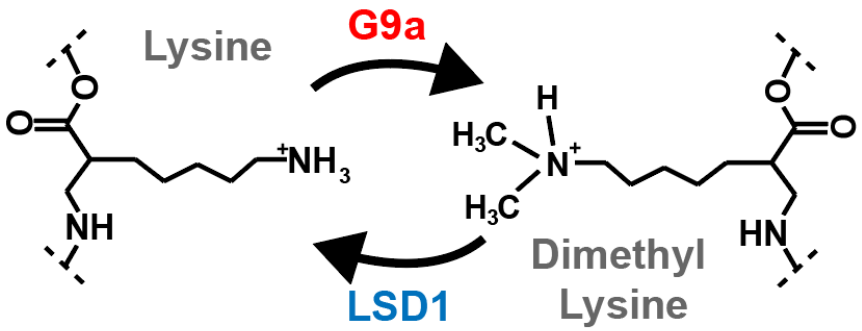

Figure 17. Depiction of lysine methylation/demethylation. G9a is the histone methyl-transferase that facilitates the addition of two methyl (CH_3) groups onto lysine 9 of histone H3 (H3K9). The demethylation of H3K9me2 is accomplished by LSD1 (lysine-specific demethylase 1)

al., 2004; Metzger et al., 2005). Without H3K9me2 present at the *FosB* gene in high levels, transcriptional machinery has a higher likelihood of binding, increasing the expression of FosB isoforms in more cells, and at higher levels. H3K9me2 was also found to be decreased at the *FosB* gene in NAc of rats that were exposed to chronic cocaine and a withdrawal period, regardless of exposure to a challenge dose of cocaine (Damez-Werno et al., 2012). H3K4me3 and H3K27me3, permissive and repressive histone marks respectively, were also examined in the same rats, but no changes were found, indicating that *FosB* induction may be occurring specifically due to the decrease in H3K9me2 in the promoter region. Together with the data in this chapter, this points towards a potential universal mechanism by which cocaine exposure may mediate the induction of ΔFosB.

The data presented in this chapter provide evidence that cocaine induces ΔFosB in the CA1 subregion of the hippocampus by increasing the number of cells expressing ΔFosB and the level to which they express it. As discussed above, it is possible that this increase is due to a cocaine-mediated decrease in the repressive histone modification, H3K9me2, along the *FosB* gene. Recent work from our group has examined the role of ΔFosB specifically in the ventral CA1 projections to the NAc and found that knockout of the *FosB* gene in this circuit impairs cocaine CPP, indicating that induction of FosB isoforms in this circuit is especially important in cocaine-

related behaviors (Eagle, 2016). Moreover, knockout of *FosB* in ventral CA1 neurons projecting to a separate brain region, the amygdala, did not impair CPP, again demonstrating the necessity of *FosB* expression in the ventral CA1-NAc circuit for this behavior. Overall, these results support a model in which cocaine-environment associations alter histone modifications, inducing the expression of ΔFosB in the CA1 subregion of the hippocampus, which is dynamically regulating drug-related learning.

HISTONE POSTTRANSLATIONAL MODIFICATIONS MEDIATE *FOSB* INDUCTION AND HIPPOCAMPAL-DEPENDENT BEHAVIORS

INTRODUCTION

The role of histone posttranslational modifications in learning and memory, and more recently in mediating cocaine responses, has been well established and discussed in Chapters I, and III. Briefly, histone acetylation contributes to hippocampal spine formation, synaptic plasticity, and subsequent memory formation, and knockdown of a histone deacetylase (HDAC) enzyme results in increased LTP and synapse formation (Guan et al., 2009). Histone methylation has also been implicated in memory formation. After fear training, retrieval of the memory increases the trimethylation of lysine 4 on histone H3 (H3K4me3) in dorsal CA1, but not in the basolateral amygdala, another area of the brain important for fear learning (Webb et al., 2017). On the other hand, cocaine exposure lowers global levels of H3K9me2 in the NAc, which has been shown to impact the *FosB* gene directly (Maze et al., 2010; Damez-Werno et al., 2012). The question remains whether these histone modifications are directly impacting the learning process and are important for hippocampal function.

In this chapter, the effects of inducing histone modifications at the *FosB* gene are explored as it relates to general and drug-related learning. Recent studies have developed tools to manipulate histone modifications in a spatially and temporally specific manner – restricted to a certain gene in one brain region and expressed during a certain time frame. This was piloted by our collaborator, Dr. Elizabeth Heller, who performed studies in which zinc-finger fusion proteins were packaged into viral vectors and could be administered to various brain regions (Heller et al., 2014b). As described in Chapter I, the zinc-finger proteins (ZFP) are artificially designed to bind only a specific genetic locus and are fused to histone modifying enzymes, which allow for direct localization of the enzyme to a specific sequence in the genome. This grants a specificity to the

enzyme that was not established by the previous method of overexpression of histone modifying enzymes. Thus, this chapter will use these previously validated tools to investigate the effects of H3K9me2 on hippocampal *FosB* induction in the context of general and cocaine-related learning.

RESULTS

ZFP-G9a impairs cocaine-mediated hippocampal FosB induction:

The induction of *FosB* after chronic cocaine exposure appears to be facilitated by a decrease in the repressive histone modification, H3K9me2. To determine whether the decrease in H3K9me2 is necessary for cocaine-mediated ΔFosB induction and the subsequent hippocampal-dependent behavioral outputs, we used viral tools that target epigenetic changes to the *FosB* promoter (Heller et al., 2014b). The Herpes Simplex Virus (HSV) encodes for the expression of a ZFP specifically targeting the *FosB* promoter and which is fused to the catalytic domain of the histone methyltransferase, G9a, thus leading to the enrichment of H3K9me2 exclusively along the *FosB* promoter (**Figure 18**). To validate our viral-mediated locus-specific histone modification, we measured ΔFosB immunofluorescence after chronic cocaine exposure and saw a significant decrease in the intensity of ΔFosB signal in neurons transfected with HSV-*FosB*-ZFP-G9a virus compared to HSV-GFP (**Figure 18**). This suggests that enrichment of H3K9me2 at the *FosB* promoter inhibits the cocaine-mediated induction of ΔFosB.

Effects of ZFP-G9a on hippocampal-dependent learning:

As general silencing of ΔFosB transcriptional activity in dorsal hippocampus leads to an impairment of hippocampal-dependent learning and memory tasks, such as Morris Water Maze training and novel object recognition (Eagle et al., 2016), it was necessary to examine whether altering the epigenetic modifications at the *FosB* promoter led to similar impairments. I further investigated any differences between dorsal and ventral hippocampal *FosB* and its role in

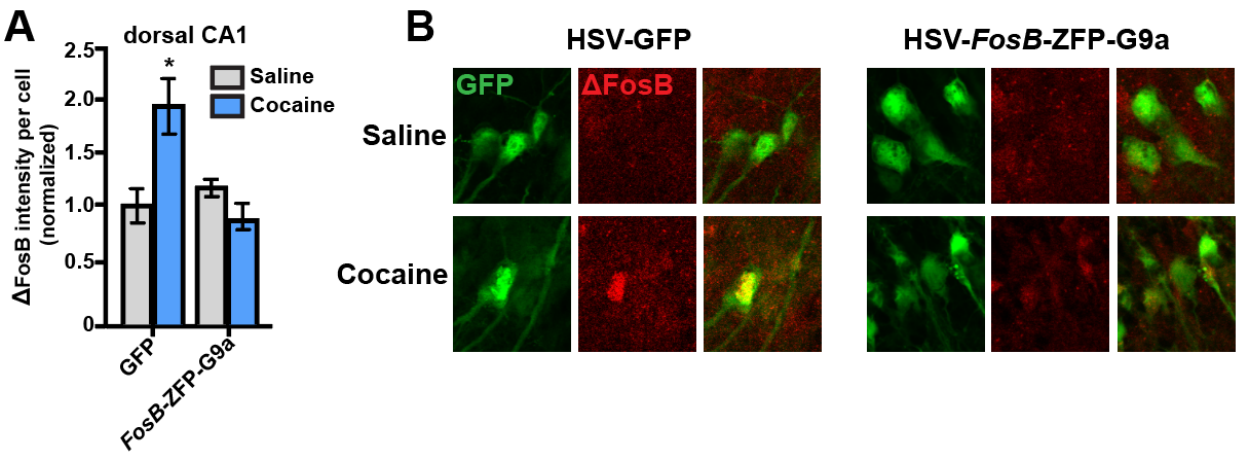

Figure 18. Histone post-translational modifications at the *FosB* gene influence cocaine-mediated ΔFosB induction. A. Quantified ΔFosB intensity per cell in dorsal hippocampus CA1 neurons via immunofluorescence after chronic cocaine exposure with either HSV-GFP or HSV-*FosB*-ZFP-G9a expressed (n = 60-130, *p < 0.05). **B.** Representative images of dorsal CA1 pyramidal neurons with ΔFosB in red and GFP in green.

hippocampal-dependent learning. I injected either HSV-GFP alone or HSV-*FosB*-ZFP-G9a into dorsal hippocampus (**Figure 19**) and saw similar impairments in learning and memory to those previously observed with viral overexpression of ΔJunD, the transcriptional silencer of ΔFosB, in the same region (Eagle et al., 2015). Mice exhibited an impairment in novel object recognition (**Figure 19A**) with no changes in contextual fear conditioning or locomotor activity (**Figure 19B,C**). When the *FosB*-ZFP-G9a virus was targeted to the ventral hippocampus, we also saw impairments in novel object recognition (**Figure 20A**) with no changes in contextual fear conditioning or locomotor activity (**Figure20B,C**). These data suggest that H3K9me2-mediated repression of ΔFosB in the dorsal and ventral hippocampus can impair the formation of memories during hippocampal-dependent behavioral tasks.

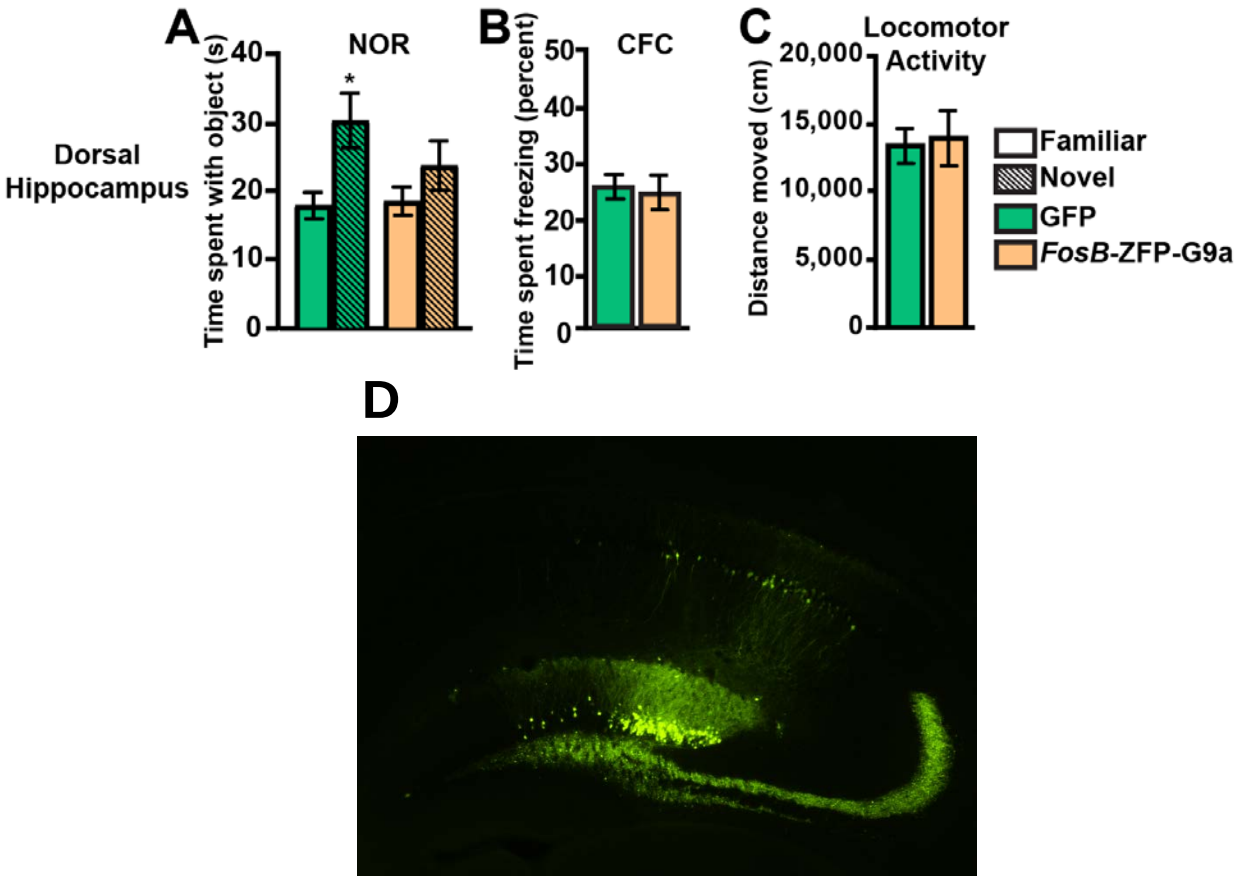

Figure 19. ZFP-G9a in dorsal hippocampus impairs novel object recognition, but not contextual fear conditioning. A. Mice received stereotaxic dorsal hippocampal infusions of HSV-GFP (green, n=8) or HSV-*FosB*-ZFP-G9a (yellow, n=5). GFP mice spent significantly more time with the novel object (striped) than the familiar object (solid) (*$p < 0.05$), whereas *FosB*-ZFP-G9a mice spent an equal amount of time with both objects (novel object recognition, NOR). **B.** Contextual fear conditioning (CFC) freezing behavior after dorsal hippocampus HSV-GFP (n=8) or HSV-*FosB*-ZFP-G9a (n=5). *FosB*-ZFP-G9a mice spent the same amount of time freezing as GFP controls. **C.** Locomotor activity was assessed in dorsal hippocampus expression of HSV-GFP (n=8) or HSV-*FosB*-ZFP-G9a (n=5) and there was no difference in the amount of movement between groups. **D.** Representative image showing GFP expression after virus infusion. Cells in the DG, CA1, CA3 are expressing GFP

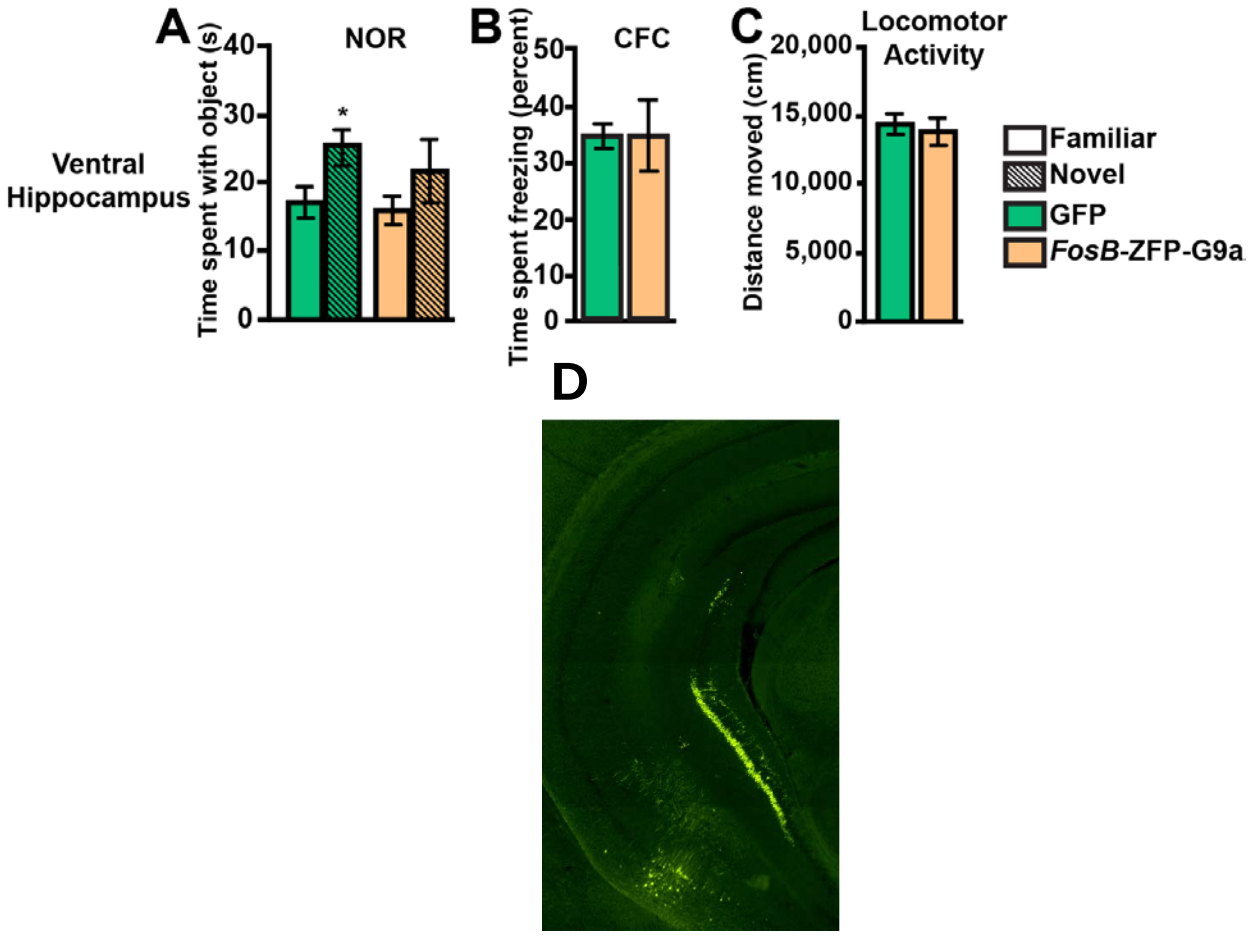

Figure 20. ZFP-G9a in ventral hippocampus impairs novel object recognition, but not contextual fear conditioning. A. Mice received stereotaxic ventral hippocampal infusions of HSV-GFP (green, n=16) or HSV-*FosB*-ZFP-G9a (yellow, n=6). GFP mice spent significantly more time with the novel object (striped) than the familiar object (solid) (*$p < 0.05$), whereas *FosB*-ZFP-G9a mice spent an equal amount of time with both objects (novel object recognition, NOR). **B.** Contextual fear conditioning (CFC) freezing behavior after ventral hippocampus HSV-GFP (n=16) or HSV-*FosB*-ZFP-G9a (n=6). *FosB*-ZFP-G9a mice spent the same amount of time freezing as GFP controls. **C.** Locomotor activity was assessed in ventral hippocampus expression of HSV-GFP (n=16) or HSV-*FosB*-ZFP-G9a (n=6) and there was no difference in the amount of movement between groups. **D.** Representative image showing GFP expression after virus infusion, targeted area is the ventral CA1 subregion which appears at the bottom of the image.

Effects of ZFP-G9a on cocaine-related behavior:

As the above experiments further demonstrate that H3K9me2-mediated repression of hippocampal ΔFosB can prevent learning, I was interested in investigating whether this modification was also important in cocaine-related behaviors, such as cocaine conditioned place preference (CPP). As cocaine facilitates a decrease in H3K9me2 at the *FosB* gene throughout the hippocampus, I hypothesized that increasing this modification with *FosB*-ZFP-G9a expression would prevent normal cocaine CPP by interrupting the ability to form a preference for the cocaine-paired side. Mice were injected with HSV-GFP or HSV-*FosB*-ZFP-G9a into dorsal hippocampus,

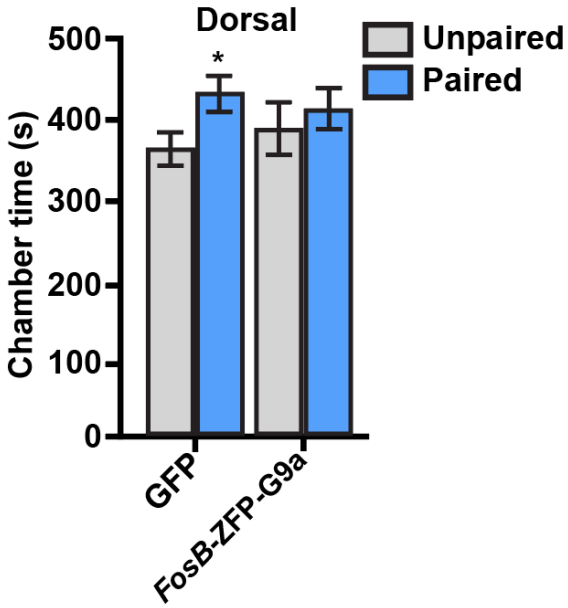

Figure 21. Epigenetic remodeling by *FosB*-ZFP-G9a in dorsal hippocampus impairs cocaine conditioned place preference (CPP). Mice were injected in dorsal hippocampus with HSV-GFP (n=16) or HSV-*FosB*-ZFP-G9a (n=6) and were subjected to cocaine CPP at a moderate dose (5 mg/kg, i.p.). Control mice formed a significant preference for the cocaine-paired chamber, which was blocked by the dorsal hippocampus injection of HSV-*FosB*-ZFP-G9a (*$p < 0.05$).

allowed to recover for 24 hours, and then exposed to a cocaine- or saline-paired chamber for two days. Mice that received *FosB*-ZFP-G9a into the dorsal hippocampus had an impairment in cocaine CPP (**Figure 21**). It will be critical to now examine the effects of the same viruses in ventral hippocampus.

DISSCUSSION

This chapter demonstrates the use of a novel viral tool allowing for the manipulation of hippocampal histone posttranslational modifications in a locus-specific manner. The induction of ΔFosB in the CA1 subregion after chronic cocaine exposure was eliminated upon viral overexpression of the *FosB*-ZFP-G9a fusion protein. This indicates that the effect of enriching H3K9me2 at the *FosB* promoter was enough to suppress expression. Repression of the *FosB* gene was most likely accomplished by the binding of heterochromatin protein 1 (HP1) to the specific H3K9me2 modification. This effect led to subsequent impairment in hippocampal-dependent learning and memory, as seen in novel object recognition.

While novel object recognition has been contested as a hippocampal-independent task, as it is a non-spatial form of learning, evidence from lesion studies, as well as from specific inhibition of dorsal CA1, clearly indicate the necessity for the hippocampus in performance of this task (Broadbent et al., 2004; Cohen et al., 2013). Additionally, silencing of dorsal hippocampal FosB/ΔFosB activity by overexpression of a dominant negative version of its binding partner, ΔJunD, impaired novel object recognition, contextual fear conditioning, temporally dissociative passive avoidance, and Morris water maze training (Eagle et al., 2015). The silencing also led to a decrease in dendritic spine formation in dorsal CA1, suggesting that ΔFosB plays an important role in regulating synaptic plasticity changes (Eagle et al., 2015). Therefore, the silencing of *FosB* via the specific enrichment of H3K9me2 at the promoter in dorsal hippocampus was predicted to

impair learning and memory. Interestingly, use of ΔJunD to silence FosB/ΔFosB activity impaired contextual fear conditioning, causing the mice to freeze less when exposed to the fear context (Eagle et al., 2015), while use of the *FosB*-ZFP-G9a virus did not result in impaired fear learning. This may have occurred due to the nature of overexpressing proteins. Overexpression of ΔJunD causes an overwhelming amount of this protein to be made and bind to FosB isoforms throughout the neuron. Overexpression of the ZFP-G9a does not unnecessarily affect anything other than the *FosB* gene, making it a subtler effect on the system.

While it does appear that the dorsal and ventral hippocampus are unique in their function, lesioning studies examining both regions suggest that they are necessary for spatial learning, although perhaps to varying degrees (Ferbinteanu et al., 2003). However, it is also possible that the task of novel object recognition may be eliciting an emotional/rewarding response. As the home cages of these mice are not enriched in any manner, aside from nesting material, exposure to a novel environment with novel objects may provide a rewarding experience. Thus, the impaired novel object recognition after expression of the *FosB*-ZFP-G9a in the ventral CA1 subregion suggests that ΔFosB may in part be mediating the role of the ventral hippocampus in recognition memory, or that ΔFosB in those neurons projecting from the ventral CA1 to NAc has a role in reward-related memory.

The hippocampus, as it is essential in the formation of contextual and spatial memories, has been previously examined in cocaine CPP, and lesioning experiments reveal that dorsal, but not ventral, hippocampus is necessary for cocaine CPP (Meyers et al., 2003). However, lesion studies and changes to locus-specific epigenetic modifications are extremely different in their effects. Furthermore, recent work from our group has revealed that use of the transcriptional silencer of ΔFosB, ΔJunD, impairs cocaine CPP when virally transduced into the dorsal and ventral hippocampus (**Figure 22**) (Eagle, 2016). Similarly, use of *FosB*-ZFP-G9a in the dorsal hippocampus blocks cocaine CPP, and we are currently examining effects in the ventral

hippocampus. As mentioned briefly in Chapter III, our lab found that ΔFosB in the ventral CA1-NAc projections, but not ventral CA1-amygdala was necessary to form cocaine CPP, further solidifying the importance of ventral CA1 ΔFosB in cocaine-related memories and behaviors (Eagle, 2016). Thus, another important future experiment may be to express HSV *FosB*-ZFP-G9a specifically in the ventral CA1-NAc projection and determine any effects on cocaine CPP.

The studies in this and the previous chapter provide strong evidence for the importance of histone modifications at the *FosB* gene in hippocampal induction of ΔFosB by cocaine and the critical role of this epigenetic regulation in subsequent drug responses and drug-associated memories. However, in order to provide a clinically relevant context for the interpretation of this work, it is

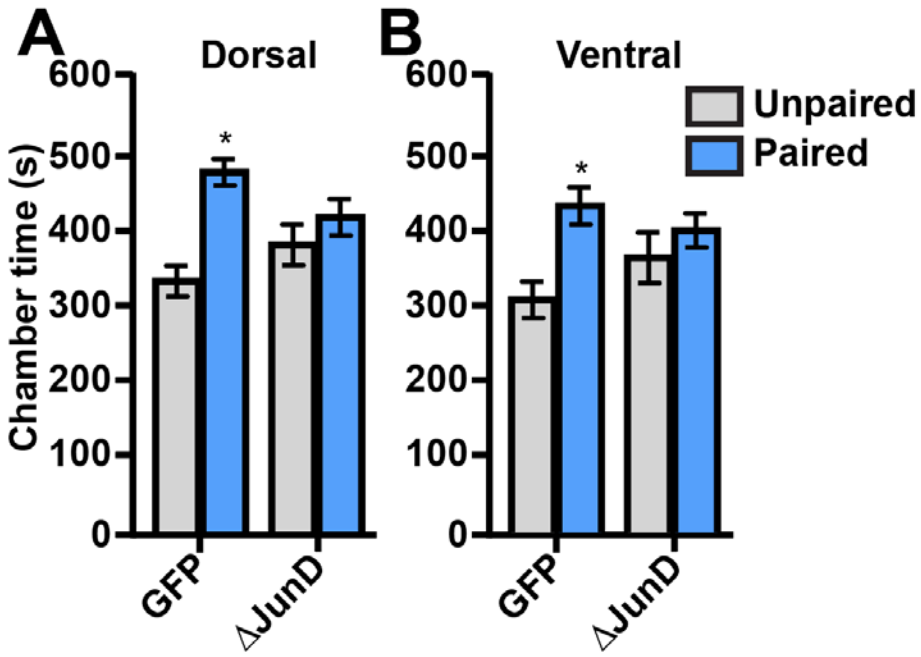

Figure 22. Silencing of FosB transcriptional activity in hippocampus impairs cocaine conditioned place preference (CPP). **A.** Mice were injected in ventral hippocampus with AAV-GFP or AAV-ΔJunD and after 2 weeks were subjected to cocaine CPP at a moderate dose (5 mg/kg, i.p.). Control mice formed a significant preference for the cocaine-paired chamber, which was blocked by the ventral hippocampus injection of AAV-ΔJunD (n=8, *p < 0.05). **B.** Mice were injected in dorsal hippocampus with AAV-GFP or AAV-ΔJunD and after 2 weeks were subjected to cocaine CPP at a moderate dose (5 mg/kg, i.p.). Control mice formed a significant preference for the cocaine-paired chamber, which was blocked by the dorsal hippocampus injection of AAV-ΔJunD (n=8, *p < 0.05).

necessary to determine whether ΔFosB is present in the human hippocampus and whether its expression is regulated by drug addiction. In the following chapter, I will address these questions using human post-mortem samples.

DIFFERENTIAL EXPRESSION OF FOSB PROTEINS AND POTENTIAL TARGET GENES IN SELECT BRAIN REGIONS OF ADDICTION AND DEPRESSION PATIENTS

Note: Figures and parts of the text were previously published in (Gajewski et al., 2016). *Permission has been obtained from the publishers to include these materials in the current publication.*

Author contributions:
Conceived and designed the experiments: Gajewski, Robison
Performed the experiments: Gajewski, Robison
Analyzed the data: Gajewski, Robison
Contributed reagents/materials/analysis tools: Turecki
Wrote the paper: Gajewski, Robison

INTRODUCTION

As described in the previous chapters, alterations in gene expression in the NAc and the brain regions that exert top-down control over NAc function, like prefrontal cortex (PFC) and hippocampus, have been implicated in the pathogenesis of addiction and depression by many studies in both model organisms and in post-mortem human brain (Robison and Nestler, 2011; Fass et al., 2014; Keralapurath et al., 2015; Loureiro et al., 2015; Menard et al., 2015). Many current treatments for depression operate through chronic enhancement of serotonergic and/or dopaminergic signaling, and virtually all drugs of abuse affect dopamine signaling. Moreover, addiction and depression are highly comorbid, with nearly one third of patients with major depressive disorder also having substance use disorders and comorbidity yielding higher risk of suicide and greater social and personal impairment (Davis et al., 2008; 2010a). Taken together, these data suggest that chronic maladaptations in the mesolimbic dopamine circuit and connected structures may underlie both addiction and depression, and that changes in gene expression are likely to play a crucial role in these maladaptations.

As both depression and addiction develop over time and may be linked to chronic exposure to

stress and/or drugs of abuse (Cadet and Bisagno, 2015; Tafet and Nemeroff, 2015), and because typical antidepressants that target serotonergic and dopaminergic signaling require weeks of treatment to be effective (Blier, 2003), it seems likely that the pathogenesis of these diseases and the mechanisms of their treatment may be linked to *long-term* changes in gene expression. Such changes could result from transcription factors, particularly stable transcription factors induced by chronic neuronal activation. Rodent models implicate ΔFosB expression in NAc in both addiction (Kelz et al., 1999a; Robison et al., 2013b) and depression (Covington et al., 2010; Robison et al., 2013a), and recent studies suggest a role for ΔFosB in these diseases in PFC (Vialou et al., 2014) and hippocampus (Eagle et al., 2015). In the NAc, ΔFosB expression promotes increased psychomotor sensitization to and reward from psychostimulants in rodents (Kelz et al., 1999a; Robison et al., 2013b). NAc ΔFosB also acts as a pro-resilience factor in the mouse chronic social defeat model of depression, and its expression there is required for antidepressant function (Vialou et al., 2010). In contrast, expression of ΔFosB in PFC promotes susceptibility to social defeat stress in mice (Vialou et al., 2014), suggesting that ΔFosB plays very different roles in the reward circuit and the brain regions that innervate it. Finally, ΔFosB is induced in the mouse dorsal hippocampus by learning and its function there is required for normal spatial memory formation (Eagle et al., 2015), providing a possible mechanism for the cognitive deficits often accompanying chronic drug exposure and/or depression (Evans et al., 2014; Wood et al., 2014; Papakostas and Culpepper, 2015).

As ΔFosB is a transcription factor, it is commonly presumed that it exerts its biological effects through modulation of the expression of select target genes, and many of those target genes have been implicated in depression and addiction. ΔFosB regulates the expression of multiple subunits of AMPA- and NMDA-type glutamate receptors (Hiroi et al., 1998; Kelz et al., 1999a; Covington et al., 2010), and these receptors have been directly implicated in addiction (Luscher, 2013; Pierce and Wolf, 2013), depression (Graybeal et al., 2012; Duman, 2014), and antidepressant

function (Zarate et al., 2013; Duman, 2014). ΔFosB also regulates the expression of signaling molecules, like calcium/calmodulin-dependent protein kinase II α (CaMKIIα), which has been linked to many psychiatric disorders (Robison, 2014), and this regulation of CaMKII expression in mice drives psychomotor sensitization to cocaine (Robison et al., 2013b) and antidepressant function (Robison et al., 2013a). In addition, ΔFosB regulates the expression of cyclin-dependent kinase 5 (cdk5) (Schenk and Partridge, 2000), which is induced in striatum by psychostimulant exposure and stress (Seiwell et al., 2007; Bignante et al., 2008; Mlewski et al., 2008) and regulates the psychomotor and motivational responses to cocaine (Taylor et al., 2007). Thus, there is strong evidence in rodent models that induction of ΔFosB in multiple brain regions by stress, antidepressants, and drugs of abuse may regulate behaviors related to depression and addiction by modulating the expression of select target genes in discreet brain regions.

Although preclinical models of addiction and depression have been quite fruitful, it is essential to support findings from animal models with evidence from human studies if we expect to translate potential molecular mechanisms into novel treatment options. ΔFosB is upregulated in the NAc of human cocaine addicts (Robison et al., 2013b) and reduced in the NAc of depressed humans (Vialou et al., 2010). However, regulation of *FosB* gene product expression in hippocampus and PFC, critical regulators of NAc neuronal activation, has not previously been studied in human brain, nor has regulation of potential ΔFosB target gene expression. I therefore examined the expression of *FosB* gene products, as well as the expression of potential ΔFosB target genes, in the PFC and hippocampus of patients suffering from major depressive disorder or cocaine addiction.

RESULTS

Characterization of human FosB isoforms:

Recent studies indicate that the three major products of the *FosB* gene in brain, full-length FosB (~50 kDa), ΔFosB (~35-37 kDa), and Δ2ΔFosB (~25 kDa), are differentially induced in mouse brain reward-associated regions in response to stress and antidepressant treatment (Vialou et al., 2015), and other Fos-related antigens likely produced by the *FosB* gene have also been observed in mouse brain (Hope et al., 1994b; Nye et al., 1995; Chen et al., 1997). Therefore, I first sought to determine whether human brain expresses a pattern of *FosB* gene products similar to that found in mouse brain. I compared a typical hippocampus sample from a cocaine addicted individual (**Table 3**) to the dorsal hippocampus from a mouse given chronic cocaine (15 mg/kg,

Table 3. Demographics of human cocaine addicts, depression patients, and matched controls

Group (number of subjects)	Age	% Male	Brain pH	Postmortem Interval (h)
Control (18)	33.05 ± 3.193	95%	6.569 ± 0.062	36.08 ± 4.515
Cocaine Dependent (19)	39.80 ± 2.153 p = 0.11	95%	6.546 ± 0.072 p = 0.48	42.78 ± 4.661 p = 0.31
Control (11)	41.58 ± 3.241	83%	6.508 ± 0.074	31.75 ± 5.899
Depressed Non-Medicated (14)	48.14 ± 3.061	71%	6.721 ± 0.055	39.04 ± 6.478
Depressed Medicated (13)	45.75 ± 2.713 p = 0.33	69%	6.671 ± 0.083 p = 0.14	40.66 ± 7.357 p = 0.65

All values are mean +/- standard error. P values are calculated using two-tailed students t-test for cocaine samples and one-way ANOVA for depression samples. 100% of participants were Caucasian. This table was adapted from: (Gajewski et al., 2016)

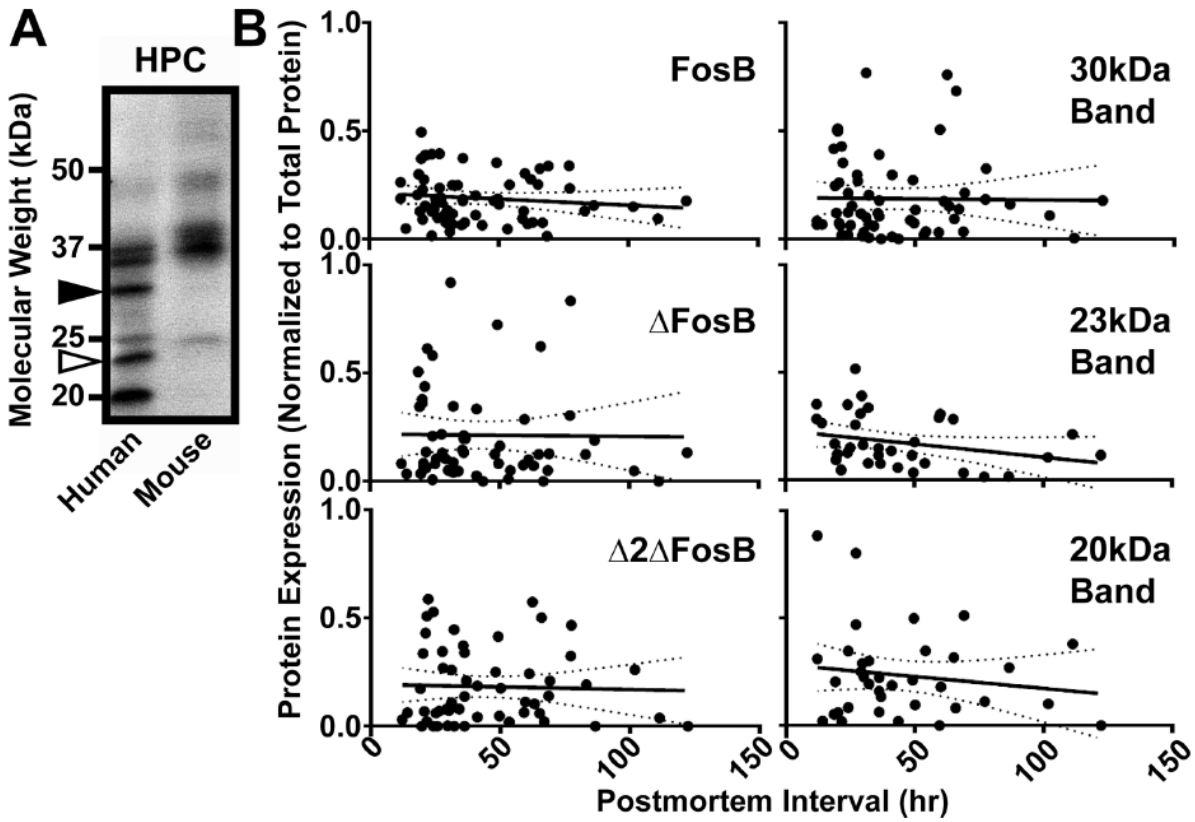

Figure 23. Comparison of human and mouse FosB proteins. A. Western blot of hippocampal (HPC) proteins with FosB antibody reveals multiple additional bands in typical human cocaine addict HPC sample compared to a chronic cocaine-treated (15 mg/kg for 7 days) mouse HPC. Novel bands are apparent at 20 kDa, 23 kDa (white arrow), and 30 kDa (black arrow). **B.** Correlation and linear regression plots of protein expression for each band in the human samples with the postmortem interval (time between death and brain freezing) for each human sample. Dotted lines represent 95% confidence interval; no linear regression slope differed significantly from 0.

i.p. for 7 days). All three major *FosB* gene products were found in both mouse and human brain tissue, but additional bands were observed in the human sample compared to mouse (**Figure 23A**). Most prominently, bands at ~30 kDa, ~23 kDa, and ~20 kDa appeared in human samples, but were not observed in mouse samples. These bands may represent proteolytic products resulting from degradation of FosB or ΔFosB due to the extended post-mortem interval (PMI) in our human samples (**Table 3**). However, no correlation was found between the intensity of these novel bands and PMI (**Figure 23B**), or between PMI and the major gene products, FosB, ΔFosB,

and Δ2ΔFosB (**Figure 23B**), i.e., none of the regression lines had a slope significantly different from zero. Thus, these novel bands may not be proteolytic degradation products resulting from prolonged time between death and tissue freezing.

To further investigate this, I gave mice a single injection of cocaine (15 mg/kg, i.p.) or saline and sacrificed them by cervical dislocation one hour later. The brains were then left *in situ* for zero, one, or eight hours before samples were taken. Some degradation products did appear (**Figure 24**), the most prominent being ~23 kDa, but the resulting pattern did not mimic that seen in human hippocampus samples. Taken together, these data indicate that there are additional Fos-related antigens in human brain that may represent novel *FosB* gene products and are unlikely to be the result of proteolysis of FosB or ΔFosB.

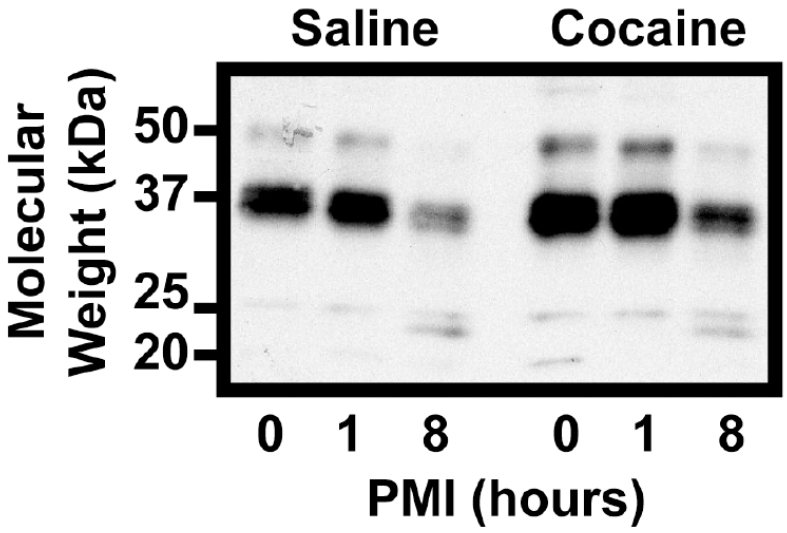

Figure 24. Expression of FosB proteins in mouse hippocampus after extended postmortem intervals. The brains of mice given an acute injection of cocaine (15 mg/kg i.p.) were left *in situ* for 0, 1, or 8 hrs after sacrifice before harvesting HPC. Western blot reveals buildup of a 23 kDa band in the 8 hr animals, but does not show other bands found in human HPC samples.

Expression of FosB isoforms in hippocampus and PFC in human cocaine addicted and depressed patients:

I next sought to determine whether cocaine dependence, untreated depression, or depression coupled with exposure to antidepressant medication are associated with changes in *FosB* gene products in human hippocampus or PFC. Patients and control subjects were chosen such that there were no significant differences in average age, gender, brain pH, or PMI (**Table 3**). In samples from cocaine dependent patients, Western blot revealed no differences in the expression of any FosB isoform in the PFC compared to controls (**Figure 25A,B**). However, there was a marked decrease in the hippocampus of cocaine dependent individuals in full-length FosB, ΔFosB, as well as in all three novel bands, 30 kDa, 23 kDa, and 20 kDa, and a trend toward a decrease in Δ2ΔFosB. Similarly, in samples from patients suffering from depression, there were no differences in the expression of any FosB isoform in the PFC, while the hippocampus showed decreases in full-length FosB and ΔFosB, as well as in the 23 kDa band and the 20 kDa band (**Figure 26A,B).** These data suggest that *FosB* gene expression in hippocampus is reduced in multiple psychiatric conditions while PFC expression is unaffected.

Expression of FosB target genes in hippocampus and PFC in human cocaine addicted and depressed patients:

Direct evidence for gene targets of ΔFosB transcriptional regulation in hippocampus is scant, with only cyclin-dependent kinase 5 (*cdk5*) a confirmed target after electroconvulsive stimulation in mice (Schenk and Partridge, 2000). However, many other genes are known targets for ΔFosB transcriptional regulation in other brain regions, particularly in NAc. These include a number of genes essential for hippocampal cell function and synaptic plasticity, such as *GRIA2* (gene encoding GluA2) (Kelz et al., 1999b) and *CaMKII* (Robison et al., 2013b). Therefore, Western blotting was used to assess the levels of potential gene targets of ΔFosB in the hippocampus and

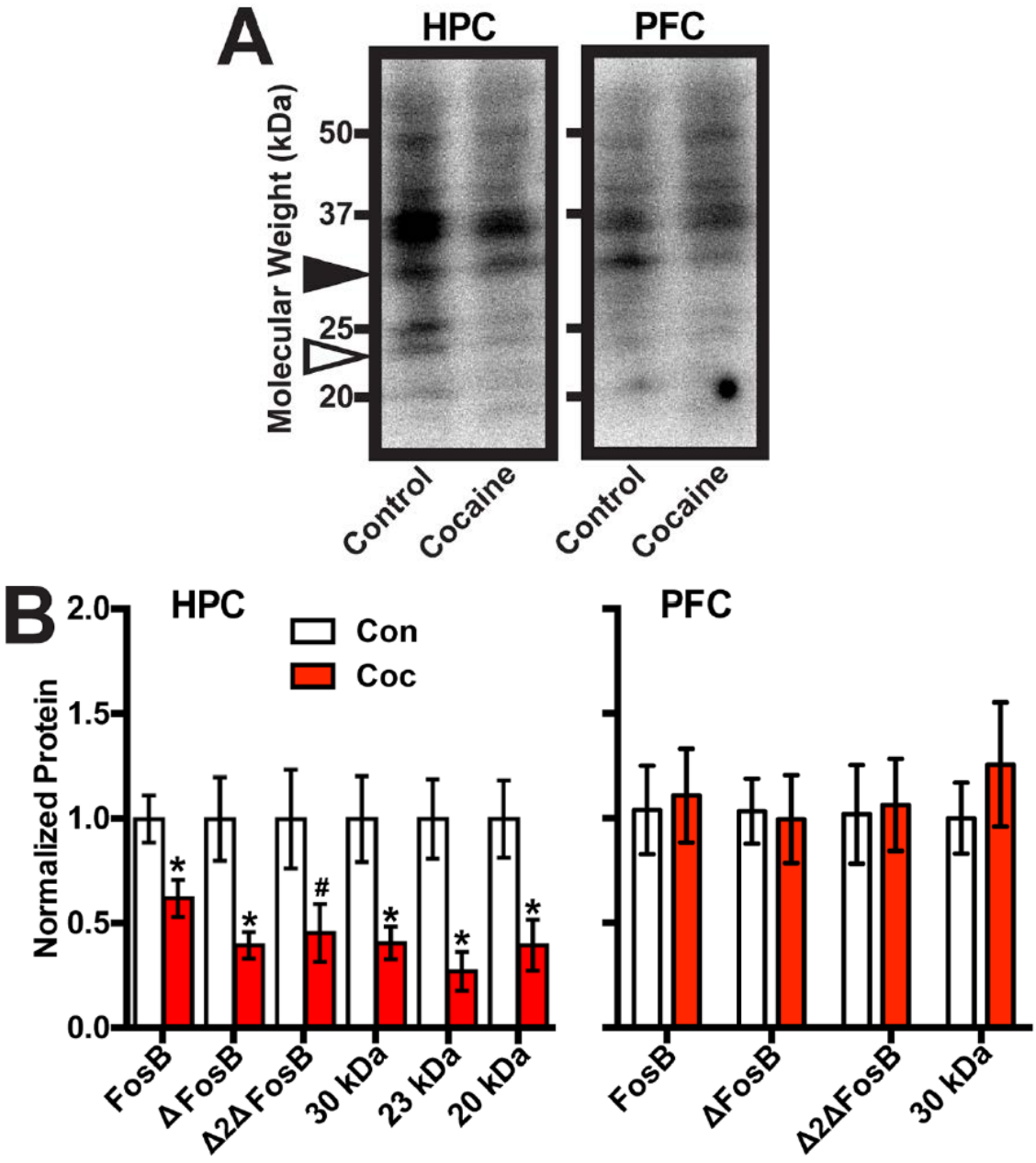

Figure 25. Expression of FosB proteins in hippocampus and PFC of human cocaine addiction. A. Western blot of FosB proteins from the hippocampus (HPC) and PFC of human cocaine addicts (Coc) and controls (Con). 23 kDa band (white arrow), and 30 kDa band (black arrow). **B.** Quantitation reveals a cocaine-dependent decrease in many FosB proteins in the HPC but not PFC (*:$p<0.05$, #:$p=0.05$). Error bars indicate mean +/- SEM.

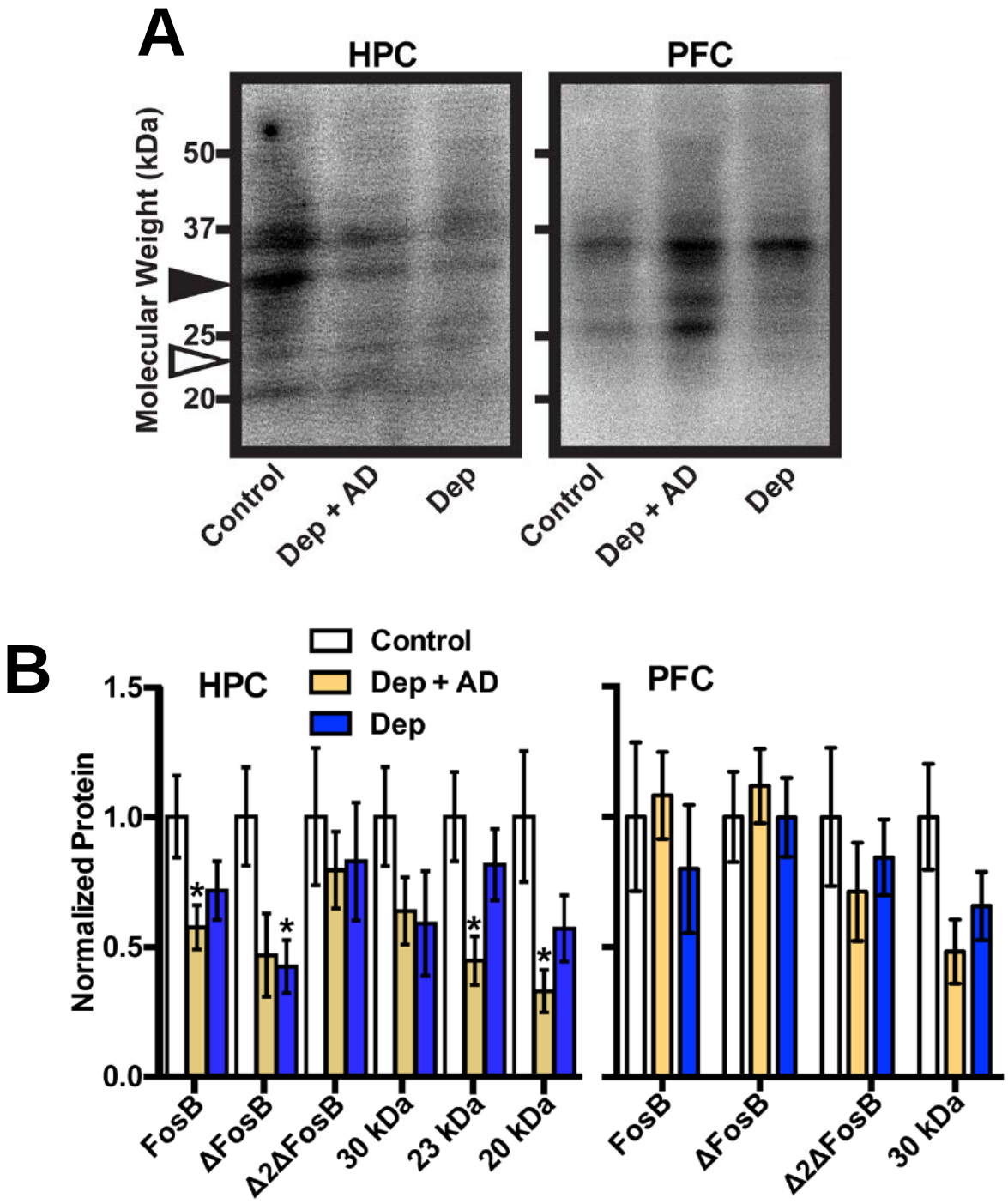

Figure 26. Expression of FosB proteins in hippocampus and PFC of human depression patients. A. Western blot of FosB proteins from the hippocampus (HPC) and PFC of human depression patients off (Dep) or on antidepressants (Dep + AD) and controls (Con). 23 kDa band (white arrow), and 30 kDa band (black arrow). **B.** Quantitation reveals a depression-dependent decrease in some FosB proteins in the HPC but not PFC (*:$p<0.05$). Error bars indicate mean +/- SEM.

PFC of cocaine dependent and depressed patients. There were no significant differences in the protein levels of the candidate target genes in the PFC of cocaine dependent individuals, while the hippocampus showed a significant decrease in GluA2 and a strong trend toward decrease in CaMKII levels expression, while cdk5 remained unchanged (**Figure 27A,B**). In the PFC and hippocampus of depressed patients there were no changes in expression of the ΔFosB target genes (**Figure 28A,B**). These data suggest that ΔFosB may be regulating the expression of potential target genes in human hippocampus, and this regulation may be brain region and disease specific.

DISSCUSSION

This chapter presents the first compilation of *FosB* gene product and ΔFosB-target protein analysis in the hippocampus and prefrontal cortex of cocaine addicts and depressed patients. These brain regions play key roles in the pathophysiology of these diseases, and the use of human post-mortem samples allows us to: 1) determine whether the molecular alterations found in well-studied rodent models of these diseases are recapitulated in humans; and 2) identify novel pathways for study in rodent models for potential therapeutic intervention. The analyses focused on expression of *FosB* gene products, as their expression in these regions has been suggested to play a role in depression and is induced by cocaine exposure in rodent models (Perrotti et al., 2008; Vialou et al., 2014; Vialou et al., 2015). When initially examining FosB protein levels in the human samples, it was clear that our FosB antibody detected more bands than have previously been reported in rodent brain samples by our group and many others (Robison and Nestler, 2011; Vialou et al., 2015). Because human brains are frozen hours after death, while mouse samples are removed and frozen within two minutes of sacrifice, I left mouse brains *in situ* after sacrifice for up to eight hours to determine whether similar bands would emerge due to possible degradation. However, because I did not observe the same pattern of FosB proteins found in the human samples, and because I also found no correlation between the length of PMI and the levels

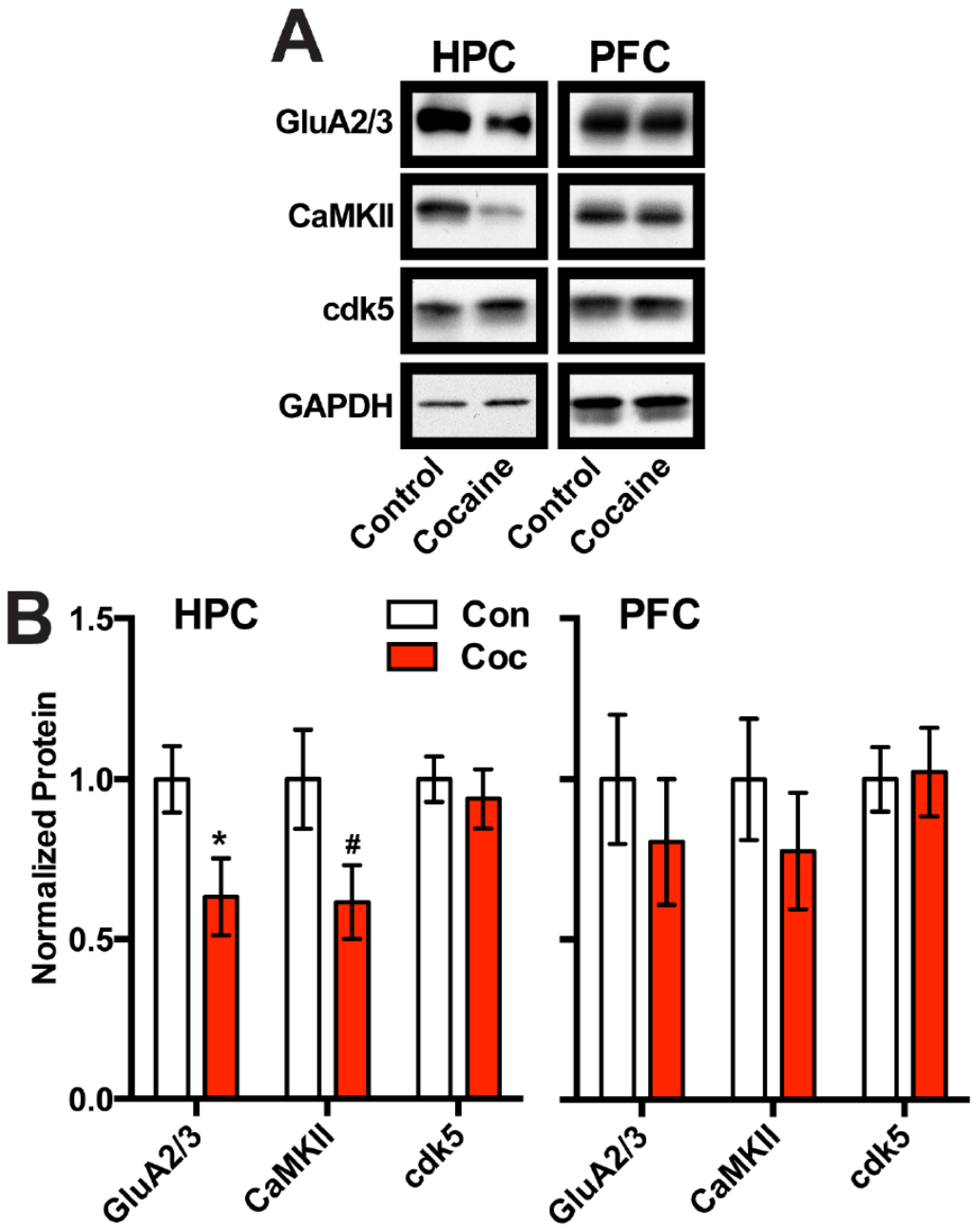

Figure 27. Expression of possible ΔFosB gene target proteins in hippocampus and PFC of human cocaine addiction. A. Western blot of potential ΔFosB gene target proteins from the hippocampus (HPC) and PFC of human cocaine addicts (Coc) and controls (Con). **B.** Quantitation reveals a cocaine-dependent decrease in GluA2 and CaMKII in the HPC but not PFC (*:p<0.05, #:p=0.05).

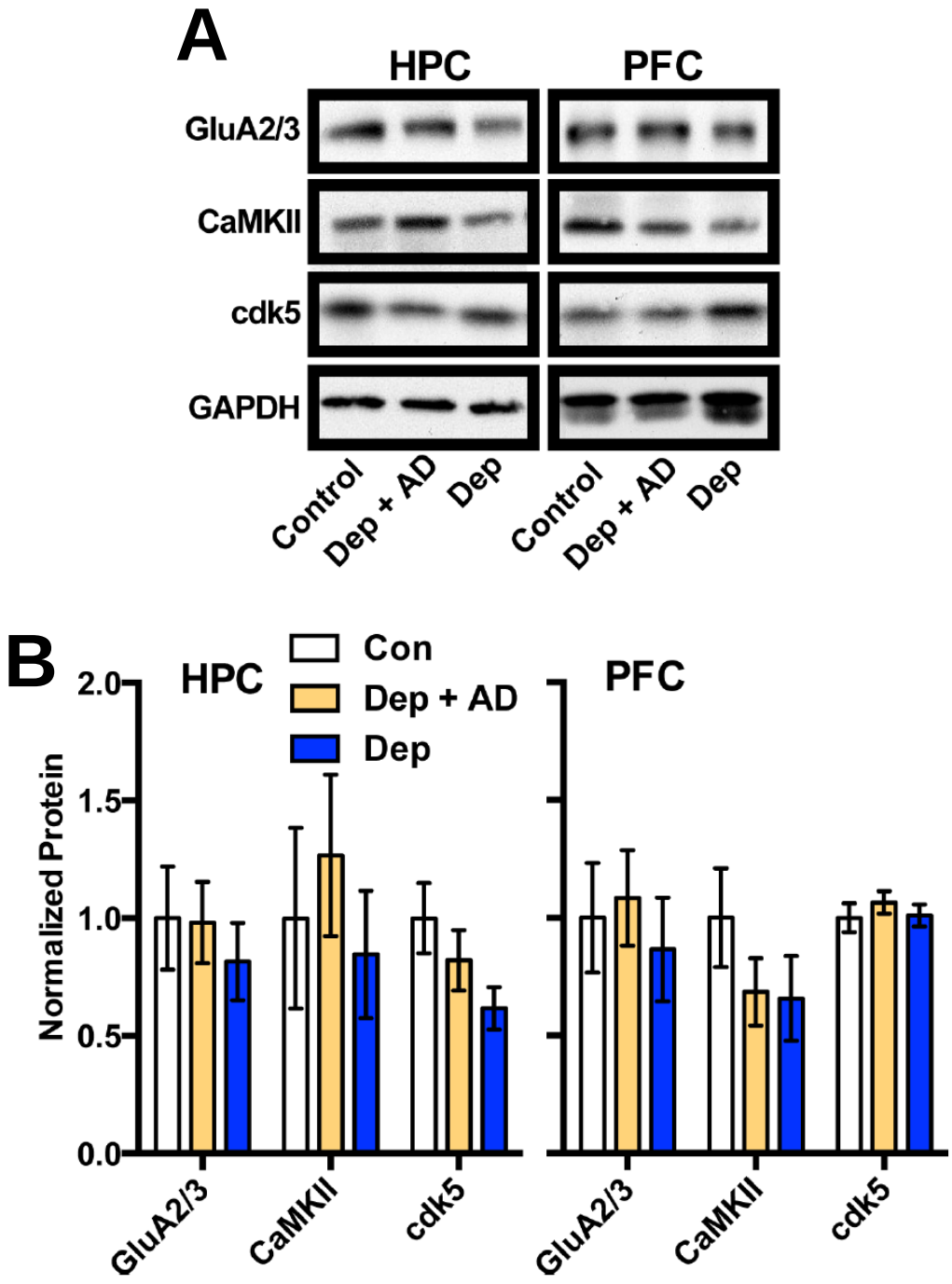

Figure 28. Expression of possible ΔFosB gene target proteins in hippocampus and PFC of human depression patients. A. Western blot of potential ΔFosB gene target proteins from the hippocampus (HPC) and PFC of human depression patients off (Dep) or on antidepressants (Dep + AD) and controls (Con). **B.** Quantitation reveals no depression-dependent changes. Error bars indicate mean +/- SEM.

of the various bands in human samples, I conclude that many of the bands in the human brain samples are unlikely to be the result of proteolytic degradation of larger FosB isoforms. Although differences in proteolytic machinery between species cannot be ruled out, other possibilities suggest themselves: 1) some of the human bands may result from differential splicing of the FosB mRNA; or 2) the human brain contains other proteins encoded by other genes that share antigenicity with FosB, and the bands are thus not specific to *FosB* gene products. Future studies from our group will address this question using PCR on cDNA derived from human and mouse brain.

Previous results from rodent studies have found an increase in FosB isoforms in hippocampus and PFC after chronic cocaine (See Chapter III and (Perrotti et al., 2008)). However, from the cohort of cocaine dependent individuals I found a decrease in all FosB isoforms in hippocampus, with no change in PFC compared to control individuals. This may be due to the inherent differences between rodent studies and cases of human addiction. Studies of cocaine addiction only last for a small fraction of the rodent's life, and no ΔFosB induction studies to date have gone beyond 14 days of continuous cocaine exposure (Robison and Nestler, 2011; Robison et al., 2013b). Human cocaine users can be addicts for much longer time periods, which may induce homeostatic effects, causing the *FosB* gene to be repressed in the hippocampus. Moreover, many studies have demonstrated that long-term addiction to psychostimulants is accompanied by reduced cognitive function (Buchta and Riegel, 2015; Cadet and Bisagno, 2015). Recent work from our lab demonstrates that hippocampal ΔFosB plays a critical role in learning (Eagle et al., 2015), and thus the decrease in hippocampal *FosB* gene expression in cocaine addicts shown here may represent a mechanism for cognitive decline in psychostimulant addiction. With decreased expression of the *FosB* gene in hippocampus, I observed a decrease in the protein levels of candidate ΔFosB target genes, GluA2 and CaMKII, and both of these molecules are also critical for hippocampal function and learning (Shonesy et al., 2014) and have been previously

linked to addiction (Loweth et al., 2014; Robison, 2014).

In the depressed patients, there was a decrease in multiple FosB proteins, including the 23 and 20 kDA unknown isoform bands, depending on whether patients were taking antidepressants. This may indicate that antidepressants have differential effects on the splicing or stability of *FosB* gene products, though previous studies in rodents did not reveal any such differences (Vialou et al., 2015). However, there were no differences in the expression of potential target genes in either the hippocampus or PFC of these patients. Although major depression is often accompanied by cognitive problems (Culpepper, 2015), it is likely that hippocampal ΔFosB is not the only factor altered in response to depression. While the cocaine addicts showed changes in hippocampal ΔFosB and in target gene expression, depression may be leading to different compensatory mechanisms that prevent reduction in GluA2 or CaMKII expression. Thus, future studies will elucidate whether changes in hippocampal gene expression in depression and addiction arise from similar mechanisms.

It is critical to note that the human populations used for this study lack the homogeneity of preclinical rodent or primate models. For instance, five of the depressed patients suffered from alcoholism, and two had opiates on board at time of death. Similarly, six of the cocaine-dependent individuals had used antidepressants in the three months prior to death. Although this is not surprising, since depression and addiction have a high level of comorbidity (Davis et al., 2008; 2010a), it does complicate interpretation of results. There were no significant differences in any of the biochemical measures between cocaine-dependent subjects who had antidepressants on board and those that did not, nor do were there differences between depressed patients who had substance dependence and those who did not (data not shown). However, this does not rule out overlapping or synergistic effects of depression and addiction on the above measures. On the contrary, as there are similar decreases in hippocampal FosB isoform expression with depression and addiction, it is possible that reduction in hippocampal *FosB* gene expression is a common

mechanism between the two conditions and may contribute to comorbidity. Investigation of this hypothesis will require much larger cohorts of human subjects and additional preclinical studies.

In conclusion, multiple *FosB* gene products are downregulated in the hippocampus, but not the PFC, of humans suffering from addiction and depression. Although I cannot make an etiological connection between this phenomenon and the disease states, it is possible that decreases in hippocampal ΔFosB and/or other FosB isoforms may in part underlie the cognitive deficits associated with depression and addiction, or contribute to the comorbidity of these psychiatric disorders.

SUMMARY AND FUTURE DIRECTIONS

SUMMARY

The work presented in this thesis contributes to the fields of addiction and epigenetics in multiple ways. First, these data describe a novel role for the epigenetic regulation of ΔFosB in hippocampal-dependent behaviors. Work from our lab indicated the necessary induction of dorsal hippocampus ΔFosB after spatial learning by illustrating that silencing and overexpression of ΔFosB in dHPC impairs learning and memory (Eagle et al., 2015). ΔFosB is not only induced after spatial learning, but also after chronic exposure to cocaine in a novel environment, suggesting that the drug-environment associations are indeed a form of hippocampal-dependent learning. The induction occurred in the same subregion as the one implicated in spatial learning, the CA1 (Eagle et al., 2015). Therefore, the dorsal CA1 may be activated in response to the novel context, while the ventral CA1 may be activated in response to the rewarding effects of cocaine. As previously stated, ΔFosB is also induced in the NAc in response to chronic cocaine, and regulation of the *FosB* gene was investigated to reveal roles for transcription factors, serum response factor (SRF) and CREB, as well as changes in histone posttranslational modifications that promote transcription (Damez-Werno et al., 2012; Vialou et al., 2012). The experiments in Chapters III and IV describe similar changes in histone modifications in hippocampus and their role in ΔFosB induction and subsequent behavior, with specific decreases in the repressive modification, demethylation of lysine 9 on histone H3 (H3K9me2).

To investigate the role of these changing histone modification, we collaborated with Dr. Elizabeth Heller, who developed the tools to modify histone marks in a locus-specific manner (Heller et al., 2014b). By combining a specific zinc finger protein (ZFP) able to recognize only a sequence contained within the *FosB* promoter, and the histone methytransferase enzyme, G9a, Dr. Heller designed a protein which would specifically increase H3K9me2 at the *FosB* promoter without

influencing the histone modifications elsewhere in the genome. This novel construct, packaged into a viral vector, allows for temporal, brain region, and gene-locus specificity in histone posttranslational modifications. Validation of this viral tool involved the examination of heterochromatin protein 1 (HP1) binding and found a specific increase at the *FosB* promoter (Heller et al., 2014b). HP1 binding has been known to recruit other enzymes to further modify histone residues, but further analysis revealed that this was not occurring at the *FosB* promoter, indicating that it was the enrichment of H3K9me2 that was leading to silencing of the *FosB* gene. Use of this virus in the above experiments revealed that epigenetic modification at the *FosB* promoter did indeed impair cocaine-mediated induction of ΔFosB and hippocampal-dependent learning. However, there remains one important study to repeat: examining the effects of HSV-ZFP-G9a in vHPC on cocaine CPP. In my initial experiments, the viral spread was not as robust in the ventral hippocampus as is typical, and I am repeating this experiment with new virus and a new injection protocol now. This may have occurred due to the amount of virus infused (0.6µl in dorsal and 0.5 µl in ventral) or in the differences in injections (an injection of 0.3µl near the dorsal DG and withdrawal for another 0.3µl near dorsal CA1, while 0.5µl is injected all at the same site for ventral CA1). Therefore, the effect of H3K9me2 at *FosB* in the ventral CA1 subregion is still being investigated. Currently, work is being conducted to investigate the role of the *FosB* gene in the ventral hippocampus projection to either NAc or amygdala and evidence is accumulating for specific influence in cocaine-, and anxiety-related behaviors, respectively (Eagle, 2016).

Another major contribution of my thesis is to highlight the differences between our current rodent models and human postmortem samples. This is an especially important area of study, as the translational aspect of basic science research helps to further the progression of clinical research and therapeutic interventions. Investigation into the molecular underpinnings of neuropsychiatric diseases is a difficult task to achieve without the use of model organisms, as there are ethical and practical obstacles in examining the living human brain (Nestler and Hyman, 2010). However, there are certain aspects of neuropsychiatric disorders that have not yet been fully modelled in

animals, such as sadness and hallucinations, making molecular analysis of post-mortem human samples essential. The results described in Chapter III reveal subregion-specific increases in hippocampal ΔFosB after chronic cocaine exposure, while Chapter V demonstrates a decrease in FosB isoforms in the hippocampus of human cocaine addicts. Differences in protein levels between rodent and human brains have been documented before, even under basal conditions (Le Maitre et al., 2013). The differences could originate from the conditions under which the cocaine was taken – for our mice, it was experimenter-administered for 10 days, while for the human subjects the amount of cocaine and length of exposure is more ambiguous and variable, and the cocaine is self-administered. Furthermore, as the hippocampus is essential in learning, and cocaine addicted individuals display cognitive deficits, the decrease in FosB isoforms may be due to a general decrease in hippocampal activity that occurs as a result of years of chronic exposure (Buchta and Riegel, 2015; Cadet and Bisagno, 2015), something not modeled in the mouse experiments in this thesis.

While the findings in the above chapters provide further evidence for the unique involvement of the dorsal and ventral hippocampus in drug-related behaviors, it is clear that the mechanisms behind this involvement are complex. In order to gain a more complete understanding of hippocampal ΔFosB and its role in drug-environment associations, many more experiments will be necessary. Three particular lines of experimentation suggest themselves when considering how best to explore hippocampal ΔFosB in the future: 1) identification of ΔFosB gene targets; 2) assessment of gene therapy and its ability to curb the drug-environment associations that may drive addiction; 3) whether ΔFosB-specific expression differences provide a mechanism for addiction predisposition.

ΔFOSB GENE TARGETS

Finding hippocampal ΔFosB gene targets:

ΔFosB influences the morphology and function of neurons, as overexpression in multiple brain regions, including the hippocampus, increases dendritic spine formation (Eagle et al., 2015). 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 or in D2-type neurons (Robison et al., 2013b), demonstrating the cell- and region-specific nature of ΔFosB function. In addition, ΔFosB overexpression increases *GRIA2* transcription by over 50% in the NAc (Kelz et al., 1999a), and it is enriched at the *GRIA2* promoter in the NAc and drives expression of the gene product (the GluA2 subunit of the AMPA receptor) in response to stress (Vialou et al., 2010). Furthermore, using cDNA array analysis, *Cdk5* was found to be a target of ΔFosB in both NAc and hippocampus (Chen et al., 2000; Bibb et al., 2001). 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 (Vialou et al., 2010; Robison et al., 2014). These are all promising and functionally significant candidates for ΔFosB binding and regulation in the hippocampus due to their roles in synaptic strength and plasticity as well as learning and memory. However, they may not be ΔFosB targets in this region, and even if they are, they are unlikely to be the only targets. Thus, it will be critical to employ unbiased approaches to uncover ΔFosB target genes in hippocampus.

When considering methods to identify ΔFosB target genes, a number of strategies present themselves. First, although the antibody is not currently validated for chromatin immunoprecipitation (ChIP), Cell Signaling Technologies recently began marketing a ΔFosB-

specific antibody (e.g., one that does not recognize full-length FosB), which would allow for the identification of ΔFosB binding locations with either a candidate gene approach or unbiased sequencing (ChIP-seq). Additionally, a FosB antibody that recognizes all isoforms is validated for ChIP, and there are methods which were applied in Chapter III that could be utilized to enrich for ΔFosB-bound genes. By utilizing the unique stability of ΔFosB and waiting 24 hours after the last stimulation (i.e. exposure to cocaine), it is possible to largely eliminate the presence of FosB, as it has very transient expression, leading the antibody to provide information preferentially about ΔFosB. A method of activating the hippocampal neurons would need to be done before the ChIP, as basal levels of ΔFosB are very low, and only accumulate after chronic activation. This could be achieved by spatial learning, cocaine exposure, or electroconvulsive seizures, with subsequent removal of whole hippocampus for ChIP.

ΔFosB ChIP-seq has previously been performed in NAc tissue, and a pathway analysis revealed binding to genes associated with extracellular stimulation, signal transduction, nervous system development, synaptic proteins, and ion channels (unpublished data from Robison, Feng, and Nestler). This further implicates ΔFosB in regulating synaptic plasticity and neuronal excitability.

An additional method utilizing transgenic mice could also uncover ΔFosB target genes. As the above-mentioned antibodies are either not validated in ChIP or are notoriously difficult to use in ChIP, creating a mouse with a tagged *FosB* gene could assist in this respect. Well characterized and validated epitope tags, such as FLAG, c-Myc, and HA, are used to isolate proteins of interest, as antibodies for these tags are very common and have been proven effective. Insertion of a DNA sequence coding for one of these short peptide tags into the gene of interest creates a fusion between the protein of interest and the tag. Others in the lab are currently breeding mice, generously provided by Dr. Eric Nestler, that have an HA-tagged *FosB* gene which can aid in the identification of FosB/ΔFosB target genes when conducting ChIP. However, with this tool, the fusion will occur with both FosB isoforms, and thus we again run into the caveat of distinguishing the target genes of each isoform. Thankfully, the unique stability of ΔFosB puts it in a position of

accessibility regardless of all isoforms being recognized.

Determining FosB gene targets regulated by cocaine:

FosB null mice (Brown et al., 1996) display impaired neurogenesis in the adult hippocampus as well as spontaneous epilepsy (Yutsudo et al., 2013). However, mice that retain expression of ΔFosB but lack full-length FosB do not have these phenotypes (Yutsudo et al., 2013). 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 ΔFosB function specifically in hippocampus, or lack of *FosB* expression in other regions or during early development that then contributes to hippocampal dysfunction. Thus, our lab has a colony of *floxed FosB* mice which lose expression of the *FosB* gene in the presence of Cre, which can be delivered by viral vector or cell-type-specific Cre-expressing mouse lines, allowing for a more region-specific knockout of *FosB* expression. *Floxed FosB* mice can be crossed with a Cre-dependent L10-GFP reporter, which expresses GFP fused to a ribosomal protein. Since antibodies specific to GFP are widely used and validated, this allows for translating ribosomal affinity purification (TRAP): immunoprecipitating ribosomes and their associated mRNAs only from Cre-positive cells (Heiman et al., 2014) (**Figure 29**). When treated with chronic cocaine or saline, we can determine genes whose cocaine-mediated expression in hippocampus is mediated by ΔFosB.

With the help of our collaborator, Dr. Ian Maze, a preliminary TRAP study was performed on transgenic mice that contained only the Cre-dependent L10-GFP reporter and were wildtype for *FosB*, or also contained the *floxed FosB* gene. After they received a retrograde HSV-Cre injection into the NAc, so that projections from the ventral CA1 would express Cre, ventral hippocampus tissue was collected using a fluorescent dissecting scope. After the formation of cDNA libraries from the pulled down mRNA, it was determined that the deletion of *FosB* changed the expression

of many genes involved in hippocampal excitability. Some examples of these new unpublished data are given here (**Table 4**). Further investigation will reveal genes specifically related to cocaine exposure, and these will be cross-referenced with the ΔFosB gene targets to determine genes regulated by cocaine-induced ΔFosB. Such experiments will elucidate the transcriptional mechanisms by which the drug is changing hippocampal synaptic plasticity and aiding in the formation of the strong, long-lasting drug-environment associations.

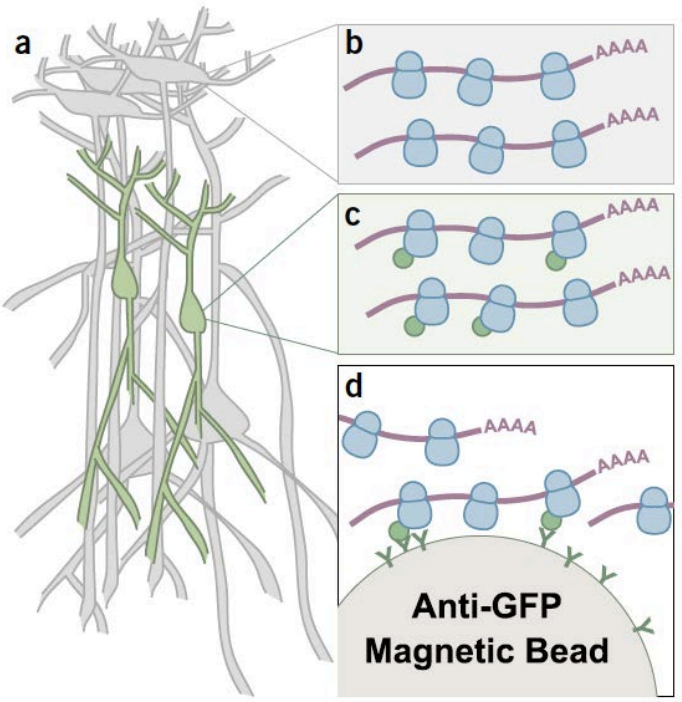

Figure 29. The translating ribosome affinity purification (TRAP) strategy. A. The cell type of interest is targeted with genetic elements to expression the EGFP-L10a transgene (NtsR2 cells in our condition). **B,C.** Translating polyribosomes originating from nontargeted cells (gray cells, B) do not have an EGFP tag on their ribosomes, whereas those originating from targeted cells (green cells, C) do. Lysis of all cells releases tagged and nontagged ribosomes. **D.** Only the tagged ribosomes are captured on an anti-GFP affinity matrix, which can be used for purification of the cell type-specific mRNA associated with tagged ribosomes. Figure adapted from: **(Heiman et al., 2014)**.

Table 4. Gene targets of FosB.

Gene Target	Gene Function	Citations
Kcnab2	Voltage-gated potassium channel subunit β-2; related to hyperexcitability in amygdala neurons	(Perkowski and Murphy, 2011)
Snap25	Synaptosomal-associated protein 25; associated with voltage-gated calcium channel activity in hippocampus	(Condliffe et al., 2010)
Hip1r	Huntingtin-interacting protein 1-related protein; plays a role in dendrite formation and excitatory synapse formation in hippocampus	(Peng et al., 2017)
Etl4	Enhancer trap locus 4; may be related to sex differences seen after stress (upregulated in males but downregulated in females)	(Pfau et al., 2016)

Selected genes identified by RNA-seq on mRNA extracted from translating ribosome affinity purification (TRAP) on vHPC-NAc neurons. These are four examples out of hundreds that were downregulated in the absence of *FosB* expression. These examples were selected based on their potential involvement in regulating hippocampal neuron excitability.

GENE THERAPY

Targeting splice variants:

Distinguishing between FosB, ΔFosB, and Δ2ΔFosB, has been a general difficulty throughout the study of the *FosB* gene and its function. Antibodies typically recognize all three isoforms, which can only truly be identified when separated out by molecular weight. Tools affecting the *FosB* gene, whether it be insertion of flox sites, utilization of CRISPR/Cas technology, or epigenetic modifications at the promoter (see Chapter IV), lead to changes in the expression of all three isoforms. These methods make it difficult to isolate the changes initiated by a single isoform. Therefore, if a protein is able to bind and influence the splicing of the *FosB* mRNA, it could allow for experiments into the separate function of each isoform.

RNA-binding domains exist in many forms – RNA recognition motifs (RRMs), zinc-finger proteins (ZFP), the hnRNP K homology (KH) domain, and a few others. ZFPs, while well-known DNA-binding domains (see Chapter IV), have also been observed to bind folded RNA (Lu et al., 2003).

However, it is currently unknown whether they can bind single-stranded RNA (ssRNA), which does not make them good candidates for binding *FosB* mRNA. Both RRMs and KH domains bind ssRNA, four nucleotides being recognized by each domain, and endogenous proteins can contain multiple RRMs or KH domains to increase specificity and affinity of RNA binding (Michelotti et al., 1996; Allain et al., 2000; Clery, 2000-2013). Similar to the ZFP-G9a fusion protein used in Chapter IV, if a RRM or KH domain was synthesized to bind only to *FosB* mRNA, it could influence splicing if it was then fused to certain splicing machinery. An RNA binding protein containing 3-4 of the domains could be made to bind at the end of exon IV, which would place it close to the splice acceptor site in the middle of the exon for ΔFosB mRNA splicing. On the N-terminal, upstream of the RNA binding protein, the protein responsible for binding to the 3' AG splice acceptor site, $U2A^{35}$ (U2 snRNP auxiliary factor), could be fused (Merendino et al., 1999; Wu et al., 1999). This would create a constant presence of $U2A^{35}$ near the 3' splice site within exon IV, favoring splicing producing ΔFosB over splicing producing FosB (**Figure 30**). $U2A^{35}$ is a promising candidate for fusing to an RNA binding protein because of its binding domains that recruit other 3' splicing proteins to the area (Wu and Maniatis, 1993; Zuo and Maniatis, 1996). Localization of $U2A^{35}$ would therefore increase the likelihood of splicing machinery cleaving the *FosB* mRNA at this site, creating more ΔFosB-encoding message. The RNA binding fusion protein could then be packaged into viral vectors for validation in multiple models (i.e. cell culture, model organisms such as rats and mice). Ultimately, if more ΔFosB is what is necessary for certain brain regions, specifically in the hippocampus where we see a decrease in ΔFosB in cocaine addicted individuals (see Chapter V), in order to combat or aid in the cognitive deficits or specific drug-related memories, this fusion protein could change endogenous levels of *FosB* mRNA to favor ΔFosB splicing and translation.

Other mechanisms used to influence splice variant expression are antisense oligonucleotides, which have been used in clinical trials for Duchenne Muscular Dystrophy, as targeted exon

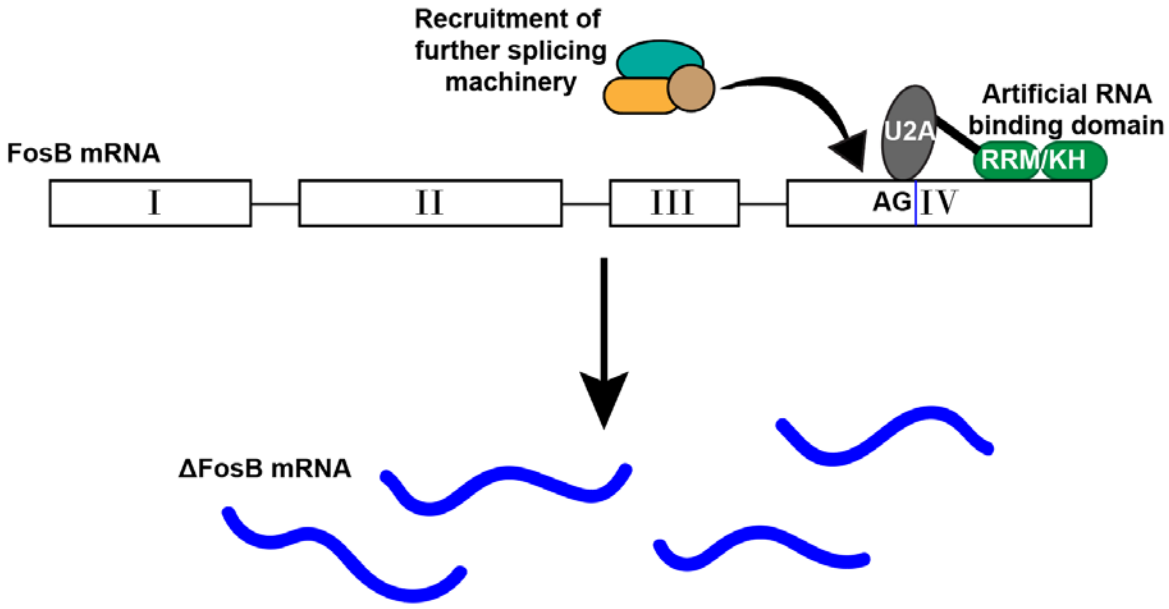

Figure 30. Diagram depicting end of the *FosB* mRNA and possible binding of RNA domains. Pictured are the four exons of *FosB* mRNA. Within the fourth exon, there is an alternative splice site indicated by the blue line (identified by splicing machinery that recognizes base pairs A and G), which forms the truncated ΔFosB isoform. An RNA binding domain could be synthesized from either RNA recognition motifs (RRM) or a KH domain to bind the end of the fourth exon. Fusion of the U2A (35) protein to this RNA binding domain could recruit splicing machinery to exclusively recognize the splice site within exon four, increasing the likelihood of ΔFosB mRNA being produced compared to the other isoforms.

skipping allows for alleviation of premature stop codons generated by mutations in the very long *Dystrophin* gene (Koo and Wood, 2013). However, the *FosB* gene only contains four exons, with the fourth one containing the splice site of interest, so the exon skipping properties of antisense oligonucleotides may not provide the best chance of elevating ΔFosB splicing. Alternatively, splice-switching oligonucleotides can create a blockade at a splice site, inhibiting the binding of RNA-binding proteins, like splicing machinery (Havens and Hastings, 2016). Instead of causing degradation of the resulting double stranded RNA complex (dsRNA is typically targeted for rapid degradation, this is the mechanism by which RNA interference works (Tang et al., 2001)), the splice switching oligos are modified to be RNase H-resistant. Clinical trials for Duchenne Muscular Dystrophy and Spinal Muscular Atrophy are utilizing these oligos. However, they are being

administered in a largely systemic manner (intravenously, intrathecal injection), which might not be the best delivery system for treating cocaine addiction (Hache et al., 2016; Mendell et al., 2016). Stereotaxic viral vector-based expression of splice-switching oligonucleotides may be a viable method to uncover the separate roles of FosB and ΔFosB in specific cells and brain regions. Recent experiments looking to utilize gene therapy as a therapeutic intervention for addiction have examined proteins like dopamine receptors and cocaine metabolism enzymes (Thanos et al., 2008; Murthy et al., 2014). While the delivery of viral vectors containing supplemental DNA is still considered experimental and only at the clinical trial stage, gene therapy remains a very interesting and promising future mechanism for treating neurophysiological disorders.

PREDISPOSITION TO ADDICTION

Hippocampal function:

Addiction presents many challenges to treatment and prevention, as even people who cease drug use and maintain long-term abstinence for decades struggle with craving and possible relapse (O'Brien et al., 1992). Identifying risk factors for addiction is essential to understanding why certain individuals are more likely to develop an addiction to drugs of abuse than others. Elucidating the role of the hippocampus in addiction could help explain the long-lasting drug associations and memories, and could help in the identification of risk factors that may predict drug taking/seeking. The dentate gyrus (DG) subgranular zone of the hippocampus has the ability to produce new neurons throughout life (adult neurogenesis), and this process is impaired by cocaine self-administration (Deschaux et al., 2014). Additionally, suppression of adult hippocampus neurogenesis enhances the vulnerability of rats to self-administer cocaine without changing any aspect of the self-administration of a palatable reward, sucrose (Noonan et al., 2010). However, studies investigating the role of hippocampal neurogenesis in general hippocampal learning and memory remain contradictory (Castilla-Ortega et al., 2011). This suggests that the role of adult

neurogenesis in memory formation may differ based on the nature of the memory, and that drug-environment associations and learning involving drug seeking and taking may be particularly reliant on neurogenesis. Additionally, *fosB*-null mice, but not mice lacking full-length FosB but expressing ΔFosB, exhibit impaired hippocampal neurogenesis (Yutsudo et al., 2013). This, along with the decreases in hippocampal ΔFosB seen in cocaine addicted individuals (see Chapter V), suggests that dysregulation of ΔFosB may reduce adult neurogenesis in cocaine addicts, driving further drug seeking and impairing cognition. Unpublished work from our lab in collaboration with Dr. Gina Leinninger also suggests a role for hippocampal ΔFosB in both developmental and adult neurogenesis, and this will be the subject of future research in our groups. Currently, examination of hippocampal neurogenesis in human adults is still under investigation using magnetic resonance imaging (MRI) and requires further pre-clinical research before we can accurately image and quantify the process of adult neurogenesis in a living human (Sierra et al., 2011). This makes it pertinent to obtain suitable fixed postmortem samples for immunohistochemistry of markers associated with neuronal differentiation to uncover whether cocaine is in fact altering hippocampal neurogenesis.

Genetic factors:

Predisposition or vulnerability to cocaine addiction may be rooted in allelic differences. As detailed in the above chapters, gene expression changes can drastically alter the function of the hippocampus. The existence of single nucleotide polymorphisms, deletions, insertions, etc., in individuals, especially with respect to neuron-specific genes, may provide a mechanism for functional differences in neuronal activation or connectivity conferring risk for addiction. Indication that genetic factors are influencing the vulnerability to addiction have been found in twin studies (Tsuang et al., 2001). Certain genes appeared very influential to cocaine dependence, such as dopamine receptor, *D2* (*D2DR*), *serotonin transporter* (*SERT*), and *preprodynorphin* (Noble et al.,

1993; Patkar et al., 2001; Chen et al., 2002). With this in mind, the genetic basis could be further investigated using candidate gene approaches in animal models. The above-mentioned genes are all expressed in the hippocampus, suggesting that their dysfunction may result in impaired hippocampal function, in addition to the various other affected brain regions (Goldsmith and Joyce, 1994; Wang et al., 1994; Morozova et al., 2014). Indeed, cocaine does lead to an increase in dopamine in the hippocampus, and blockage of serotonin transmission to the dorsal hippocampus reduces cocaine seeking in rats (Kramar et al., 2014; Kohtz and Aston-Jones, 2017). Future experiments should characterize cocaine-mediated changes in the above-mentioned genes in both dorsal and ventral hippocampus, as well as in the specific subregions, as Chapter III and others indicate that the hippocampal subregions are unique in their response to drugs of abuse.

The induction and behavioral role of the *FosB* gene has been examined in depth in NAc and now in the hippocampus in response to many drugs of abuse, but has yet to come up in a genome-wide association study (GWAS) when examining addicted individuals. Genome-wide analysis of genetic linkage to substance dependence revealed that each individual signal likely had a small effect but that many of these signals together may contribute to the heterogeneity of the addiction phenotype (Straub et al., 1999). There remains the possibility that *FosB* is so critical for general neuronal function that mutations affecting the activity of ΔFosB are prohibitive, and thus variation on a genetic level may not accumulate in the population. Additionally, examining the sequence directly as a candidate gene may reveal specific changes in the *FosB* gene that may not have been apparent in the threshold given for GWAS. Further analysis should then be conducted on the known ΔFosB target genes to gain more insight into the possible changes in neuronal excitability and how it relates to a potential vulnerability to addiction.

Epigenetic factors:

Data in chapter IV and others have shown that cocaine exposure can change the epigenetic landscape of the brain (Damez-Werno et al., 2012; Sadri-Vakili, 2015). It is also possible that basal differences exist within the population in the expression of certain enzymes involved in epigenetic modifications. A very revealing study into the behaviors of rats demonstrated that selective breeding could produce rats that had a higher or lower propensity to take drugs (high and low responders, respectively), such as self-administration of cocaine (Piazza et al., 1989; Piazza et al., 1998). Importantly, these rats were actually bred based on their locomotor response to a novel environment and later determined to have similar behaviors to those found in human addiction. Basal differences between these two lines exist in the form of histone posttranslational modifications affecting mRNA expression of FGF2 (fibroblast growth factor) and D2DR in the NAc (Flagel et al., 2016). Lower FGF2 and D2DR mRNA levels in the low responders were associated with enrichment of the repressive histone modification, H3K9me3, at the *FGF2* and *D2DR* promoters. FGF2 is a neurotrophic growth factor that is essential for neuronal survival, growth, and differentiation (Palmer et al., 1995; Raballo et al., 2000) and has been implicated in psychostimulant locomotor sensitization and drug-mediated plasticity changes (Flores et al., 2000; Fumagalli et al., 2006; Mueller et al., 2006). Furthermore, D2DR was found to have a positive association with cocaine addiction in the candidate gene studies performed in the above-mentioned genetic screens (Noble et al., 1993). Therefore, it will be necessary to examine the cause of the increased H3K9me3 at these promoters and if it exists throughout the genome. The enzymes responsible for writing the mark are histone methyltransferases, SUV39H1 and SETDB1 (Rea et al., 2000; Ryu et al., 2006). Antibodies for both of these enzymes exist and if their expression is specifically increased in the low responding rats' NAc, it might reveal a possible therapeutic intervention. However, if the levels are not increased cell-wide, but instead they are

being recruited to the *FGF2* and *D2DR* promoters specifically, this would provide further evidence for the importance of locus-specific epigenetic investigation in the context of addiction.

Hippocampal FGF2 is important in regulating anxiety and fear learning, as rats who experience lower than normal anxiety and those who freeze less after fear conditioning exhibit higher levels of hippocampal FGF2 (Perez et al., 2009; Graham and Richardson, 2016). When comparing basal levels of FGF2 in the hippocampus, low responders (discussed above), who demonstrate more anxious behavior, have decreased levels compared to high responders, and knockdown of FGF2 resulted in high responders becoming more anxious (Chaudhury et al., 2014). This suggests that hippocampal FGF2 may be regulating anxiety/emotional responses. Future experiments should determine hippocampus subregion specific changes in the basal state of low versus high responders, as well as projection specific differences – specifically projections from the ventral CA1 to NAc. Differences in the basal state, before exposure to drugs of abuse, could further implicate hippocampal functional differences between those who have varying propensities to seek illicit substances.

Dopamine is also involved in the formation of hippocampal-dependent memories (Rossato et al., 2009) and both D1- and D2-type dopamine receptors are engaged in this process (Lemon and Manahan-Vaughan, 2006; Franca et al., 2015). Whether the hippocampal neurons receiving dopamine are the same ones that are influencing NAc activity requires further investigation, and it will be interesting to determine whether these are D1DR or D2DR expressing neurons. Much more needs to be elucidated when it comes to hippocampal D2DR and its regulation, but changes at the level of epigenetics makes for attractive future therapeutics to neuropsychiatric diseases.

FINAL SUMMARY

This thesis presents evidence that hippocampal ΔFosB is epigenetically regulated by chronic cocaine exposure, that this regulation is critical for cocaine responses, and that expression of

ΔFosB is dysregulated in the hippocampus of human addicts. These findings allow us to form a more complex and accurate model of ΔFosB's role in general hippocampal-dependent behavior and drug-related memories. These data will hopefully provide the groundwork for further exploration of the role of epigenetic changes facilitated by drug addiction, as well as the role of long-term drug exposure in rodent models, and contribute to our understand of drug-associated learning and memory, ultimately leading to a better understanding of the etiology, and perhaps treatment, of drug addiction.

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