

# Epigenetic Regulation of Hippocampal *FosB* Expression Controls Behavioral Responses to Cocaine

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Drug addiction results in part from maladaptive learning, including the formation of strong associations between the drug and the circumstances of consumption. However, drug-induced changes in gene expression underlying the saliency of these associations remain understudied. Consolidation of explicit memories occurs within the hippocampus, and we have shown that spatial learning induces expression of the transcription factor  $\Delta$ FosB in hippocampus and that this induction is critical for learning. Drugs of abuse also upregulate  $\Delta$ FosB in hippocampus, but the mechanism of its induction by cocaine and its role in hippocampus-dependent cocaine responses is unknown. We investigated differences in mouse dorsal and ventral hippocampal  $\Delta$ FosB expression in response to chronic cocaine, because these regions appear to regulate distinct cocaine-related behaviors. We found that cocaine-mediated induction of  $\Delta$ FosB was subregion-specific, and that  $\Delta$ FosB transcriptional activity in both the dorsal and ventral hippocampus is necessary for cocaine conditioned place preference. Further, we characterize changes in histone modifications at the *FosB* promoter in hippocampus in response to chronic cocaine and found that locus-specific epigenetic modification is essential for *FosB* induction and multiple hippocampus-dependent behaviors, including cocaine place preference. Collectively, these findings suggest that exposure to cocaine induces histone modification at the hippocampal *FosB* gene promoter to cause  $\Delta$ FosB induction critical for cocaine-related learning.

**Key words:**  $\Delta$ FosB; cocaine; epigenetics; hippocampus; histone; transcription

## Significance Statement

Although cocaine addiction is driven in part by the formation of indelible associations between the drug and the environment, paraphernalia, and circumstances of use, and although this type of associative learning is dependent upon changes in gene expression in a brain region called the hippocampus, the mechanisms by which cocaine alters hippocampal gene expression to drive formation of these associations is poorly understood. Here, we demonstrate that chronic cocaine engages locus-specific changes in the epigenetic profile of the *FosB* gene in the hippocampus, and that these alterations are required for cocaine-dependent gene expression and cocaine–environment associations. This work provides novel insight into addiction etiology and potential inroads for therapeutic intervention in cocaine addiction.

## Introduction

The hippocampus is essential for the formation of drug-related memories, specifically drug–environment associations, and the establishment of these associations requires differential activa-

tion 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 (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). Many previous studies have focused on cocaine-mediated epigenetic changes in the nucleus accumbens (NAc), a component of the brain's reward cir-

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cuitry, 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 dimethyltransferase called G9a, is decreased in NAc by repeated cocaine exposure leading to a decrease in the repressive histone modification, lysine 9 dimethylation of histone H3 (H3K9me2), and this decrease is necessary for plasticity changes in the NAc and subsequent cocaine-related behavior (Maze et al., 2010b; Benevento et al., 2015). Similarly, epigenetic modifications are also important for maintaining normal hippocampal function and plasticity (Sweatt, 2013; Srivas and Thakur, 2017), indicating a potential mechanism by which cocaine may cause changes in plasticity-related hippocampal gene expression. Thus, 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-lasting changes in synaptic morphology and function in the hippocampus (Keralapurath et al., 2014), implicating persistent regulation of gene expression.  $\Delta$ FosB is a stable transcription factor induced by chronic stimuli and studied extensively in the context of addiction (Robison and Nestler, 2011).  $\Delta$ FosB is a splice variant originating from the *FosB* gene but lacking two degron domains, making it uniquely resistant to proteolysis, with a half-life of up to 8 d in the brain (Ulery-Reynolds et al., 2009).  $\Delta$ FosB is induced in many brain regions in response to chronic stimuli, including drugs of abuse (Perrotti et al., 2008), and the mechanism of its induction in the NAc has recently focused on changes in epigenetic factors, specifically H3K9me2 by G9a (Núñez et al., 2010; Damez-Werno et al., 2012; Heller et al., 2014; Hamilton et al., 2018). Additionally, much of what we know about the target genes of  $\Delta$ FosB comes from addiction and depression studies in the NAc, and many of those NAc target genes, such as *CaMKII $\alpha$* , *GluA2*, and *Cdk5* (Kelz et al., 1999; Chen et al., 2000; Robison et al., 2013), regulate synaptic plasticity in the hippocampus. Though it has long been known that  $\Delta$ FosB is induced in dorsal hippocampus by chronic exposure to drugs or stress (Perrotti et al., 2004, 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  $\Delta$ FosB induction in hippocampus by cocaine and the role of hippocampal  $\Delta$ FosB in cocaine reward. Our results provide novel insight into cocaine-mediated epigenetic and transcriptional changes in the hippocampus and how those changes regulate drug-related memories and behaviors, indicating the potential for targeting hippocampal epigenetic and transcriptional mechanisms in the treatment of addiction.

## Materials and Methods

**Animals.** 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 were approved by the Institutional Animal Care and Use Committee at Michigan State University. For chromatin immunoprecipitation (ChIP), immunohistochemistry, and Western blot experiments, mice received daily intraperitoneal (i.p.) injections of either saline or cocaine (20 mg/kg) and were placed into a novel environment (1 chamber of a conditioned place preference apparatus from San Diego Instruments) for 30 min for 10 consecutive days, and were killed 1 or 24 h after the last injection (ChIP, qRT-PCR, Western blot: 1 h; immunohistochemistry: 24 h). Additionally, 30–40 min before any behavioral assay took place, mice were placed into the behavioral room to habituate to the

environment. Open field, novel object recognition, and contextual fear conditioning were performed in battery in that order over 5 d. All other behavioral experiments were performed on individual cohorts of mice.

**Surgery.** Stereotaxic surgery was performed to inject viral vectors into the dorsal or ventral hippocampus of adult male mice. Thirty gauge needles (Hamilton) were bilaterally placed at the following coordinates for dorsal hippocampus: 10° angle, –2.2 mm anteroposterior (AP),  $\pm$ 2.5 mm mediolateral, virus was infused (0.6  $\mu$ l) separately (0.3  $\mu$ l/infusion) over 3 min periods at two sites: –2.1 mm dorsoventral (DV) and –1.9 mm DV; coordinates for ventral hippocampus: 3° angle, –3.2 mm AP,  $\pm$ 3.4 mm mediolateral, virus was infused (0.5  $\mu$ l) over 5 min period at one site: –4.8 DV. After infusion, the needles remained at the injection site for 5 min to allow for diffusion of the viral particles. Previously validated (Robison et al., 2013; Heller et al., 2014) viral vectors included the following: herpes simplex virus (HSV) expressing GFP alone (HSV-GFP), HSV expressing GFP and  $\Delta$ JunD (HSV- $\Delta$ JunD), or HSV expressing GFP and *FosB*-ZFP-G9a (HSV-*FosB*-ZFP-G9a); adeno-associated virus (AAV) expressing GFP alone (AAV-GFP) or AAV expressing GFP and  $\Delta$ JunD (AAV- $\Delta$ JunD). HSVs were obtained from the Massachusetts Institute of Technology Viral Core Facility (now located at Massachusetts General Hospital) and AAVs were obtained from the University of North Carolina Vector Core. Location of injection sites were confirmed using perfusion, brain slicing, and postmortem GFP fluorescence following behavioral analyses. Animals lacking clear GFP fluorescence bilaterally in the target regions were excluded from analysis (n.b., this is the reason for different group sizes in some experiments).

**Open-field activity.** Animals were placed in an open box as previously described (Eagle et al., 2015) and tested for 60 min, which occurred 48 h after stereotaxic surgery. Activity was recorded with a digital CCD camera connected to a computer running automated video tracking software package (Clever Sys). Time spent within the center of the box (the center starts ~9.5 cm from the edge of the wall) and distance moved were measured.

**Novel object recognition.** Novel object recognition (NOR) was assessed using a 3-d paradigm as described previously (Eagle et al., 2015) on the same cohort that underwent open-field (OF) analysis. Briefly, mice which were first habituated to the OF apparatus (see above method), were exposed to two identical objects placed in opposite corners of the OF box 24 h later and were allowed to explore the apparatus for 30 min. Four hours (see Fig. 5D, Short-Term) or 24 hours (all other figures) after initial exposure to the objects, one object was removed and replaced with another dissimilar object, and mice were allowed to freely explore the apparatus for 5 min. Time spent with the objects was scored by a blind observer. Object investigation was defined as the nose of the mouse pointing toward the object when the mouse was no >1 cm away. Automated video tracking software (Clever Sys) was used to analyze total distance moved over the course of the 5 min exploration.

**Contextual fear conditioning.** Contextual fear conditioning (CFC) consisted of a 2 d procedure as described previously (Eagle et al., 2015). The conditioning portion took place in the afternoon following the NOR test (thus, the same cohort of mice were used for OF, NOR, and CFC). For conditioning, mice were placed into an operant chamber for a total of 6 min. Three mild electric footshocks (0.8 mA, 1 s duration), each separated by a 60 s interval, were administered. Twenty-four hours after conditioning, mice were placed back into the conditioning chamber for 8 min. 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 s. Data were analyzed as total time spent freezing (displayed in figures) as well as in 1 min individual blocks across the assay. Groups did not differ in either method of analysis.

**Elevated plus maze.** Elevated plus maze (EPM) behavior was performed as previously described (Eagle et al., 2015). Briefly, 48 h after stereotaxic surgery mice were placed into the center of the maze facing an open arm and behavior was video recorded for 5 min. The percentage of time spent in the open arms and the percentage of entries into the open arms were quantified using the automated video tracking software (Clever Sys).

**Cocaine conditioned place preference.** Conditioned place preference (CPP) consisted of a 4 d protocol as previously described (Iníguez et al.,

2015). Briefly, mice were evaluated for cocaine place preference using three-chambered CPP San Diego Instrument boxes and software. The mice were placed into the three-compartment apparatus on preconditioning day and allowed to freely explore all three chambers for 15 min to obtain baseline preference. The preconditioning trial took place 1–2 d before any stereotaxic surgery to eliminate any stress-evoked biases. Groups were balanced and adjusted for any pre-existing chamber bias, such that total preference for each group was very close to zero before conditioning. Conditioning trials (30 min, 2 per day) were given on 2 consecutive days and began 48 h after stereotaxic surgery. During the conditioning trials mice received an intraperitoneal injection of saline in the morning and were confined to one chamber for 30 min. Four hours later mice received an intraperitoneal injection of cocaine (5 mg/kg) and were confined to the opposite chamber for 30 min. On test day, which took place 24 h after the last conditioning trial, mice were allowed to freely explore all three chambers for 15 min and amount of time spent in each chamber was recorded and used to assess preference. Data were captured by recording movements with a 4 × 6 photobeam array and analyzed using PAS software and the Place Preference Reporter.

**Immunohistochemical assay for FosB.** After 10 d of chronic cocaine (or saline) treatment (see *Animals* section in Materials and Methods), mice were perfused transcardially with ice-cold PBS followed by 10% formalin 24 h after the last injection. Brains were postfixed 24 h in 10% formalin, cryopreserved in 30% sucrose, and then sliced into 35  $\mu$ m sections. Immunohistochemistry was performed using rabbit anti-FosB primary antibody [1:500; FosB (5G4); Cell Signaling Technology, 2251S], and biotinylated goat anti-rabbit secondary (1:1000; BA-1000, Vector Laboratories), and visualized by 3,3'-diaminobenzidine staining (Vector Laboratories).

**Immunofluorescence for FosB.** Mice were perfused transcardially with ice-cold PBS followed by 10% formalin. Brains were postfixed for 24 h in 10% formalin and cryopreserved in 30% sucrose overnight, and then sliced into 35  $\mu$ m sections. Immunofluorescence was performed using anti-FosB primary antibody [FosB (5G4), 1:1000; Cell Signaling Technology, 2251S] and corresponding secondary antibody (Donkey anti-rabbit Cy5, 1:200; Jackson ImmunoResearch, 711-175-152). 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.

**Chromatin immunoprecipitation.** ChIP was performed essentially as described previously (Damez-Werno et al., 2012). Briefly, 1 h after the last injection, freshly dissected whole hippocampi were pooled from two mice for each ChIP and crosslinked with formaldehyde. The material was further sheared and immunoprecipitated using sheep anti-mouse magnetic beads (11201D, Invitrogen) conjugated to an antibody that specifically recognizes H3K9me2 (ab1220, Abcam). Immunoprecipitated DNA was subjected to qRT-PCR analysis to examine enrichment along the *FosB* gene. Levels of chromatin modifications are compared with those for input DNA as described previously (Maze et al., 2010a). Primers used to assess the enrichment of histone posttranslational modifications along the *FosB* gene were as follows:

–1200 Forward: ATGGGACTCAGGTGTCAGG  
 –1200 Reverse: AGCCAGGGCTACACAGAGAA  
 –900 Forward: AGGCTTCCAACACCATCAAG  
 –900 Reverse: TTCTGTGTGACTCGGTGAGC  
 –700 Forward: GCTCACCGAGTACACAGAA  
 –700 Reverse: GCTGGTGAAAAGGCAAGG  
 –500 Forward: GAGTTGCACCTTCTCCAACC  
 –500 Reverse: GGCCCAGTGTGTTGTTGGTA  
 –250 Forward: ATGGCTAATTGCGTCCACAGG  
 –250 Reverse: ACCTCCAAACTCTCCCTTC  
 +1 Forward: GCTCCCGGTTTCATTCATAA  
 +1 Reverse: GCAAAAAGGAAACCCACAAA  
 +250 Forward: TTCTTTCTGTGGCTTCTGG  
 +250 Reverse: TTTTCGATGCAAGTTCCCTCT  
 +500 Forward: CTACTCCGGACTCGCATCTC  
 +500 Reverse: GAAGGAGTCCACCGAAGACA  
 +700 Forward: CGCGAGTCTCAGTACCTGT  
 +700 Reverse: AACCAAAGTGCAAACCGAAC

+1100 Forward: TCCACCCAGCAAGAGTTAGG  
 +1100 Reverse: CTCGGCGTCCAAAATAGAA  
 +1300 Forward: GCTTTAGCCAATCAGCGTTC  
 +1300 Reverse: CAGGTCCACACAAGGTCTCT

**Western blotting.** One hour after the last injection, brains were rapidly extracted on ice and then sliced into 1 mm sections. Whole dorsal hippocampus, ventral dentate gyrus, or ventral CA1 were removed with a 12-gauge punch and immediately frozen on dry ice. Samples were 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, 2251S) and assayed for total protein using Swift Membrane Stain (G Biosciences). Protein was quantified using NIH ImageJ software.

**RNA isolation and qRT-PCR.** One hour after the last injection, brains were rapidly extracted on ice and then sliced into 1 mm sections. Whole dorsal hippocampus, ventral dentate gyrus, or ventral CA1 were removed with a 12-gauge punch and immediately frozen on dry ice. RNA isolation, qRT-PCR and data analysis were performed as described previously (Covington et al., 2009). Briefly, RNA was isolated by TRIzol reagent (Invitrogen) according to the manufacturer's instructions and purified using the RNeasy micro kit (Qiagen, 74004). Quality was checked using a NanoDrop spectrophotometer (ThermoFisher Scientific) with purity of samples confirmed with a 260:280 ratio between 1.8 and 2.1. cDNA was made using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, 4368814). qRT-PCR was conducted with the Bio-Rad CFX Connect RT PCR system with the following parameters: 10 min at 95°C; 40 cycles of 95°C for 1 min, 60°C for 30 s, 72°C for 30 s; graded heating to 95°C to generate dissociation curves for confirmation of single PCR products. Data were normalized to *Gapdh* levels measured in parallel. Data were analyzed by comparing  $C(t)$  values of the treatment condition (saline vs cocaine exposure) with the  $\Delta\Delta C(t)$  method (Robison et al., 2014).

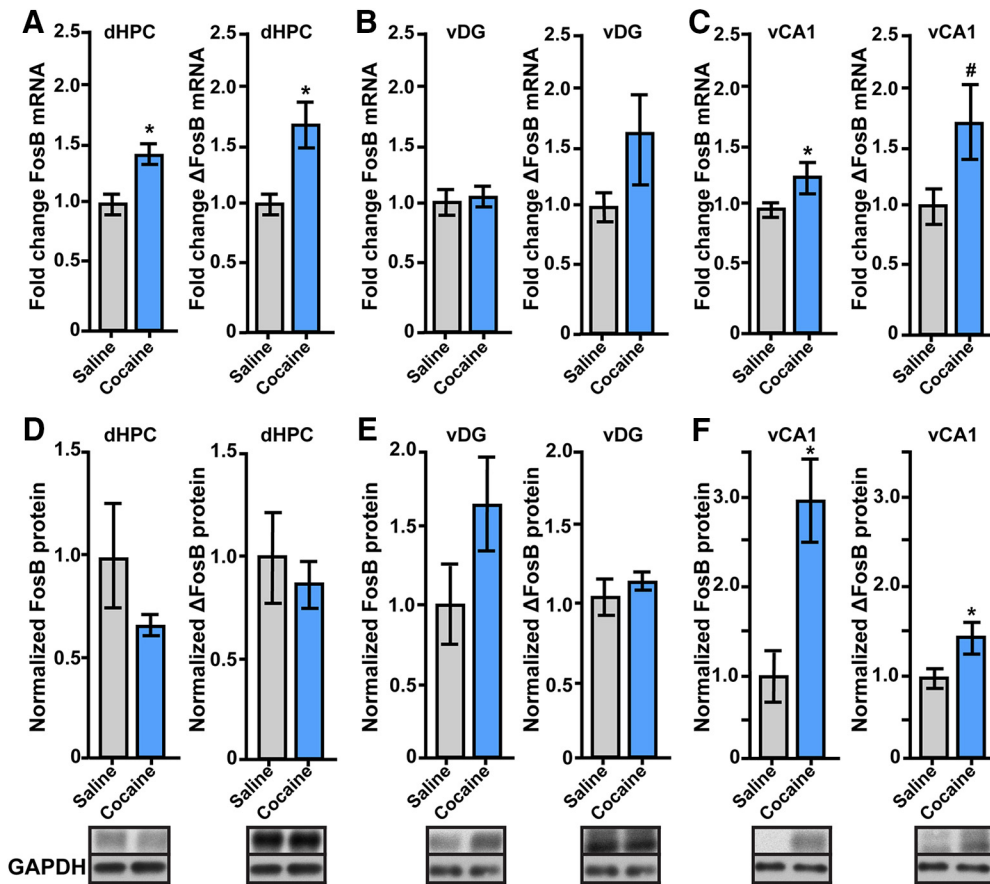
FosB Forward: GTGAGAGATTTGCCAGGGTC  
 FosB Reverse: AGAGAGAAGCCGTCAGGTTG  
 $\Delta$ FosB Forward: AGGCAGAGCTGGAGTCGGAGAT  
 $\Delta$ FosB Reverse: GCCGAGGACTTGAACCTCACTCC  
 GAPDH Forward: AGGTCGGTGTGAACGGATTG  
 GAPDH Reverse: TGTAGACCATGTAGTTGAGGTCA

**Statistical analyses.** All statistical analyses were performed using Prism software (GraphPad Software). Anxiety phenotypic behavior (OF activity and EPM), CFC freezing, protein and mRNA quantification, and locomotor activity were analyzed using unpaired Student's *t* tests. NOR, ChIP, and cocaine CPP were analyzed using two-way (with or without repeated measures) ANOVAs followed by Holm–Sidak-corrected *post hoc* comparisons for simple effects. Alpha criterion of 0.05 was set for all analyses.

## Results

### Induction of $\Delta$ FosB in hippocampal subregions and role in cocaine-related behaviors

Induction of  $\Delta$ FosB has been observed in the hippocampus in response to drugs of abuse such as morphine and cocaine (Perrotti et al., 2008). However, to date 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 min/d for 10 d and compared the expression of FosB isoforms in tissue punches of subregions of dorsal and ventral hippocampus. We were able to separate the ventral CA1 (vCA1) subregion from the rest of the ventral hippocampus [containing primarily CA3 and dentate gyrus (vDG)] because of the size of the ventral hippocampus, thus enriching for hippocampal cells that project from vCA1 to other regions of the brain critical for emotional learning and drug responses (Cooper et al., 2017). Unfortunately, this technique does not allow for the same separation of the dorsal hippocampus, because this region is much smaller. mRNA



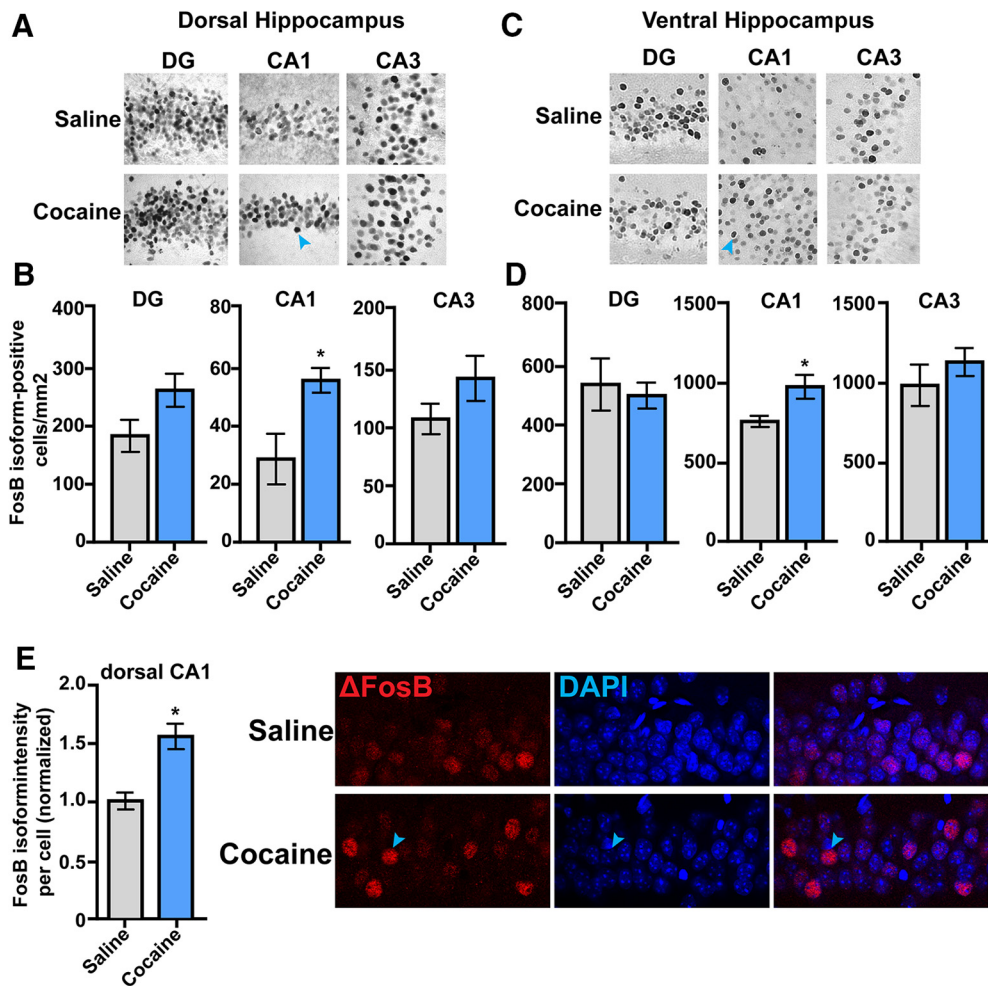
**Figure 1.** Cocaine effects on hippocampal expression of FosB isoforms. **A**, Mice were killed 1 h after the last exposure to saline or cocaine paired with a novel environment. qPCR reveals cocaine-dependent increases in the transcript levels for FosB and  $\Delta$ FosB in whole dorsal hippocampus (dHPC). **B**, Ventral hippocampus was microdissected to isolate the vDG, which displayed no changes in full-length FosB or  $\Delta$ FosB mRNA. **C**, Specific microdissection of vCA1 revealed a trend toward an increase in both FosB and  $\Delta$ FosB mRNA. **D**, dHPC displayed no changes in either FosB or  $\Delta$ FosB protein. **E**, **F**, vDG did not show any significant changes in FosB or  $\Delta$ FosB (**E**), and vCA1 showed significant induction of both FosB and  $\Delta$ FosB (**F**). At the bottom of each graph are representative images from Western blots showing FosB or  $\Delta$ FosB, and GAPDH ( $n = 8$ /group; \* $p < 0.05$ , # $p \leq 0.06$ ).

was isolated to determine expression of each isoform in response to cocaine. There was significantly more FosB and  $\Delta$ FosB mRNA in whole dorsal hippocampus after chronic cocaine exposure (Fig. 1A; FosB  $t_{(14)} = 3.307$ ,  $p = 0.005$ ,  $\Delta$ FosB  $t_{(13)} = 2.608$ ,  $p = 0.021$ ). We also found strong trends toward increases in both isoforms in vCA1 (Fig. 1C; FosB vCA1  $t_{(14)} = 2.112$ ,  $p = 0.053$ ;  $\Delta$ FosB vCA1  $t_{(14)} = 2.026$ ,  $p = 0.062$ ), though changes in vDG were weaker and more variable (Fig. 1B). We processed tissue from the same animals for Western blot to determine whether the relevant protein products were similarly regulated, and we found no significant changes in whole dorsal hippocampus FosB or  $\Delta$ FosB protein levels between cocaine- and saline-treated mice (Fig. 1D). However, in vCA1, both FosB and  $\Delta$ FosB were increased (Fig. 1E; FosB  $t_{(12)} = 3.658$ ,  $p = 0.003$ ,  $\Delta$ FosB  $t_{(12)} = 2.167$ ,  $p = 0.05$ ).

Tissue dissection and Western blot do not allow resolution of cell type and can only crudely enrich for hippocampal subregion. To better assess changes in the neurons of subregions of the hippocampus, we completed the same cocaine exposure protocol and performed immunohistochemistry or immunofluorescence on coronal brain slices. As the antibody we use recognizes both full-length FosB and  $\Delta$ FosB, we killed animals 24 h after the last drug exposure to enrich for the stable  $\Delta$ FosB isoform (Perrotti et al., 2008; Ulery-Reynolds et al., 2009). We found an increase in the number of  $\Delta$ FosB-positive neurons in the CA1 subregion of

dorsal hippocampus after chronic cocaine exposure (Fig. 2A, C;  $t_{(7)} = 3.001$ ,  $p = 0.019$ ), but no significant changes in the dorsal DG or CA3 (Fig. 2A, C). In ventral hippocampus, there was also a significant increase in the number of  $\Delta$ FosB-positive cells in CA1 with no change in DG and CA3 (Fig. 2B, D;  $t_{(12)} = 2.37$ ,  $p = 0.035$ ). We also examined the intensity of  $\Delta$ FosB fluorescence signal per neuron in the dorsal CA1 and found an increase in fluorescence intensity per cell (Fig. 2E;  $t_{(93)} = 4.364$ ,  $p < 0.0001$ ). This suggests that drugs of abuse selectively induce  $\Delta$ FosB in specific hippocampal subregions, both in the number of neurons expressing  $\Delta$ FosB and the amount of  $\Delta$ FosB found in each neuron. We thus hypothesized that this induction may contribute to the formation of the strong associations between drugs and environment.

To determine whether the activity of hippocampal  $\Delta$ FosB is necessary for cocaine–environment associations, we used AAV-mediated expression of  $\Delta$ JunD, a dominant-negative version of  $\Delta$ FosB's obligate binding partner, JunD, to silence  $\Delta$ FosB's transcriptional activity (Robison et al., 2013; Eagle et al., 2015). AAVs expressing GFP alone or GFP and  $\Delta$ JunD were targeted to the dorsal or ventral hippocampus (Fig. 3A, B). Our laboratory has previously examined  $\Delta$ FosB in dorsal hippocampus-mediated general learning and memory and found that  $\Delta$ JunD produced impairments in dorsal hippocampal-dependent tasks (Eagle et al., 2015), but viral expression of  $\Delta$ JunD in ventral hippocampus



**Figure 2.** Cocaine effects on FosB-isoform neuronal expression in dorsal and ventral hippocampal subregions. **A, B**, Representative  $40\times$  images of coronal sections of dorsal (**A**) and ventral (**B**) hippocampus stained for FosB isoforms (and enriched for  $\Delta$ FosB) from mice given saline or cocaine in a novel environment. Arrowheads indicate examples of FosB-isoform+ cells. **C, D**, Cocaine increased FosB-isoform+ cells specifically in the CA1 subregion of dorsal (**C**) and ventral (**D**) hippocampus ( $n = 5$  mice;  $*p < 0.05$ ). **E**, Quantified FosB-isoform intensity per cell in dorsal hippocampus CA1 neurons after chronic cocaine exposure ( $n = 25$ – $60$  cells;  $*p < 0.05$ ). Representative images show FosB-isoforms in red and DAPI in blue. Arrowhead indicates an example cell in which FosB isoforms are induced at high levels following chronic cocaine exposure.

has not been examined. To this end, we first examined the effects of viral expression of  $\Delta$ JunD in ventral CA1 in hippocampal-dependent learning and memory. We observed impairment in NOR (Fig. 3C;  $t_{(22)} = 3.002$ ,  $p = 0.0131$ ), but no change in freezing behavior after CFC (Fig. 3D;  $t_{(25)} = 0.7992$ ,  $p = 0.4317$ ). Freezing behavior was assessed in 1 min bins and no differences were found at any time point (data not shown). There was no significant difference in total distance moved in the NOR test (GFP:  $1104.7 \pm 111.1$  cm;  $\Delta$ JunD:  $1117.8 \pm 64.9$  cm;  $p = 0.92$ ). Because the ventral hippocampus projects to the amygdala (McDonald and Mott, 2017), we also tested anxiety-like behavior using OF and EPM. We observed no change in percentage time spent in center of the OF or percentage time in open arms of the EPM (Fig. 3E,F; OF:  $t_{(22)} = 1.271$ ,  $p = 0.217$ ; EPM:  $t_{(17)} = 0.7788$ ,  $p = 0.4468$ ), indicating that general inhibition of ventral hippocampus  $\Delta$ FosB does not affect anxiety, though this does not rule out a role for  $\Delta$ FosB in the specific projection neurons connecting these regions. We next examined the effect of  $\Delta$ JunD in dorsal or ventral hippocampus on cocaine CPP and saw that expression in either region impaired CPP, because  $\Delta$ JunD-expressing mice did not display a preference for the cocaine-paired chamber, whereas GFP controls did (Fig. 3G,H; dorsal:

$F_{(1,58)} = 5.634$ ,  $p < 0.0001$ ; ventral:  $F_{(1,40)} = 9.9095$ ,  $p < 0.01$ ). These data suggest that  $\Delta$ FosB function in both dorsal and ventral hippocampus is critical for mediating conditioned place preference.

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

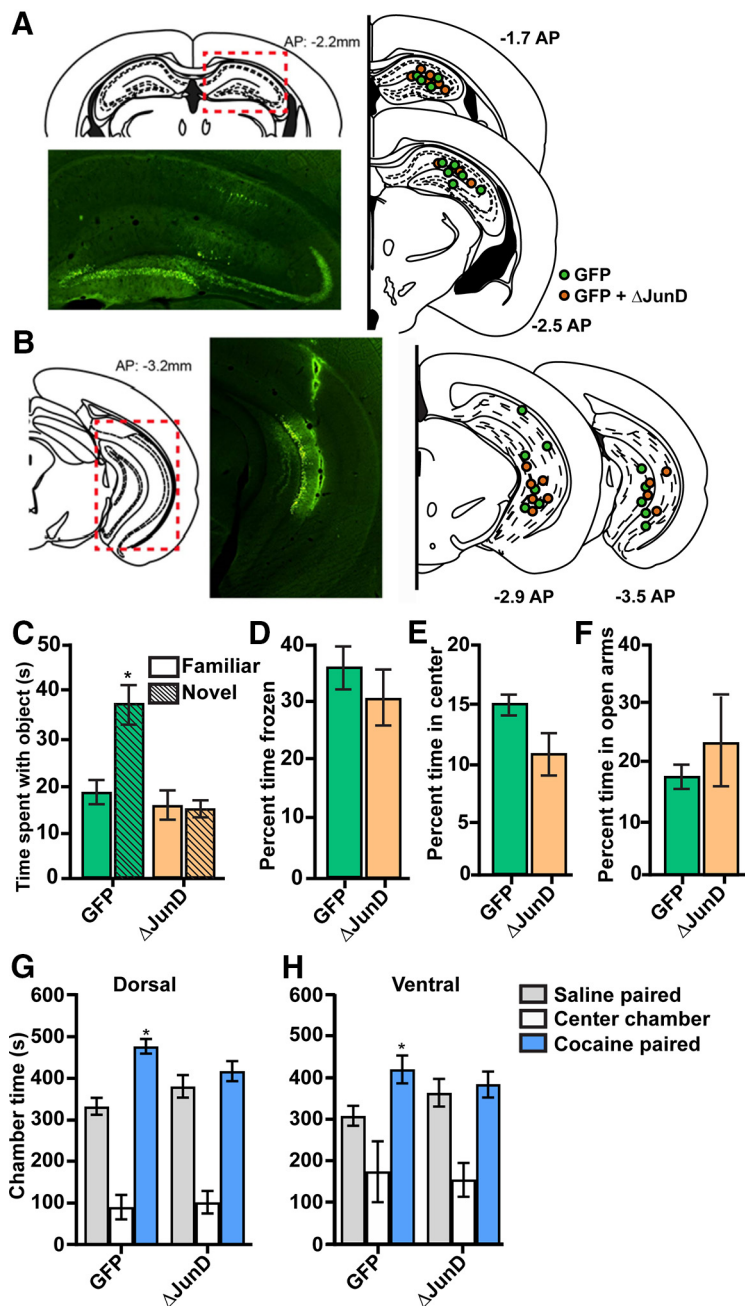
Induction of  $\Delta$ FosB in the NAc after cocaine exposure is mediated in part by a decrease in H3K9me2 at the *FosB* gene (Damez-Werno et al., 2012). Therefore, we assessed whether cocaine-mediated hippocampal  $\Delta$ FosB induction was also facilitated by this modification. We conducted ChIP from whole hippocampus and found a cocaine-mediated decrease in H3K9me2 at multiple points along the *FosB* promoter and gene body (Fig. 4A; RM ANOVA,  $F_{(1,11)} = 8.296$ ,  $p = 0.015$  for cocaine vs saline main effect). This suggests that the *FosB* gene is typically enriched with H3K9me2, and that this mark is reduced after cocaine exposure to induce transcription (Fig. 4B, top).

To determine whether changes in H3K9me2 are necessary for cocaine-mediated hippocampal  $\Delta$ FosB induction and the subsequent hippocampal-dependent behavioral outputs, we used a viral vector that catalyzes H3K9me2 specifically at the *FosB* pro-

motor (Heller et al., 2014). This HSV expresses a zinc finger protein (ZFP) designed to bind solely at the *FosB* promoter fused to the catalytic domain of the histone dimethyltransferase, G9a (Fig. 4B, bottom), and decreases *FosB* gene expression in NAc (Heller et al., 2014; Hamilton et al., 2018). To validate this effect in hippocampus, we measured  $\Delta$ FosB immunofluorescence after either HSV-GFP or HSV-*FosB*-ZFP-G9a viral infection into the dorsal hippocampus and saw that HSV-*FosB*-ZFP-G9a prevented the cocaine-mediated increase in the intensity of  $\Delta$ FosB signal (Fig. 4C,D;  $F_{(5,192)} = 14.29$ ,  $p < 0.0001$ ). This suggests that enrichment of H3K9me2 at the *FosB* promoter inhibits the cocaine-mediated induction of  $\Delta$ FosB.

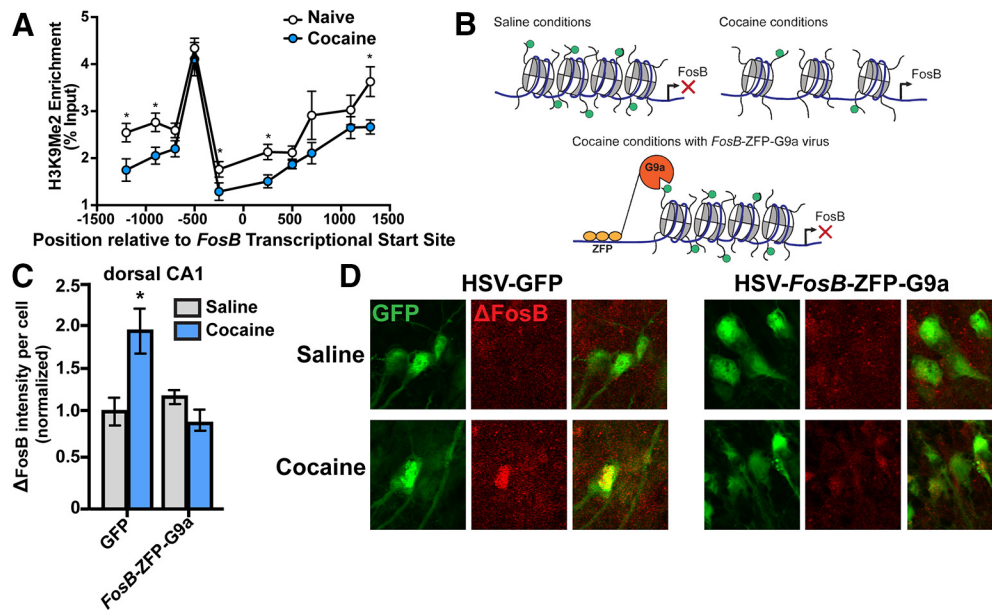
General silencing of  $\Delta$ FosB transcriptional activity in dorsal hippocampus using the dominant-negative  $\Delta$ JunD leads to an impairment of hippocampal-dependent learning and memory tasks (Eagle et al., 2016). We injected HSV-*FosB*-ZFP-G9a into dorsal hippocampus and saw similar impairments in learning and memory to those observed with  $\Delta$ JunD. Mice exhibited an impairment in novel object recognition (Fig. 5A;  $t_{(16)} = 2.817$ ,  $p = 0.012$ ) with no changes in CFC or locomotor activity (Fig. 5B,C; CFC:  $t_{(11)} = 0.1657$ ,  $p = 0.8714$ ; activity:  $t_{(14)} = 0.6456$ ,  $p = 0.5290$ ). There was no significant difference in total distance moved in the NOR (GFP:  $1093.6 \pm 106.1$  cm; G9a:  $997.1 \pm 55.2$  cm;  $p = 0.43$ ). We also show that HSV-*FosB*-ZFP-G9a injection into dorsal hippocampus had no effect on short-term memory, because novel object recognition performed only 4 h after familiarization with objects was unaffected (Fig. 5D). When the *FosB*-ZFP-G9a virus was targeted to the ventral hippocampus, we also saw impairments in novel object recognition (Fig. 5E;  $t_{(36)} = 2.386$ ,  $p = 0.022$ ) with no changes in CFC or locomotor activity (Fig. 5F,G; CFC:  $t_{(7)} = 0.1109$ ,  $p = 0.9148$ ; activity:  $t_{(9)} = 0.2173$ ,  $p = 0.8328$ ). Freezing behavior was assessed in 1 min bins, and no significant differences were found at any time points (data not shown). There was no significant difference in total distance moved in the NOR (GFP:  $1194.8 \pm 76.3$  cm; G9a:  $1037.1 \pm 118.8$  cm;  $p = 0.28$ ). These data suggest that increasing H3K9me2 at the *FosB* promoter can prevent *FosB*/ $\Delta$ FosB-driven hippocampus-dependent behaviors.

Because cocaine mediates a decrease of H3K9me2 at the *FosB* gene in hippocampus, we investigated whether H3K9me2 deposition at the *FosB* gene is sufficient to impair hippocampus-dependent cocaine–environment associations. Mice were injected with HSV-GFP or HSV-*FosB*-ZFP-G9a into dorsal or ventral hip-

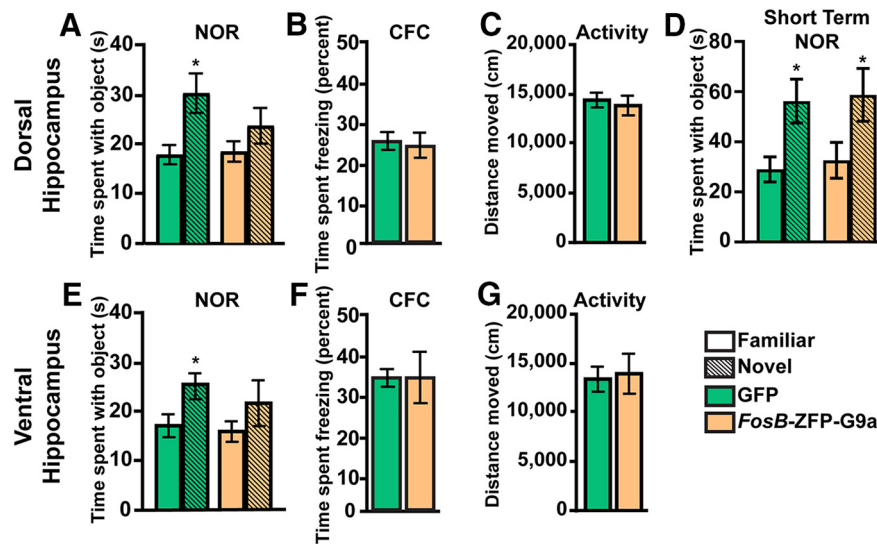


**Figure 3.** Transcriptional silencing of hippocampal  $\Delta$ FosB impairs cocaine conditioned place preference. **A**, Example of dorsal hippocampal targeting of viral-mediated GFP expression (left) and injection sites of a representative cohort of dorsal hippocampal surgeries with AAV-GFP or AAV- $\Delta$ JunD (right). **B**, Example of ventral hippocampal targeting (left) and injection sites (right). **C**, Mice received ventral hippocampal infusions of AAV-GFP or AAV- $\Delta$ JunD. GFP mice spent significantly more time with the novel object than the familiar object, whereas the  $\Delta$ JunD mice showed impaired novel object recognition. There was no difference between ventral hippocampus AAV-GFP or AAV- $\Delta$ JunD in CFC freezing behavior (**D**), center zone time in OF (**E**), or open arm time in EPM (**F**;  $n = 6-7$ ; \* $p < 0.05$ ). Mice were injected in dorsal (**G**) or ventral (**H**) hippocampus with HSV-GFP or HSV- $\Delta$ JunD and subjected to cocaine CPP (5 mg/kg, i.p.). Control mice formed a significant preference for the cocaine-paired chamber, which was blocked by HSV- $\Delta$ JunD in either hippocampal region ( $n = 8$ , \* $p < 0.05$ ).

pocampus, allowed to recover for 24 h, and then exposed to cocaine and saline conditioning for 2 d. Mice that received *FosB*-ZFP-G9a in either the dorsal or ventral hippocampus displayed impaired cocaine CPP (Fig. 6A,B; dorsal:  $F_{(1,42)} = 5.436$ ,  $p = 0.025$ ; ventral:  $F_{(1,36)} = 4.048$ ,  $p = 0.0496$ ). Thus, cocaine-mediated epigenetic changes at the *FosB* promoter in both dorsal and ventral hippocampus appear necessary for mediating cocaine conditioned place preference.



**Figure 4.** Reduction of H3K9me2 at the *FosB* promoter is required for cocaine induction of  $\Delta$ FosB in ventral hippocampus. **A**, Mice were injected with cocaine (20 mg/kg, i.p.) or saline and placed in a novel environment for 30 min/d for 10 d. Chromatin immunoprecipitation for H3K9me2 was conducted on microdissected hippocampus, which was taken 1 h after the last injection. Significant decreases in H3K9me2 enrichment were found at multiple sites along the *FosB* gene ( $n = 8$ ;  $*p < 0.05$ ). **B**, Diagram of histone posttranslational modifications at the *FosB* gene before and after cocaine exposure, and depicting locus-specific H3K9me2 deposition at the *FosB* promoter. Graphs (**C**) and representative images (**D**) of ventral hippocampus CA1 neurons in saline and cocaine-treated mice.  $\Delta$ FosB (red) was significantly increased by cocaine in neurons of mice injected with HSV-GFP, but not HSV-*FosB*-ZFP-G9a (green;  $n = 16$ –30;  $*p < 0.05$ ).



**Figure 5.** Epigenetic remodeling by *FosB*-ZFP-G9a in dorsal and ventral hippocampus impairs general learning and memory. **A**, Dorsal hippocampus HSV-GFP mice spent significantly more time with the novel object than the familiar object, whereas *FosB*-ZFP-G9a mice spent a similar amount of time with both objects ( $n = 8$ –18;  $*p < 0.05$ ). **B**, After CFC, *FosB*-ZFP-G9a mice spent the same amount of time freezing as GFP controls. **C**, There was no difference locomotor activity between groups. **D**, Short-term memory, as measured by time with the novel object only 4 h after familiarization, was not affected *FosB*-ZFP-G9a expression in dorsal hippocampus. **E**, Ventral hippocampus HSV-GFP mice spent significantly more time with the novel object than the familiar object, whereas *FosB*-ZFP-G9a mice spent a similar amount of time with both objects. There were no differences in CFC (**F**) or locomotor activity (**G**;  $n = 8$ –18;  $*p < 0.05$ ). **G**, There was no difference locomotor activity between groups.

## Discussion

One of the critical obstacles in treatment of addiction is the strength and persistence of drug-associated memories, implicating a role for dysregulated hippocampal activity in addiction. Here, we present the first quantitative evidence for cocaine-mediated induction of *FosB* isoforms within specific subregions of the hippocampus and altered histone posttranslational modifications

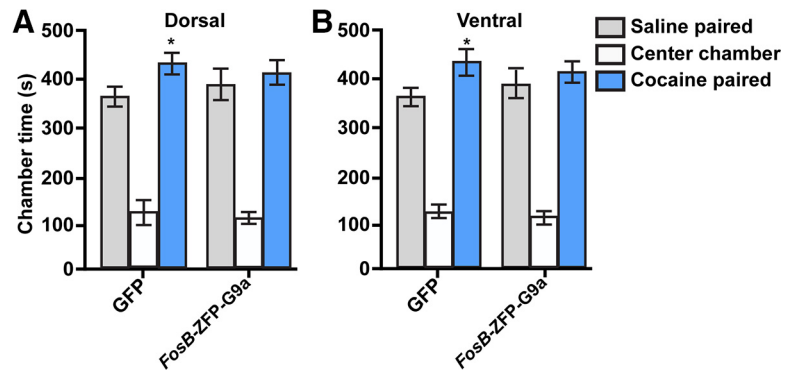
associated with this induction. Both  $\Delta$ FosB induction and cocaine CPP were blocked by viral overexpression of the *FosB*-ZFP-G9a fusion protein in hippocampus. This indicates that enrichment of H3K9me2 at the *FosB* promoter is sufficient to suppress  $\Delta$ FosB expression and subsequent cocaine-environment associations.

The hippocampus is essential in the formation of associations concerning cues and events in a drug context (Fuchs et al., 2005). 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), and indeed, drug-associated stimuli can induce craving and relapse even during abstinence (Gawin and Kleber, 1986). 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.

Induction of full-length *FosB* and  $\Delta$ FosB in the dorsal hippocampus has been previously reported with both experimenter- and self-administered cocaine, as well as stress and antidepressants (Perrotti et al., 2008; Vialou et al., 2015). Here, we used 20 mg/kg cocaine intraperitoneal injection to assess induction of *FosB* and  $\Delta$ FosB in dorsal and ventral hippocampus subregions, because this dose is well known to cause robust induction throughout the brain (Perrotti et al., 2008; Robison et al., 2013). It is important to note that the lower dose of cocaine (5 mg/kg) used in CPP was chosen to allow

us to observe either increased or decreased cocaine preference, because a ceiling effect is reached when mice are given higher doses of cocaine (Kelz et al., 1999). Induction in both dorsal and ventral hippocampus CA1 by chronic intraperitoneal cocaine is unsurprising as CA1 neurons undergo a variety of changes in response to cocaine (Miguéns et al., 2015; Kerala-purath et al., 2017), and also play key roles in circuitry underlying associations of salient stimuli with environmental cues. The dorsal CA1 is especially important for contextual encoding and contextual-dependent retrieval compared with the other subregions (Ji and Maren, 2008; Zhu et al., 2014). Ventral CA1 projects to reward circuitry, including NAc and parts of the cortex, and these circuits regulate many addiction-related behaviors including cue- and context-induced drug seeking, from heroin (Wang et al., 2018) to cocaine (Pascoli et al., 2014). Thus, the CA1 may be uniquely positioned to modulate the role of memory in drug addiction (Zornoza et al., 2005).

Using immunohistochemistry, previous studies showed qualitative increases in *FosB* gene products in dorsal hippocampus after cocaine (Perrotti et al., 2008). Our initial Western blots did not reveal  $\Delta$ FosB induction in dorsal hippocampus after cocaine exposure. However, transcript levels of *FosB* and  $\Delta$ FosB were increased in whole dorsal hippocampus, 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. It is also possible that current *FosB* antibodies lack the sensitivity and/or specificity to uncover subtle changes in  $\Delta$ FosB levels, and production of better antibodies for *FosB* isoforms is a current goal for the field. Moreover, the *FosB* gene is expressed in many subtypes of neurons as well as in microglia (Nomaru et al., 2014), so it is possible that at the time of kill (1 h after the last injection), protein levels may be increased in one cell type, whereas mRNA is increased in a different cell type. Furthermore, the use of tissue punches and qRT-PCR or Western blot do not allow us to distinguish between cell type or subregion, and thus the use of immunohistochemistry was critical to determine whether the changes in protein we observed were occurring in neurons relevant to the behaviors we are studying (i.e., CA1 pyramidal neurons). Increases in both *FosB* and  $\Delta$ FosB protein became apparent when ventral CA1 was specifically targeted for dissection, and further examination using immunohistochemistry revealed increases in  $\Delta$ FosB-positive cells in the dorsal and ventral CA1, presumably in pyramidal neurons based on the location of the staining. The dorsal CA1 subregion makes up a relatively small portion of the whole dorsal hippocampus, which may explain the lack of induction of *FosB*/ $\Delta$ FosB seen in the Western blot analysis. Increases in the number of  $\Delta$ FosB+ cells in the dorsal CA1 has also been observed after mice go through spatial learning via the Morris water maze (Eagle et al., 2015), suggesting that both general and drug-related learning may be mediated by dorsal CA1  $\Delta$ FosB. It is important to note that the immunohistochemical staining experiments were performed on animals killed 24 h after their last cocaine injection, whereas ChIP, qPCR, and Western blot experiments were performed on animals killed 1 h after the last injection. The 24 h time point was chosen for staining as it allows for relative enrichment for  $\Delta$ FosB over other *FosB* isoforms (Perrotti



**Figure 6.** Epigenetic remodeling by *FosB*-ZFP-G9a in dorsal and ventral hippocampus impairs cocaine CPP. Mice were injected in dorsal or ventral hippocampus with HSV-GFP or HSV-*FosB*-ZFP-G9a and were subjected to cocaine CPP (5 mg/kg, i.p.). Control mice formed a significant preference for the cocaine-paired chamber, which was blocked by injection of HSV-*FosB*-ZFP-G9a in dorsal (A) or ventral (B) hippocampus. Center time is also displayed (white bar), with no significant differences between the groups ( $n = 6-15$ ; \* $p < 0.05$ ).

et al., 2008; Ulery-Reynolds et al., 2009; Cates et al., 2014; Vialou et al., 2015). It is possible that differential transcriptional and epigenetic machinery could be engaged at these two time points, but the induction of  $\Delta$ FosB at both time points is relevant, especially as the hippocampus plays a significant role in cocaine withdrawal-related behaviors (Ary and Szumlinski, 2007).

Induction of  $\Delta$ FosB in ventral CA1 may be indicative of increased activity of those neurons that project to reward circuitry. Exposure to repeated cocaine and subsequent stimulation of the ventral hippocampus enhances dopamine release in NAc, suggesting that ventral CA1 neurons may regulate the effects of cocaine exposure on reward processing (Barr et al., 2014). Additionally, ventral hippocampal stimulation induces cocaine seeking in a self-administration paradigm even after extinction (Vorel et al., 2001), and synaptic plasticity at ventral CA1–NAc synapses is critical for cocaine seeking (Pascoli et al., 2014). Because we know that  $\Delta$ FosB regulates glutamatergic synapse function in NAc MSNs (Grueter et al., 2013), it is possible that cocaine induction of  $\Delta$ FosB ventral CA1 neurons projecting to NAc may alter synaptic function to regulate drug-related learning.

Although we have known for decades that chronic exposure to cocaine induces  $\Delta$ FosB in the brain, the mechanisms of that induction are only now becoming clearer. H3K9me2 is decreased at the *FosB* gene in the NAc of rats after withdrawal from chronic cocaine, 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 in NAc may be occurring specifically because of the decrease in H3K9me2 in the promoter region. Critically, H3K9me2 at the *FosB* gene is indeed sufficient to impair cocaine-mediated induction of *FosB* isoforms in NAc (Heller et al., 2014), and our data support the same conclusion in hippocampus. This histone tail modification is written by the histone methyltransferase G9a, which is also regulated in NAc by cocaine (Maze et al., 2010b), and which plays an important role in hippocampal gene activation and silencing during memory consolidation (Gupta-Agarwal et al., 2012). H3K9me2 is typically recognized by the silencing factor heterochromatin protein 1 (HP1), which then modifies the structure of local chromatin to regulate gene expression (Nishibuchi and Nakayama, 2014), but the role of HP1 in hippocampal function has not been well characterized. Future studies will investigate whether these upstream and downstream mediators of H3K9me2 are regulated in hippocampus in response to



cocaine, and whether interference with their function affects *FosB* gene induction or cocaine–environment associations.

Previous work from our laboratory silenced dorsal hippocampal *FosB*/ $\Delta$ *FosB* activity by overexpression of  $\Delta$ JunD, and this caused impairment in a variety of learning tasks (Eagle et al., 2015). Interestingly, whereas  $\Delta$ JunD impaired CFC, causing the mice to freeze less when exposed to the fear context (Eagle et al., 2015), the *FosB*-ZFP-G9a virus did not result in impaired fear learning (though both impaired recognition memory). This may be because of subtle differences in the two methodologies. For example, whereas  $\Delta$ JunD inhibits all  $\Delta$ *FosB* in the cell, overexpression of ZFP-G9a affects the *FosB* gene, thus preventing new  $\Delta$ *FosB* from being produced, leaving existing  $\Delta$ *FosB* available; a critical point, because  $\Delta$ *FosB* is quite stable (Ulery-Reynolds et al., 2009). This suggests that existing  $\Delta$ *FosB* may be sufficient for fear memory formation, whereas new  $\Delta$ *FosB* production may be necessary for recognition memory. Additionally, overexpression of  $\Delta$ JunD in the ventral hippocampus showed similar behavioral impairments to those observed with expression of ZFP-G9a-*FosB* in that region, with impairments in novel object recognition, and no changes in freezing behavior after CFC. The lack of change in fear memory is a surprising result, because the ventral hippocampus has projections to the amygdala that influence fear memory (Xu et al., 2016). However, the effects of  $\Delta$ *FosB* may differ by projection, or the transduction of many cell types throughout the ventral hippocampus by our viral vectors may be offsetting cell-type-specific effects.

Finally, we observed that preventing  $\Delta$ *FosB* induction or function in either the dorsal or ventral hippocampus impaired cocaine CPP. In contrast, lesion experiments suggest that dorsal, but not ventral, hippocampus is necessary for cocaine CPP (Meyers et al., 2003). However, these studies remove entire brain regions and associated circuits, whereas our manipulations of  $\Delta$ *FosB*, and presumably downstream gene target expression, are much subtler and may engage circuits or mechanisms that affect CPP expression. Some of the known gene targets of  $\Delta$ *FosB*, including glutamate receptors and calcium/calmodulin-dependent protein kinase II (Vialou et al., 2010; Robison et al., 2013), are directly tied to hippocampal neuronal function and to addiction (Robison, 2014; Spencer et al., 2016). Future studies will interrogate the expression of  $\Delta$ *FosB* in specific projection neurons, as ventral CA1 projections to NAc may provide a unique substrate for the integration of memory and drug-seeking behavior (Vorel et al., 2001; Pascoli et al., 2014).

The studies presented here provide strong evidence for the importance of histone modifications at the *FosB* gene in hippocampal induction of  $\Delta$ *FosB* by cocaine and the critical role of this epigenetic regulation in subsequent drug responses. Overall, these results support a model in which exposure to cocaine alters the epigenetic landscape of the hippocampal *FosB* gene, inducing expression and driving persistent memories and behaviors that may contribute to cocaine addiction.

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