Disorders of the Nervous System

**Fosb Induction in Nucleus Accumbens by Cocaine Is Regulated by E2F3a**

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**Abstract**

The transcription factor ΔFosB has been proposed as a molecular switch for the transition from casual, volitional drug use into a chronically addicted state, but the upstream regulatory mechanisms governing ΔFosB expression are incompletely understood. In this study, we find a novel regulatory role for the transcription factor E2F3, recently implicated in transcriptional regulation by cocaine, in controlling ΔFosB induction in the mouse nucleus accumbens (NAc) following cocaine administration. We find that an E2F consensus sequence 500 bp upstream of the Fosb transcription start site is enriched for E2F3 specifically over other E2F isoforms. We further conclude that ΔFosB expression is regulated specifically by E2F3a, not E2F3b, that E2f3a expression is specific to D1 receptor-expressing medium spiny neurons, and that E2F3a overexpression in NAc recapitulates the induction of Fosb and ΔFosb mRNA expression observed after chronic cocaine exposure. E2F3a knockdown in NAc does not abolish ΔFosB induction by cocaine, a result consistent with previously published data showing that singular knockdown of upstream regulators of ΔFosB is insufficient to block cocaine-induced expression. Finally, to elucidate potential combinatorial epigenetic mechanisms involved in E2F3a’s regulation of Fosb, we explore H3K4me3 enrichment at the Fosb promoter and find that it is not enhanced by E2F3a overexpression, suggesting that it may instead be a pre-existing permissive mark allowing for E2F3a to interact with Fosb. Together, these findings support a role for E2F3a as a novel, upstream regulator of the addiction-mediating transcription factor ΔFosB in NAc.

**Key words:** addiction; cocaine; E2F3a; ΔFosB; nucleus accumbens; transcription

**Significance Statement**

A hallmark question in the neuroscience of addiction is how repeated drug use is translated into long-lasting changes in neural regulation. Long-lasting changes in behavior are mediated in part by intracellular transcriptional mechanisms which affect gene expression and neural circuitry. Therefore, pinpointing signaling pathways downstream of abused drugs is a high priority for understanding the addiction process. To this end, we identify and characterize E2F3a as a novel regulator of ΔFosB, a transcription factor widely studied as a molecular switch for the transition from casual drug use to addiction.

**Introduction**

Drug addiction is a partly heritable disorder, with ~50% of risk explained by genetic sequence variations (Meyre and Mayhew, 2017). Thus, many preclinical and clinical studies have focused on epigenetic effects and gene expression differences induced by chronic exposure to drugs of abuse. The transcription factor ΔFosB, an alter-
native splice variant encoded by the \textit{Fosb} gene, accumu-
lates in nucleus accumbens (NAC) after repeated exposure to virtually all abused substances and thus has been proposed as a molecular switch for the transition from casual drug use into the chronically addicted state by reorganizing gene expression \cite{Nestler, 2015}. This phenomenon positions ΔFosB and its encoding gene \textit{Fosb} as unique therapeutic targets.

Numerous studies have demonstrated the necessity and sufficiency of ΔFosB for molecular and behavioral responses to cocaine and other stimulants, and have focused on downstream genes controlled by ΔFosB induction in stimulant addiction \cite{Hiroi et al., 1997; Carlezon et al., 1998; Kelz et al., 1999; McClung and Nestler, 2003; Renthal et al., 2008, 2009; Maze et al., 2010; Grueter et al., 2013; Heller et al., 2014}. However, little is known about how ΔFosB induction itself is regulated down-stream of drug action. Clarifying mechanisms of ΔFosB induction in the context of stimulant addiction is critical for further understanding how this transcription factor contributes to the pathogenesis of addiction. To this end, this study uses cocaine exposure to investigate a putative transcriptional mediator of ΔFosB induction in NAC.

A prior study that used ChIP-seq and RNA-seq in parallel to investigate unbiased, genome-wide transcriptional mechanisms underlying persistent changes in gene expression in mouse NAC after repeated cocaine administration identified the E2F family of transcription factors as among the most highly implicated upstream regulator of gene expression in the context of stimulant addiction \cite{Feng et al., 2009}. A more recent study showed that E2F3a, one of two E2F3 isoforms, mediates a substantial portion of changes in gene expression and alternative splicing in NAC after chronic cocaine exposure as well as promotes behavioral actions of the drug \cite{Cates et al., 2018b}. Given these findings, we hypothesized that E2F3a might also regulate ΔFosB expression and splicing.

ΔFosB arises via an alternative splicing event of \textit{Fosb} mRNA in which Exon IV is truncated \cite{Alibhai et al., 2007; Fig. 1A}. Because two degradation domains are cleaved in the truncation of Exon IV, ΔFosB is unusually stable and accumulates on repeated \textit{Fosb} induction \cite{Nye and Nestler, 1996; Carle et al., 2007}. Furthermore, phosphorylation of Ser27 in ΔFosB following cocaine exposure leads to further enhanced stability \cite{Ulery-Reynolds et al., 2009}. Although some factors leading to ΔFosB’s induction and accumulation have been elucidated—polypyrimidine tract binding protein 1 (PTB; encoded by \textit{Ptbp1}), cAMP response element binding protein (CREB), serum response factor (SRF), and early growth response factor 3 (EGR3; Marinescu et al., 2007; Vialou et al., 2012; Chandra et al., 2015)—many of the upstream regulators and mechanisms governing ΔFosB expression following drug exposure remain to be clarified. Here, we examine the influence of E2F3a on ΔFosB expression in four brain regions related to processing the molecular and behavioral effects of cocaine: NAC, caudate–putamen (CPu), ventral tegmental area (VTA), and prefrontal cortex (PFC). Our goal is to gain insight into the regulation of this key upstream regulator of cocaine-mediated neural and behavioral changes.

E2F transcription factors are involved in chromatin modification, gene regulation, and RNA processing via direct interaction with and recruitment of histone modifying enzymes and splicing machinery, as well as binding directly to splice sites and influencing the expression of splicing factors \cite{Lees et al., 1993; Ahlander and Bosco, 2009}. E2F3 has two alternative splice forms, E2F3a and E2F3b, which have been shown to be activating or repressive under different conditions \cite{Leone et al., 2000}. Like all E2F family members, E2F3a and E2F3b share a canonical consensus sequence “TTTCGCGC” \cite{Reichel et al., 1987; Hume et al., 2015}. Here, we demonstrate that E2F3a is a key upstream regulator of \textit{Fosb}, and induces expression of the canonical addiction-mediating transcription factor, ΔFosB. Specifically, we show that E2F3 binds at an E2F consensus sequence 500 bp upstream of the \textit{Fosb} transcription start site (TSS) and that E2F3a, but not E2F3b, expression is increased in dopamine receptor type 1 (D1)-expressing medium spiny neurons (MSNs) following cocaine exposure. Further, overexpression of E2F3a, but not E2F3b, recapitulates induction of ΔFosB as seen with repeated cocaine administration. These data establish a novel role for E2F3a in the brain in regulating the molecular response to cocaine, specifically in regulating expression of the transcription factor ΔFosB in NAC.

Materials and Methods

Animals and treatments

C57BL/6J male mice \cite{The Jackson Laboratory}, 7–8 weeks old and weighing 25–30 g, were habituated to the animal facility 1 week before use and maintained at 22–25°C on a 12 h light/dark cycle. All animals had access to food and water \textit{ad libitum}. All animal procedures were performed in accordance with guidelines of the Institutional Animal Care and Use Committee at the Icahn School of Medicine at Mount Sinai.

For quantitative chromatin immunoprecipitation (qChIP) assays and gene expression measures following fluorescence activated cell sorting (FACS), mice were injected intraperitoneally with saline or cocaine (20 mg/kg) once daily for 7 days in their home cage. Mice were then euthanized 24 h following the final cocaine injection.

For assaying the effects of viral-mediated E2F3a knockdown, mice were injected intraperitoneally with saline once daily for 4 d beginning 2 d post-viral infusion. On the fifth day, animals were either injected with a final dose of saline or a single, acute dose of cocaine (20 mg/kg) and euthanized 1 h later.
Viral-mediated gene transfer

Mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) and prepared for stereotactic surgery. Thirty-three gauge syringe needles (Hamilton) were used to bilaterally infuse 0.5 μl virus in NAc at a rate of 0.1 μl/min at 1.6 mm anterior, 1.5 mm lateral, and 4.4 mm ventral from bregma at a 10° angle, or in PFC at 1.8 mm anterior, 0.75 mm lateral, and 2.7 mm ventral from bregma at a 15° angle. We used bicistronic p1005 HSV vectors expressing GFP alone or GFP plus E2f3a or E2f3b for overexpression experiments, or HSV vectors that expressed GFP as well as microRNAs targeted against LacZ or E2f3a for knockdown experiments. In these systems, GFP expression is driven by a cytomegalovirus promoter, whereas the select gene of interest is driven by the IE4/5 promoter (Maze et al., 2010). Viral expression was confirmed during tissue collection using fluorescence microscopy (Leica) to visualize GFP and confirm targeting of NAc or PFC. The effectiveness of the overexpression and knock-out vectors in NAc has been published (Cates et al., 2018b) and replicated here (data not shown).

Tissue collection

Tissue was collected at the appropriate time point following pharmacological or viral manipulation via cervical dislocation followed by dissection of NAc with a 14-gauge blunt needle for cocaine studies, or a 15-gauge needle for viral studies to ensure only virally infected tissue was dissected. PFC was dissected with a single central 12-gauge punch for cocaine studies, or a 14-gauge punch for viral studies. Tissue was immediately frozen on dry ice. Single animal samples were used in all RNA-qPCR experiments. For qChIP experiments, animals were pooled as described below.

Quantitative chromatin immunoprecipitation

qChIP was performed on NAc or PFC punches, pooled from three mice, 72 h after viral infection, or from two mice, 24 h after the last drug treatment. qChIP methodology was modified from Ferguson et al. (2015). Chromatin was prepared by fixation in 940 μl 1% formaldehyde for 12 min followed by addition of 64 μl 2% glycine to stop the fixing reaction for 5 min. Punches were then washed 5 times in cold PBS, and passed through a 21-gauge syringe 10 times to homogenize the tissue. The homogenized tissue was centrifuged at 1250 rcf, and then resuspended and incubated in a 0.5% NP-40 cellular lysis buffer for 15 min, centrifuged at 2700 rcf and resuspended in a 1% SDS nuclear lysis buffer for 10 min. The samples were then sheared using a Diagenode Bioruptor.
Table 1. ChIP-qPCR Primers along the Fosb gene

| Location on Fosb | Forward | Reverse |
|------------------|---------|---------|
| −1250            | ATGGGACTCAGGTGGTCAGG | AGGCCAGGGCTACACAGAGAA |
| −500             | GAGTTGACCTTCTCCAACC | GGCCAGTGTTTGTTTGTGTA |
| −250             | ATGGCTAATTCGCAGTCAAG | ACCCTCCAAAACCTCCCTTCT |
| Exon IV          | CAACCTGACGCTTCTCTCT | CGGTTTTGTTTGTTTGTGTA |

These qChIP primers were used to amplify three putative E2F consensus sequences in Fosb: 500 and 250 bp upstream of the TSS, and a site within Exon IV. A sequence 1250 bp upstream of the TSS was used as a negative control.

Table 2. RT-qPCR Primers for Fosb transcripts and Gapdh for probing mRNA expression

| Transcript | Forward | Reverse |
|------------|---------|---------|
| Fosb       | GTGAGAGATTTCGCCAGGGTC | AGAGAGAGGCGTGCTAGGTTG |
| ΔFosb      | AGGCCAGAGCTGAGTCGGAG | GCCGGAGACCTTGAACTCCCTG |
| Gapdh      | AGGTCGGTTGGAAACGGGGAT | TGTAGACCATGAGGTTGAGG |

These qPCR primers were used to amplify and assess expression of Fosb and ΔFosb mRNA. Gapdh was used as a housekeeping gene in the ΔΔCt method.
expression of $\Delta$FosB mRNA (Fig. 1A). To test whether Fosb and thus $\Delta$FosB expression is regulated by E2F3 specifically, and not another E2F isoform, we performed qChIP for E2F1, E2F3, and E2F4 in NAc under both saline and repeated cocaine conditions (once daily intraperitoneal injections for 7 d). E2F2, E2F5, E2F6, E2F7, and E2F8 are sparsely expressed in the adult mouse brain (Lein et al., 2007) and were not investigated in this study. We designed primers to amplify the three identified binding sites of interest, as well as a negative control region where no binding was expected at 1250 bp upstream of the TSS. An interaction of cocaine treatment and gene position was observed for E2F3 only ($F_{(9,60)} = 13.35, p < 0.0001; N = 4–5, 3 mice/sample for all qChIP assays; row a, Table 3), with a specific increase in binding following cocaine administration at the site 500 bp upstream of the TSS ($t_{(9)} = 9.904, p < 0.0001; $Fig. 1B,C;$ row a, Table 3). Note that no available antibody can distinguish between E2F3a and E2F3b.

Next, to determine whether regulation of Fosb by E2F3 at this ~500 bp site is specific to NAc, we repeated qChIP for E2F3 in three additional regions of the reward circuitry: PFC, VTA, and CPU. In PFC, significant binding over an IgG control was observed ($F_{(9,60)} = 48.44, p < 0.0001; N = 5; row b, Table 3), but this effect was not significantly different between saline and cocaine conditions ($t_{(9)} = 1.69, p = 0.2208; $Fig. 1D;$ row b, Table 3). Modest binding was observed in VTA, but again this was not significantly different between saline and cocaine ($F_{(1,16)} = 7.024, p = 0.0243; $t_{(9)} = 0.275, p = 0.9999; N = 3–4; $Fig. 1E;$ row c, Table 3). E2F3 binding in CPU was also significantly increased over an IgG control ($F_{(1,16)} = 25.113, p < 0.0001; N = 5; row d, Table 3), with no significant effect of cocaine ($t_{(9)} = 1.451, p = 0.3324; $Fig. 1F;$ row d, Table 3). Together, these data indicate that regulation of E2F3 binding to Fosb after repeated cocaine is specific to NAc.

Expression of E2F3a, not E2F3b, is increased by cocaine in NAc D1 MSNs

To explore the cell-type-specific expression of E2F3’s two isoforms, E2F3a and E2F3b, we performed FACS on tagged D1 MSNs or D2 MSNs and analyzed the mRNA expression of each transcript. In isolated D1 MSNs, both E2F3a and E2F3b are expressed at baseline levels, but only E2F3a expression is increased by repeated cocaine exposure. Main effects of both drug treatment ($F_{(1,9)} = 9.624, p = 0.0127; row e, Table 3) and transcript variant ($F_{(1,9)} = 6.845, p = 0.028; N = 3–4; row e, Table 3) were observed and a significant increase in $\Delta$Fos3a expression only was confirmed via post hoc analysis ($\Delta$Fos3a: $t_{(9)} = 4.186, p = 0.0047; \Delta$Fos3b: $t_{(9)} = 0.3328, p = 0.9999; $Fig. 2A;$ row e, Table 3). In D2 MSNs, $\Delta$Fos3b is expressed minimally but no $\Delta$Fos3a expression was detected under control or cocaine conditions ($F_{(1,9)} = 15.489, p = 0.0034; N = 3–4; $Fig. 2B;$ row f, Table 3). Importantly, these data indicate that $\Delta$Fos3a is induced by cocaine selectively in the same neuronal population in which cocaine induction of $\Delta$FosB occurs. Thus, Lobo et al. (2013) found that $\Delta$FosB is induced by cocaine in D1 but not D2 NAc MSNs, and Grueter et al. (2013) demonstrated that selective overexpression of $\Delta$FosB in D1 but not D2 NAc MSNs enhances behavioral responses to cocaine.

Overexpression of E2F3a recapitulates cocaine-induced Fosb and $\Delta$FosB mRNA expression

To probe which E2F3 isoform regulates $\Delta$FosB expression, we performed stereotactic surgery to infuse HSV-GFP, -E2F3a, or -E2F3b into NAc. A main effect of virus was observed ($F_{(2,29)} = 9.332, p = 0.0007; N = 6; row g, Table 3), and a significant E2F3a-induced increase in both Fosb and $\Delta$FosB mRNA expression was confirmed via post hoc analysis ($\Delta$FosB: $t_{(9)} = 3.138, p = 0.0078; \Delta$FosB: $t_{(9)} = 2.868, p = 0.0185; $Fig. 2D;$ row g, Table 3). The ratio of Fosb/$\Delta$FosB mRNA was not affected by E2F3a overexpression ($t_{(9)} = 0.3142, p = 0.7605; row h, Table 3), consistent with the lack of binding of E2F3 to the Fosb splice site in Exon IV. E2F3b overexpression did not affect mRNA expression of either Fosb isoform ($\Delta$FosB: $t_{(9)} = 1.336, p = 0.5757; \Delta$FosB: $t_{(9)} = 0.575, p = 0.9999; $Fig. 2D;$ row g, Table 3).

Because significant binding of E2F3 was observed in PFC (Fig. 1E), but was unaffected by cocaine, we used this region as a control for the effect of E2F3a overexpression in NAc. E2F3a overexpression in PFC did not affect expression of either Fosb or $\Delta$FosB mRNA ($F_{(1,19)} = 1.637, p = 0.2162; N = 6; $Fig. 2E;$ row i, Table 3).

To further explore the necessity of E3F3a binding to Fosb to induce $\Delta$FosB expression, we knocked down expression of E2F3a with a miRNA and assessed $\Delta$FosB mRNA levels following an acute dose of experimenter-administered cocaine. We found a main effect of treatment ($F_{(2,29)} = 3.297, p = 0.0415; N = 5–7; row j, Table 3), and confirmed via post hoc analysis that an acute dose of cocaine induces a near-significant increase in $\Delta$FosB mRNA expression (miR-LacZ + saline vs miR-LacZ + cocaine: $t_{(9)} = 2.492, p = 0.0508; $Fig. 2F;$ row j, Table 3). On E2F3a knockdown, cocaine no longer induced $\Delta$FosB (miR-LacZ + saline vs miR-E2F3a + cocaine: $t_{(9)} = 1.223, p = 0.6855; row j, Table 3), but the difference between E2F3a knockdown and control conditions did not achieve statistical significance (miR-LacZ + cocaine vs miR-E2F3a + cocaine: $t_{(9)} = 1.3311, p = 0.9999; $Fig. 2F;$ row j, Table 3). Together, these findings indicate that E2F3a binding to the Fosb promoter is sufficient for induction of $\Delta$FosB expression but is not solely required. This is consistent with published data on two other transcription factors that regulate $\Delta$FosB: SRF and CREB. Ablation of
both of these upstream regulators simultaneously blocks the cocaine-induced increase in ΔFosB, but knockdown of either molecule alone is insufficient (Vialou et al., 2012).

Overexpression of E2F3a increases binding of E2F3 at the Fosb promoter but does not affect H3K4me3 enrichment

To determine whether this increase in Fosb expression following E2F3a overexpression is because of direct binding at the Fosb gene promoter in NAc, qChIP was performed following overexpression of E2F3a. An interaction of virus and gene position was observed following overexpression of E2F3a. An interaction of virus and gene position was observed following overexpression of E2F3a (3.619, p = 0.0224; N = 5–6, 3 mice/sample for all qChIP assays; row k, Table 3). E2F3a overexpression elicited a specific increase in E2F binding 500 bp upstream of the TSS, the same site at which binding was observed following cocaine exposure, but not at the other two putative binding sites as confirmed by post hoc analysis (3.285, p = 0.0093; Fig. 3A; row k, Table 3). This result, along with our initial cocaine qChIP results, confirms that the putative E2F binding site 500 bp upstream of the TSS is a functional E2F response element, at least in the NAc.

In the same genome-wide study that implicated E2F3 as a regulator of molecular responses to cocaine in the NAc, H3K4me3 enrichment was highly correlated with predicted E2F3 binding motifs, suggesting that this mark may be important in E2F3 action (Feng et al., 2014). We therefore tested whether overexpression of E2F3a in NAc increases H3K4me3 at this site in Fosb or if perhaps H3K4me3 is a pre-existing mark. No effect of E2F3a overexpression was observed on H3K4me3 enrichment near the E2F3 binding site 500 bp upstream of TSS (Fig. 3B), indicating that H3K4me3 may be a permissive, pre-existing mark before E2F3 regulation of Fosb.

Discussion

Here, we demonstrate that a specific isoform of the E2F3 transcription factor, E2F3a, is a novel regulator of the cocaine-elicited expression of the addiction-associated transcription factor ΔFosB. We show that in NAc specifically, E2F3 binding at the Fosb promoter at a site 500 bp upstream of the TSS is increased after repeated cocaine exposure. E2F3a overexpression in this brain region increases E2F3 binding at this same site and induces transcription of both Fosb products, Fosb and ΔFosB mRNA. Interestingly, we observed significant baseline binding of E2F3 500 bp upstream of TSS in PFC, VTA, and CPU but not regulation by cocaine. This result could point to a role for E2F3 in regulating steady-state Fosb expression in these reward circuitry regions, but one that is not affected by drug exposure. Further, using FACS, we determine that E2F3a’s regulation of ΔFosB is
restricted to D1 MSNs in NAc, based on E2f3a’s restricted baseline and cocaine-induced expression in this cell type. There was also no effect of cocaine on much lower levels of E2f3b in either D1 or D2 MSNs. Based on overexpression experiments, we deduced that E2f3 binding in NAc, which cannot be differentiated by qChIP because of antibody limitations, is likely E2f3a, given that overexpression of E2f3b has no effect on expression of either Fosb isoform, whereas E2f3a increases expression of both isoforms. By contrast, miRNA knockdown of E2f3a is not sufficient to abolish the induction of ΔFosB following cocaine. Together, these results implicate E2f3a as a novel isoform- and region-specific regulator of ΔFosB, opening a new avenue for targeting this upstream regulator of addiction-mediated behaviors and a previously unrecognized signal transduction pathway affected in NAc by cocaine exposure.

Before this investigation, two studies implicated E2f3a expression in NAc as a mediator of the transcriptional, alternative splicing, and behavioral responses to cocaine (Feng et al., 2014; Cates et al., 2018b). The first, a genome-wide study by Feng et al. (2014), included a combination of ChIP-seq and RNA-seq and identified E2f3 as one of the most prominent upstream regulators of gene expression and alternative splicing in NAc after chronic cocaine exposure. The next, a study by Cates et al. (2018b), investigated specifically which E2f3 isoform is responsible for those changes. That study found that E2f3a, not E2f3b, acts in NAc as a novel regulator of gene expression, splicing changes, and behavioral responses induced by cocaine. Because it is known that ΔFosB, like E2f3a, potentiates cocaine reward (see Introduction), these recent findings with E2f3 suggest that it might be an upstream regulator of ΔFosB.

There are three critical points of regulation in the expression of ΔFosB: transcription of Fosb, alternative splicing of Exon IV, and the cell-type specificity of these events. Our findings add E2f3 to a list of several other factors that influence ΔFosB expression at different points in its production. PTB (encoded by Ptbp1) has been shown to regulate Fosb splicing in vitro (Alibhai et al., 2007). Specifically, increased PTB leads to preferential splicing of full-length Fosb, but when mutated causes decreased PTB binding at Exon IV and increased preferential splicing of ΔFosb (Marinescu et al., 2007). We did not find any direct involvement of E2f3a in the alternative splicing of Fosb and ΔFosb, as there was no evidence of binding at the Exon IV alternative splice site and no effect on the ratio of the two isoforms (Fig. 1B). Interestingly, E2f3a has been shown to regulate alternative splicing of Ptbp1 itself (Cates et al., 2018b). Thus, it is possible that cocaine leads to alternative splicing of Ptbp1 via E2f3a, increasing expression of a specific isoform of PTB that leads to the alternative splicing event giving rise to ΔFosB. However, prior work has shown no effect of chronic cocaine on Fosb splicing (Alibhai et al., 2007).

The transcription factors, CREB and SRF, have previously been shown to bind the Fosb promoter at ~250 bp in response to cocaine exposure (Renthal and Nestler, 2008; Vialou et al., 2012). Deletion of both transcription factors together ablates the cocaine-mediated induction of ΔFosB, whereas deletion of either alone has no effect. Interestingly, deletion of both CREB and SRF reduces sensitivity to cocaine reward, whereas deletion of CREB alone enhances it, suggesting that each transcription factor differentially regulates ΔFosB expression in combination with other upstream regulators. These results are in agreement with the data presented in this study, in which viral-mediated knockdown of E2f3a is insufficient to abolish the induction of ΔFosB mRNA after an acute cocaine exposure (Fig. 2F).

E2f3a is expressed throughout the brain (Lein et al., 2007), and we observed significant binding of E2f3 at the ~500 bp site on Fosb in NAc, PFC, VTA, and CPu. However, E2f3 binding to Fosb is specifically altered by cocaine exposure in NAc only (Fig. 1), indicating a role for E2f3 in the regulation of Fosb expression after cocaine exposure that is specific to NAc. Moreover, we show that expression of E2f3a is specific to D1 MSNs in NAc (Fig. 2A,B). Previous studies of ΔFosB have shown that it is expressed at baseline in both D1 and D2 MSNs, but that following both experimenter-administered and self-administered cocaine expression increases in D1 MSNs only (Lobo et al., 2013). These past findings align directly with our data presented here that indicate a D1 MSN-specific role for E2f3a in regulating the cocaine response in NAc.

Figure 3. Viral-mediated E2f3a overexpression leads to increased E2f3 binding to Fosb promoter in NAc. A, E2f3 binding was increased at 500 bp upstream of the Fosb TSS after E2f3a overexpression (interaction of virus and gene position by two-way ANOVA: $F_{(3,35)} = 3.619, p = 0.0224$; E2f3a vs GFP at $-500$ bp via post hoc analysis: $t_{(31)} = 3.285, *p = 0.0093$). B, H3K4me3 enrichment was not affected by E2f3a overexpression (no main effect of virus observed: $F_{(1,40)} = 0.057, p = 0.8126$). $N = 5–6$, with three mice pooled per sample for all qChIP assays.
In addition, another past study of $\Delta$FosB’s function in D1 and D2 MSNs investigated the effects of overexpression in each cell type on cocaine-elicited reward. This investigation found that $\Delta$FosB overexpression in D1 but not D2 MSNs in NAc was sufficient to cause cocaine conditioned place preference at a dose of 3.75 mg/kg (Grueter et al., 2013). Here, we find no detectable expression of $\Delta$FosB in D2 MSNs in NAc at baseline, and a D1 MSN-specific increase in the transcript elicited by chronic cocaine exposure (Fig. 2A,B). Together, these results suggest that E2F3a regulates $\Delta$FosB expression in D1 MSNs following cocaine exposure, and thus may possibly play a role in $\Delta$FosB-controlled changes in behavior and gene expression. Given that no E2f3a expression was detected at baseline in D2 MSNs, how $\Delta$FosB is regulated in this cell type remains an outstanding question. Finally, our results support regional specificity for E2F3a’s function and suggest that it acts differently in distinct cell populations in different regions.

Given this MSN-specific and region-specific expression, our data support a broader understanding that ubiquitously expressed transcription factors target specific loci in different cells and specific brain regions. Despite this principle, it is interesting that we observed a non-significant decrease in E2F3 binding at the −500 bp site on Fosb in the CPu after cocaine exposure (Fig. 1F). NAc and CPu are differentially implicated in cocaine self-administration behaviors, with NAc being essential for the acquisition of lever-pressing and CPu being essential for the perseveration of behavior on more demanding schedules of reinforcement (Murray et al., 2012). $\Delta$FosB is known to be induced in both regions after cocaine administration (Lobo et al., 2013). Given our findings, it would be worthwhile to investigate E2F3a’s regulation of $\Delta$FosB expression in both NAc and CPu during different stages of rodent self-administration to determine how E2F3a and $\Delta$FosB might play a role in the transition from volitional responding to compulsive drug-seeking.

That $\Delta$FosB expression is specifically regulated in NAc by E2F3a, and not E2F3b, is also of note. The bioinformatic prediction of E2F3 as one of the most prominent upstream regulators of cocaine-induced gene expression and alternative splicing changes suggested a novel role for the E2F family in cocaine action but did not discriminate among family members or between these two E2F3 isoforms (Feng et al., 2014). E2F3a and E2F3b share the same DNA binding, nuclear localization, and transactivation domains, but E2F3b lacks an N-terminus ubiquitin-targeting domain (He et al., 2000). In proliferating tissue, E2F3a typically acts as a gene activator and E2F3b as a repressor (Leone et al., 2000). In fact, E2F3a was recently shown to be a novel regulator of cocaine elicited reward in that its overexpression enhanced cocaine reward and locomotor behaviors as well as recapitulated cocaine-induced gene expression and splicing changes (Cates et al., 2018b). Another recent study demonstrated a role for E2F3b in regulating cocaine action in the PFC (Cates et al., 2018a). Given these findings, our data on $\Delta$FosB expression in post-mitotic brain tissue is consistent with divergent transcriptional regulatory roles for these two E2F3 isoforms.

In addition to predicting E2F3 as a novel upstream regulator, Feng et al. (2014) found significant enrichment of H3K4me3, a histone modification associated with active promoters in euchromatin, associated with E2F binding motifs in NAc in response to chronic cocaine administration. This finding suggests that, in response to chronic cocaine, H3K4me3 in NAc is either deposited as a chromatin mark that could permit E2F3 binding or that H3K4me3 is recruited by E2F3 in concert with its cocaine response to activate gene promoters. We tested these possibilities by overexpressing E2F3a in NAc and found that H3K4me3 enrichment at the Fosb promoter was unaffected. This result is consistent with the former hypothesis, that H3K4me3 is a pre-existing mark in NAc neuronal euchromatin that could allow E2F3a to bind its consensus sequence 500 bp upstream of the Fosb TSS and induce transcription. It is interesting to note that H3K4me3 is also enriched at baseline at −250 bp (Fig. 3B), the site where CREB and SRF bind their response elements to induce Fosb (Vialou et al., 2012); this implicates H3K4me3’s general involvement in Fosb transcription, though this speculation deserves further investigation in follow-up studies of Fosb’s transcriptional activators.

We did not investigate any other permissive or repressive chromatin marks that might interact with E2F3a at Fosb in NAc, such as H3K4me1, H3K27me3, or H3K27ac, among many others. Likewise, we did not investigate the status of H3K4me3 enrichment at Fosb’s −500 bp site in other brain reward regions. To this point, however, histone deposition data from the Roadmap Epigenomics Consortium shows enrichment of H3K4me3 at the −500 bp site upstream of the Fosb promoter in adult mouse forebrain, hindbrain, and midbrain (Zhou et al., 2011). This observation across brain regions is consistent with our observation that the mark is also enriched at this site under both baseline and chronic cocaine conditions in the NAc. This analysis indicates that the presence of H3K4me3 alone cannot explain why E2F3a can bind to and activate Fosb in NAc but not in the other brain regions studied. More targeted investigations are therefore needed to identify the histone modifications and perhaps other chromatin-remodeling factors that are responsible for E2F3a’s unique action on Fosb expression within D1 MSNs of the NAc.

In summary, we demonstrate a novel role for E2F3a, a specific isoform of the E2F family of transcription factors, in regulating the expression of $\Delta$FosB downstream of cocaine action in NAc. $\Delta$FosB is a critical regulator of the behavioral responses to diverse substances of abuse that can lead to addiction. Thus, understanding how $\Delta$FosB is regulated by upstream transcription factors like E2F3a in the context of cocaine exposure will lead to a greater understanding of how this critical regulator might be exploited for potential therapeutic development in the treatment of addiction and other disorders related to the use of commonly abused substances.

References

Ahlander J, Bosco G (2009) The RB/E2F pathway and regulation of RNA processing. Biochem Biophys Res Co 384:280–283.
Alibhai IN, Green TA, Potashkin JA, Nestler EJ (2007) Regulation of fosb and $\Delta$fosb mRNA expression: in vivo and in vitro studies. Brain Res 1143:22–33.
Carle TL, Ohnishi YN, Ohnishi YH, Alibhai IN, Wilkinson MB, Kumar A, Nestler EJ (2007) Proteasome-dependent and -independent
mechanisms for FosB destabilization: identification of FosB degron domains and implications for ΔFosB stability. Eur J Neurosci 25:3009–3019.

Carlezon WA, Olson VG, Lane-Ladd SB, Brodkin ES, Hiroi N, Duman RS, Neve RL, Nestler EJ (1998) Regulation of cocaine reward by CREB. Science 282:2272–2275.

Cartharius K, Feuch K, Grote K, Klocke K, Haltmeier M, Klingenhoff A, Frisch M, Bayerlein M, Werner T (2005) MatInspector and beyond: promoter analysis based on transcription factor binding sites. Bioinformatics 21:2933–2942.

Cates HM, Bagot RC, Heller EA, Purushothaman I, Lardner CK, Walker DM, Peña CJ, Neve RL, Shen L, Nestler EJ (2018a) A novel role for E2F3b in regulating cocaine action in the prefrontal cortex. Neuropsychopharmacology 44:776–784.

Cates HM, Heller EA, Lardner CK, Purushothaman I, Peña CJ, Walker DM, Cahill ME, Neve RL, Shen L, Bagot RC, Nestler EJ (2018b) Transcription factor E2F3a in nucleus accumbens affects cocaine action via transcription and alternative splicing. Biol Psychiatry 84:167–179.

Chandra R, Francis CT, Konkalpatt M, Amgalan A, Gancarz AM, Dietz DM, Lobo MK (2015) Opposing role for Egr3 in nucleus accumbens cell types in cocaine action. J Neurosci 35:7927–7937.

Daily K, Patel VR, Rigor P, Xie X, Baldi P (2011) MotifMap: integrative genome-wide maps of regulatory motif sites for model species. BMC Bioinformatics 12:495.

Engmann O, Labonté B, Mitchell A, Basht rakov P, Calipari ES, Rosenbluh C, Loh YE, Walker DM, Burek D, Hamilton PJ, Issler O, Neve RL, Turecki G, Hurd Y, Chess A, Shen L, Mansuy I, Jeltsch A, Akbarian S, Nestler EJ (2017) Cocaine-induced chromatin modification associates with increased expression and three-dimensional looping of Autos. Biol Psychiatry 82:794–805.

Feng J, Wilkinson M, Liu X, Purushothaman I, Ferguson D, Vialou V, Maze I, Shao N, Kennedy P, Koo J, Dias C, Laitman B, Stockman V, LaPlant Q, Cahill ME, Nestler EJ, Shen L (2014) Chronic cocaine-regulated epigenomic changes in mouse nucleus accumbens. Genome Biol 15:R65.

Ferguson D, Shao N, Heller EA, Feng J, Neve RL, Kim HD, Call T, Magazu S, Shen L, Nestler EJ (2015) SIRT1-FOXO3a regulate cocaine-regulated epigenomic changes in mouse nucleus accumbens. Genome Biol 16:26.

FosB mutant mice: Loss of chronic cocaine induction of FosB in the brain controls sensitivity to cocaine. Nature 401:272–276.

Lees J, Ito Vidal M, Valentine M, Loo k T, Harlow E, Dyson N, Helin K (1993) The retinoblastoma protein binds to a family of E2F transcription factors. Mol Cell Biol 13:7812–7825.

Lein ES, et al. (2007) Genome-wide atlas of gene expression in the adult mouse brain. Nature 445:168–176.

Lem E, Nuckolls F, Ishida S, Adams M, Sears R, Jakoi L, Miron A, Nevins JR (2000) Identification of a novel E2F3 product suggests a mechanism for determining specificity of repression by Rb proteins. Mol Cell Biol 20:3626–3632.

Lobo MK, et al. (2013) ΔFosB Induction in striatal medium spiny neuron subtypes in response to chronic pharmacological, emotional, and optogenetic stimuli. J Neurosci 33:18381–18395.

Marinescu V, Loomis PA, Ehmann S, Beales M, Potashkin JA (2007) Regulation of retention of FosB intron 4 by PTB. PLoS One 2:e828.

Maze I, Covington HE, Dietz D, LaPlant Q, Renthal W, Russo SJ, Mechanic M, Mouzon E, Neve RL, Haggarty SJ, Ren Y, Sampath SC, Hurd YL, Greengard P, Tarakhovsky A, Schaefer A, Nestler EJ (2010) Essential role of the histone methyltransferase G9a in cocaine-induced plasticity. Science 327:213–216.

McClung CA, Nestler EJ (2003) Regulation of gene expression and cocaine reward by CREB and ΔFosB. Nat Neurosci 6:1208–1215.

Meyer D, Mayhew A (2017) Assessing the heritability of complex traits in humans: methodological challenges and opportunities. Curr Genomics 18:332–340.

Murray JE, Belin D, Everitt BJ (2012) Double dissociation of the dorsomedial and dorsolateral striatal control over the acquisition and performance of cocaine seeking. Neuropsychopharmacology 37:2456–2466.

Nestler EJ (2015) ΔFosB: a transcriptional regulator of stress and antidepressant responses. Eur J Pharmacol 753:66–72.

Nye HE, Nestler EJ (1996) Induction of chronic Fos-related antigens in rat brain by chronic morphine administration. Mol Pharmacol 49:636–645.

Reichel R, Kovesdi I, Nevins JR (1987) Developmental control of a promoter-specific factor that is also regulated by the E1A gene product. Cell 48:501–506.

Renthal W, Carle TL, Maze I, Covington HE, Truong H-T, Alibhai I, Kumar A, Montgomery RL, Olson EN, Nestler EJ (2008) Delta FosB mediates epigenetic desensitization of the c-fos gene after chronic amphetamine exposure. J Neurosci 28:7344–7349.

Renthal W, Kumar A, Xiao G, Wilkinson M, Covington HE, Maze I, Sikder D, Robison AJ, LaPlant Q, Etz D, Russo SJ, Vialou V, Chakravarty S, Kodake DJ, Stack A, Kabbaj M, Nestler EJ (2009) Genome-wide analysis of chromatin regulation by cocaine reveals a role for sirtuins. Neuron 62:335–348.

Renthal W, Nestler EJ (2008) Epigenetic mechanisms in drug addiction. Trends Mol Med 14:341–350.

Tsankova NM, Berton O, Renthal W, Kumar A, Neve RL, Nestler EJ (2006) Sustained hippocampal chromatin regulation in a mouse model of depression and antidepressant action. Nat Neurosci 9:519–525.

Ullery-Reynolds PG, Castillo MA, Vialou V, Russo SJ, Nestler EJ (2009) Phosphorylation of ΔFosB mediates its stability in vivo. Neuroscience 158:369–372.

Vialou V, Feng J, Robison AJ, Ku SM, Ferguson D, Scobie KN, Mazei-Robison MS, Mouzon E, Nestler EJ (2012) Serum response factor and cAMP response element binding protein are both required for cocaine induction of ΔFosB. J Neurosci 32:7577–7584.

Zhou X, Maricque B, Xie M, Li D, Sundaram V, Martin EA, Koebbe BC, Nielsen C, Hirst M, Farnham P, Kuhn RM, Zhu J, Sminov I, Kent WJ, Haussler D, Madden PA, Costello JF, Wang T (2011) The Human Epigenome Browser at Washington University. Nat Methods 12:989–990.