Cellular/Molecular

Threonine 149 Phosphorylation Enhances ΔFosB Transcriptional Activity to Control Psychomotor Responses to Cocaine

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Stable changes in neuronal gene expression have been studied as mediators of addicted states. Of particular interest is the transcription factor ΔFosB, a truncated and stable *FosB* gene product whose expression in nucleus accumbens (NAc), a key reward region, is induced by chronic exposure to virtually all drugs of abuse and regulates their psychomotor and rewarding effects. Phosphorylation at Ser^{27} contributes to ΔFosB's stability and accumulation following repeated exposure to drugs, and our recent work demonstrates that the protein kinase CaMKIIα phosphorylates ΔFosB at Ser^{27} and regulates its stability *in vivo*. Here, we identify two additional sites on ΔFosB that are phosphorylated *in vitro* by CaMKIIα, Thr^{149} and Thr^{180}, and demonstrate their regulation *in vivo* by chronic cocaine. We show that phosphomimetic mutation of Thr^{149} (T149D) dramatically increases AP-1 transcriptional activity while alanine mutation does not affect transcriptional activity when compared with wild-type (WT) ΔFosB. Using *in vivo* viral-mediated gene transfer of ΔFosB-T149D or ΔFosB-T149A in mouse NAc, we determined that overexpression of ΔFosB-T149D in NAc leads to greater locomotor activity in response to an initial low dose of cocaine than does WT ΔFosB, while overexpression of ΔFosB-T149A does not produce the psychomotor sensitization to chronic low-dose cocaine seen after overexpression of WT ΔFosB and abrogates the sensitization seen in control animals at higher cocaine doses. We further demonstrate that mutation of Thr^{149} does not affect the stability of ΔFosB overexpressed in mouse NAc, suggesting that the behavioral effects of these mutations are driven by their altered transcriptional properties.

Key words: accumbens; CaMKII; cocaine; δFosB; phosphorylation; transcription

Introduction

Drug addiction arises in part from altered gene expression in discrete brain regions in response to chronic exposure to drugs of abuse (Robison and Nestler, 2011). Increasing evidence suggests that a subset of these gene expression changes are mediated by ΔFosB, a Fos family transcription factor induced in multiple brain regions specifically by chronic exposure to virtually all drugs of abuse (Nestler, 2008; Perrotti et al., 2008). In nucleus accumbens (NAc), ΔFosB expression increases locomotor and rewarding responses to drugs of abuse (Kelz et al., 1999; Colby et al., 2003), whereas blockade of ΔFosB transcriptional activity reduces drug reward (McClung and Nestler, 2003; Peakman et al., 2003; Zachariou et al., 2006; Robison et al., 2013). NAc ΔFosB also regulates other forms of reward. It accumulates in NAc with sexual experience, sugar and high-fat diets, and calorie restriction, and promotes reward to these stimuli (Pitchers et al., 2010, 2013; Been et al., 2013). Additionally, NAc ΔFosB is induced by chronic stress and antidepressant treatment and mediates stress resilience and antidepressant action (Vialou et al., 2010; Robison et al., 2014).

These effects are mediated by numerous ΔFosB gene targets (McClung and Nestler, 2003). Recent work has focused on ΔFosB induction of CaMKIIα, which is specific to D1-type medium spiny neurons (MSNs) of NAc shell and mediates ΔFosB's enhanced responses to cocaine and antidepressant-like actions (Robison et al., 2013, 2014). NAc CaMKII regulates the psychomotor effects of cocaine through AMPA receptor modulation (Pierce et al., 1998), and recent work demonstrates that ΔFosB

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regulates NAc MSN glutamatergic synapse morphology and function in a cell type-specific manner (Grueter et al., 2013), a process long associated with the structural and catalytic roles of CaMKII (Hell, 2014).

ΔFosB not only regulates CaMKII expression, it is also phosphorylated by CaMKII, establishing a feedforward loop engaged by chronic cocaine that is essential for cocaine's behavioral and cellular effects (Robison et al., 2013). Previous studies demonstrate that ΔFosB is a potent *in vitro* substrate for CaMKIIα ($K_M = 5.7 \pm 2.0$ μM; $K_{CAT} = 2.3 \pm 0.3$ min^{-1}) with a stoichiometry of phosphorylation indicating at least three separate substrate sites (2.27 ± 0.07 mol/mol; Robison et al., 2013). In the same study, we identified Ser[27] as one of the CaMKII substrate sites, a site previously shown to regulate the stability of ΔFosB *in vitro* and *in vivo* (Ulery et al., 2006; Ulery-Reynolds et al., 2009). We demonstrated further that overexpression of constitutively active CaMKII promotes ΔFosB accumulation *in vivo* (Robison et al., 2013), indicating that Ser[27] phosphorylation may be regulated by CaMKII in the brain. However, the identity and function of the other CaMKII phosphorylation sites within ΔFosB, and how they might regulate ΔFosB activity and drug responses, remain unknown. Here, we uncover two novel CaMKII phospho-sites within ΔFosB, Thr[149], and Thr[180] and demonstrate that phosphorylation of Thr[149] is regulated in the brain by chronic cocaine, dramatically increases ΔFosB-mediated gene transcription, and promotes locomotor activation by cocaine in mice.

Materials and Methods

Animals. C57BL/6J male mice (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 *ad libitum*. All experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committees at Icahn School of Medicine at Mount Sinai and Michigan State University.

Mass spectrometry. Standard peptides were designed to mimic the phospho or non-phospho forms of Thr[149], Thr[180], and Ser[199] ΔFosB. After synthesis and purification, each "heavy" idiotypic peptide was dissolved in 50/50 acetonitrile/water buffer and sent for amino acid analysis to determine absolute concentration of the synthetic peptide stock solution. Each heavy peptide was then directly infused into the 4000 QTRAP mass spectrometer (MS) at Yale's Keck Center to determine the best collision energy for MS/MS fragmentation and two to four multiple reaction monitoring (MRM) transitions. Next, the neat heavy peptides were subjected to LCMS on the 4000 QTRAP to ensure peptide separation. The instrument was run in the triple quadrupole mode, with Q1 set on the specific precursor *m/z* value (Q1 is not scanning) and Q3 set to the specific *m/z* value corresponding to a specific fragment of that peptide. In the MRM mode, a series of single reactions (precursor/fragment ion transitions where the collision energy is tuned to optimize the intensity of the fragment ions of interest) were measured sequentially, and the cycle (typically 1–2 s) was looped throughout the entire time of the HPLC separation. MRM transitions were determined from the MS/MS spectra of the existing peptides. Two transitions per peptide, corresponding to high-intensity fragment ions, were then selected and the collision energy optimized to maximize signal strength of MRM transitions using automation software. Peaks resulting from standard peptides and ΔFosB samples from the brains of saline-treated or cocaine-treated mice were then compared to determine the absolute abundance of each peptide form in the samples. Data analysis on LC-MRM data is performed using AB MultiQuant 1.1 software.

Enrichment of ΔFosB from mouse brain. Mice were injected intraperitoneally with saline or cocaine (15 mg/kg) in their home cages once daily for 7 d. Twenty four hours following the final injection, mice were decapitated without anesthesia to avoid effects of anesthetics on neuronal protein levels and phospho-states. Brains were serially sliced in a 1.0 mm matrix (Braintree Scientific) and NAc (ventral striatum) and dorsal striatum were removed in PBS containing protease (Roche) and phosphatase (Sigma-Aldrich) inhibitors using a 12 gauge punch and immediately frozen on dry ice. Tissue was homogenized in PBS with 0.2% Triton X-100 and centrifuged at 10,000 × *g* for 5 min at 4°C to remove insoluble proteins. The soluble fractions from 10 mice were combined and concentrated by dialysis against 0.1 M HEPES, pH7.4, and 500 mM NaCl. The resulting concentrated protein was separated by SDS-PAGE and bands from 32 to 40 kDa were cut from the gel to enrich for ΔFosB (35–37 kDa). Protein was extracted from the gel slices and subjected to mass spectroscopic analysis as described above.

DNA constructs. The luciferase reporter construct was 4 × AP-1/RSV-Luc, which consists of a promoter region of four AP-1 consensus sequences in tandem with a minimal RSV promoter, and a luciferase reporter gene under the control of this promoter (Ulery and Nestler, 2007). We used site-directed mutagenesis (Qiagen) to generate mutant constructs encoding ΔFosB with Thr[149] or Thr[180] converted to Asp (T149D and T180D) or to Ala (T149A and T180A) in a pcDNA3.1 backbone. WT or catalytically dead (Lys[42] to Met) CaMKII was also expressed using the pcDNA3.1 backbone. All mutations were verified by dideoxysequencing.

Luciferase activity assays. Neuro2a cells (N2a; American Type Culture Collection) were cultured in EMEM (ATCC) supplemented with 10% heat-inactivated fetal bovine serum (ATCC) in a 5% CO_2 humidified atmosphere at 37°C. Cells were plated into 12-well plates. Twenty-four hours later (when cells were ~95% confluent) cells were transiently cotransfected with a combination of 4 × AP-1/RSV-Luc plasmid and pcDNA3.1 plasmids (Life Technologies) containing WT or mutant ΔFosB and/or CaMKIIα constructs using Effectene (Qiagen). A total of 200 ng DNA was transfected per well. Approximately 48 h post transfection, cells were washed twice with 1 ml PBS and whole-cell lysates were prepared using 180 μl lysis buffer provided with ONE-Glo Luciferase Assay System (Promega). Fifty microliters of the lysate was removed for Western blot analysis. The remaining lysates were incubated on ice for 5 min and the luciferase activity (luminescence) present in each sample was assayed using the substrates and protocol included in the ONE-Glo Luciferase Assay System. The luminescence of each sample was detected in triplicate using Kodak autoradiography film and quantified using ImageJ software (NIH). Luminescence was normalized to total ΔFosB expression as assessed by Western blot.

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 0.5–1.0 μl of virus into 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. We used bicistronic p1005 HSV vectors expressing GFP alone or GFP plus WT, T149D, or T149A ΔFosB. In this system, 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). In the locomotor experiment, viral expression was confirmed during tissue collection using fluorescence microscopy (Leica) to visualize GFP and ensure targeting of the NAc.

Locomotor activity assay. Locomotor activity was measured per published protocols (Lobo et al., 2010) with minor modifications. Activity was assessed in the *x*- and *y*-planes for horizontal ambulation in a 75 cm^2 chamber using EthoVision XT (Noldus). Twenty-four hours before undergoing surgery, mice were habituated to the locomotor chamber for 60 min with no injection. Three days after surgery (day 0) animals were injected intraperitoneally with saline and placed in locomotor chamber for 45 min at which time baseline locomotor was recorded. On days 4–8 after surgery (days 1–5), animals were injected with cocaine (3.75 mg/kg) and analyzed for 45 min.

Immunohistochemistry. Adult male mice were terminally anesthetized (15% chloral hydrate) and transcardially perfused with PBS followed by 4% formalin. Brains were then postfixed overnight in formalin at 4°C and cryoprotected in 30% sucrose at 4°C until isotonic. Brains were sliced in 35 μm sections on a freezing microtome and immunohistochemistry for ΔFosB expression was performed essentially as described previously (Perrotti et al., 2008). Briefly, slices were blocked for 1 h in 0.3% Triton

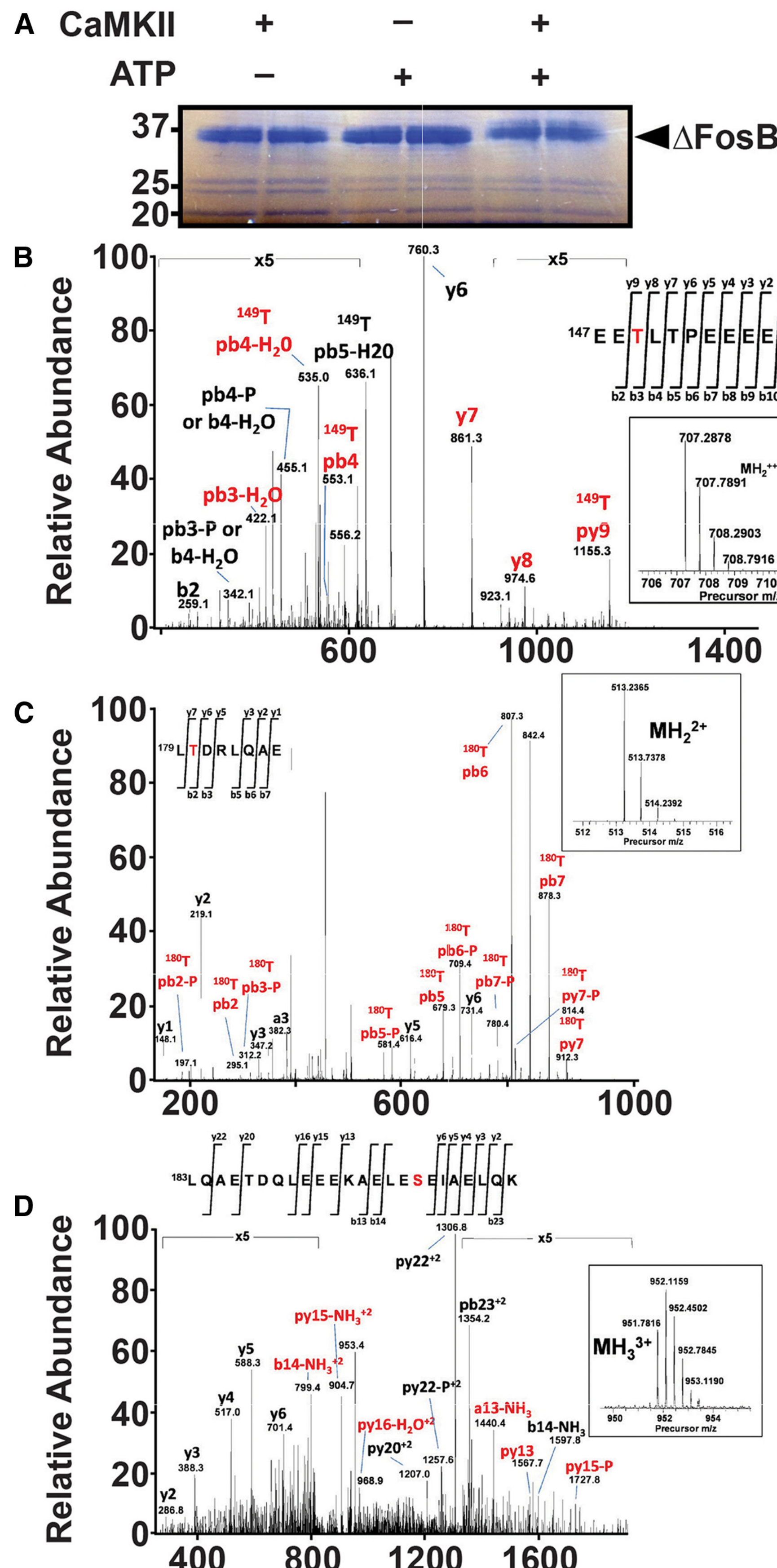


Figure 1. Identification of novel sites of ΔFosB phosphorylation *in vitro*. ***A***, Coomassie-stained SDS-PAGE gel with purified His_6-ΔFosB exposed to CaMKII alone, ATP alone, or CaMKII and ATP. Note the upward shift in electrophoretic mobility of ΔFosB when exposed to CaMKII and ATP (black arrow). ***B–D***, The precursor (inset) and fragment spectra of TiO_2-enriched phosphopeptides detected from ΔFosB after *in vitro* phosphorylation by CaMKII. After using both trypsin digestion and enrichment of the phosphopeptide samples by TiO_2, analysis reveals phosphorylation of Thr[149] (***B***), Thr[180] (***C***), and Ser[199] (***D***).

X-100 and 3% normal goat serum at room temperature then incubated overnight at 4°C in 1% normal goat serum, 0.3% Triton X-100, and pan-FosB antibody (Santa Cruz Biotechnology; sc-48, 1:1000). Sections were washed, placed for 1.5 h in a 1:200 dilution of Cy3-conjugated goat anti-rabbit IgG (Millipore), and slices were mounted under glass coverslips for visualization on a confocal microscope (Axiovert 100; LSM 510 with META emission wavelengths of 488, 543, and 633 nm; Zeiss). Images captured in both the red (FosB) and green (GFP) channels were quantified for intensity using ImageJ software (NIH).

Statistical analysis. All analysis was performed using Prism software (GraphPad). Student's *t* tests were used for all pairwise comparisons (indicated in Results where the *t* value is given), and one-way or two-way ANOVAs were used for all multiple comparisons (indicated in Results where the *F* value is given), followed by Bonferroni or Tukey *post hoc* tests where appropriate.

Results

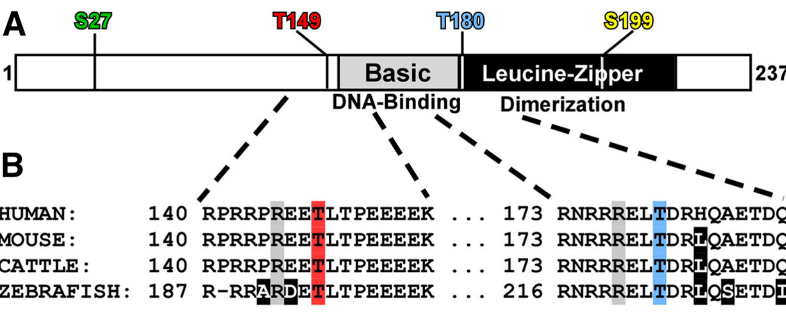
Novel CaMKII phospho-sites within ΔFosB

To identify novel CaMKII phospho-sites within ΔFosB, we performed *in vitro* phosphorylation of purified His_6-ΔFosB with purified CaMKIIα as previously described (Robison et al., 2013). Incubation of ΔFosB with CaMKII in the presence, but not absence, of ATP caused an increase in the apparent molecular weight of ΔFosB consistent with phosphorylation at multiple sites (Fig. 1*A*). MS analyses of these samples revealed phosphorylation of ΔFosB at Thr[149], Thr[180], and Ser[199] (Fig. 1*B*--*D*), along with multiple additional sites (data not shown). All three of these sites are within the leucine-zipper domain of ΔFosB (Fig. 2*A*), and thus could regulate dimerization, DNA-binding, or transcriptional activation by the AP-1 complex.

Because both Thr[149] and Thr[180] were previously predicted as possible CaMKII phospho-sites by bioinformatics analysis (Ulery et al., 2006), and the CaMKII consensus phosphorylation sequence at both sites is perfectly conserved from zebrafish through humans (Fig. 2*B*), we focused on validation of these sites as bona fide CaMKII substrates. We generated labeled synthetic peptides mimicking the phospho- and non-phospho-states of Thr[149], Thr[180],

←

and Ser[199] and then used known quantities of these peptides as standards in MRM analyses of ΔFosB before and after *in vitro* phosphorylation by CaMKII. Subsequent quantitation confirms that Thr[149] and Thr[180] are potent substrates for CaMKII, while Ser[199] phosphorylation is entirely unaffected by coincubation with CaMKII (Fig. 2*C*).



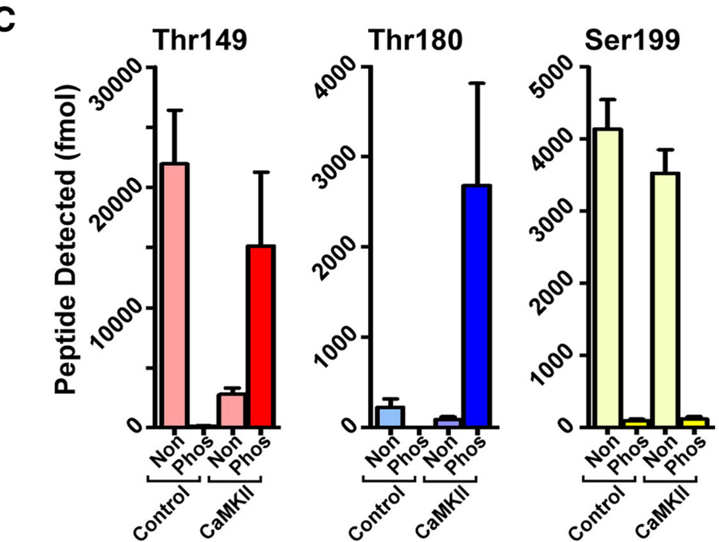


Figure 2. ΔFosB Thr[149] and Thr[180] are potent CaMKII substrates. ***A***, Schematic of ΔFosB protein structure depicting functional domains and known phosphorylation sites. ***B***, Amino acid sequence alignments showing conservation of FosB sequences surrounding Thr[149] (red) and Thr[180] (blue) in human, mouse, cow, and zebrafish, including the −3 arginine (gray) that confers CaMKII substrate specificity. ***C***, MRM analysis of ΔFosB phosphorylated *in vitro* by CaMKII reveals that Thr[149] and Thr[180], but not Ser[199], are potent CaMKII substrates. Error bars represent SEM.

ΔFosB Thr[149] phosphorylation in brain is increased by chronic cocaine

Previous studies have demonstrated that ΔFosB is a phosphoprotein in the brain (Ulery et al., 2006). Therefore, we next sought to determine whether ΔFosB is phosphorylated at Thr[149] or Thr[180] in the brain, and whether these phospho-sites are regulated by a behaviorally relevant stimulus, chronic cocaine exposure. Adult (8 weeks) male mice were administered 20 mg/kg cocaine or saline vehicle intraperitoneally once per day for 7 d. Twenty-four hours after the last injection striatum was harvested and proteins were homogenized in the presence of protease and phosphatase inhibitors, concentrated by dialysis, and proteins of ~32–38 kDa were purified by SDS-PAGE gel extraction. We then performed MRM analyses on the purified proteins using the same labeled peptides described above and observed peaks corresponding to phospho-Thr[149] and phospho-Thr[180] in striatal extracts (Fig. 3). Importantly, the amount of Thr[149] phosphopeptide was significantly higher in the proteins purified from cocaine-treated animals than in those from saline-treated controls (Fig. 3*D*; $t_{(4)} = 3.203$, $p = 0.0328$). Levels of phospho-Thr[180] were lower, and although there was a trend for an increase with cocaine, it was not significant (Fig. 3*H*). We therefore focused the remainder of our studies on Thr[149] phosphorylation.

ΔFosB T[149] phosphorylation increases AP-1 transcriptional activity

Because Thr[149] is within the basic region of ΔFosB, which is important for DNA binding (Glover and Harrison, 1995; Fig. 2*A*), we hypothesized that Thr[149] phosphorylation may regulate ΔFosB-mediated gene transcription. We constructed mutants of ΔFosB mimicking phosphorylation at Thr[149] and Thr[180] (T149D and T180D) and assayed their effects on gene transcription using an AP-1-luciferase reporter assay in Neuro2a cells. While T180D ΔFosB induces a twofold increase in AP-1-luciferase activity, which is comparable to WT ΔFosB's effect, T149D ΔFosB expression caused a dramatic 17-fold increase in AP-1 luciferase activity (Fig. 4), much stronger than that of WT or T180D ΔFosB ($F_{(6,12)} = 2.062$; $p < 0.0001$). Coexpressing WT CaMKII with WT ΔFosB increased induction of AP-1 activity to an extent similar to that observed with T149D ΔFosB, 15-fold greater than WT ΔFosB alone. However, cotransfection with catalytically dead K42R CaMKII caused a much smaller though still significant increase, suggesting that CaMKII catalytic activity is the primary but not sole means by which it regulates ΔFosB transcriptional activity. These data suggest that CaMKII-mediated phosphorylation of ΔFosB at Thr[149] robustly increases AP-1 transcriptional activity of the protein.

ΔFosB Thr[149] phosphorylation does not affect *in vivo* protein stability

Previous data demonstrate that CaMKII overexpression can enhance the stability of ΔFosB in mouse NAc *in vivo* (Robison et al., 2013), though the mechanism of this enhancement was not determined. Because phosphorylation of ΔFosB Ser[27] is known to increase ΔFosB stability *in vitro* and *in vivo* (Ulery et al., 2006; Ulery-Reynolds et al., 2009), and Ser[27] is a potent CaMKII substrate (Robison et al., 2013), we hypothesized that CaMKII phosphorylation of Ser[27] was responsible for this enhancement of stability. Nevertheless, we sought to determine whether Thr[149] phosphorylation could also regulate ΔFosB stability in mouse brain. We constructed herpes simplex virus (HSV) vectors that express GFP along with WT, phospho-absent (T149A), or phosphomimetic (T149D) ΔFosB and injected them into the NAc of adult male mice (Fig. 5). Animals were analyzed 3, 7, or 14 d after virus injection, and ΔFosB expression levels were assessed by immunofluorescence and quantitative image analysis (Fig. 6*A*). No significant difference in ΔFosB expression was found between WT ΔFosB and either mutant at any of the three time points assessed (Fig. 6*B*). Thus, unlike Ser[27], Thr[149] phosphorylation does not alter ΔFosB stability *in vivo*.

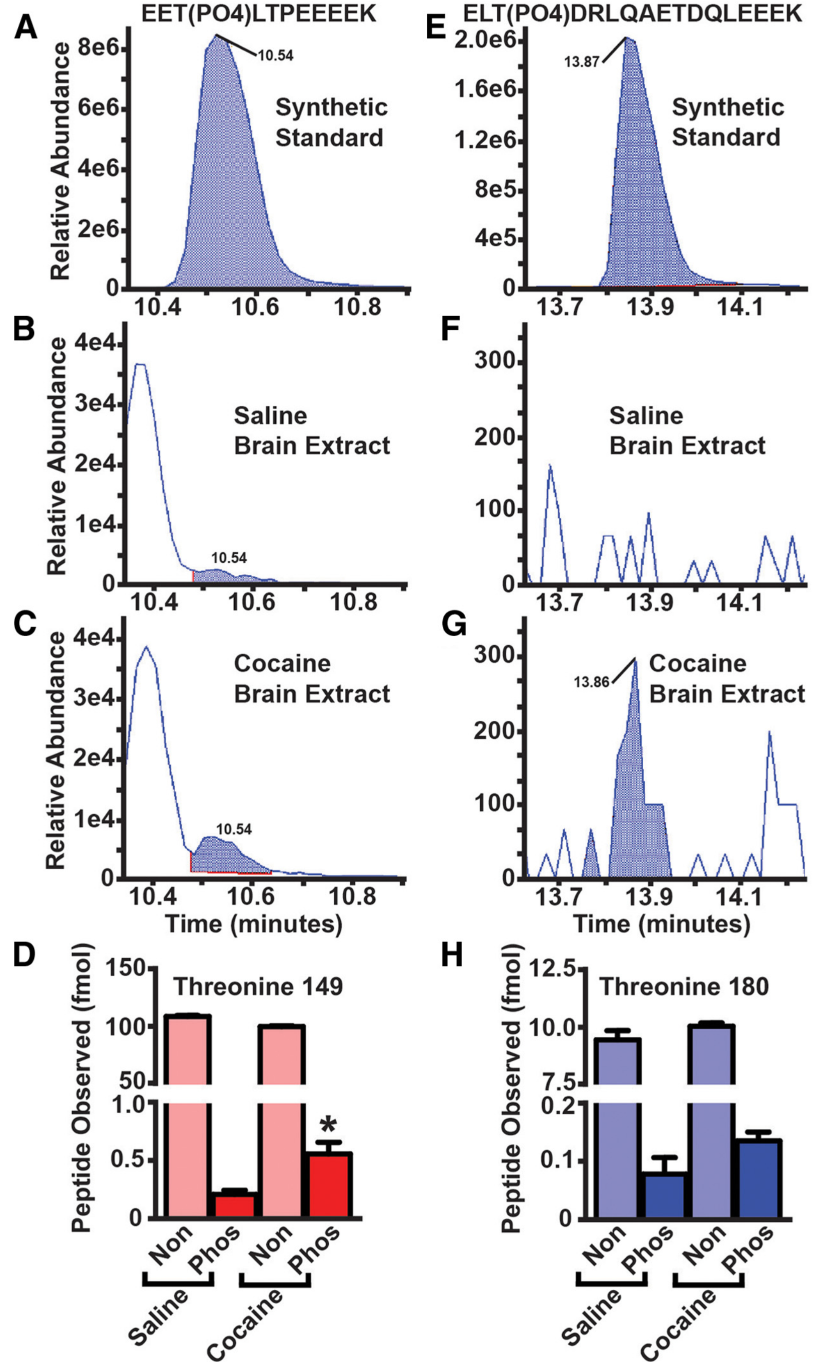


Figure 3. Phosphorylation of ΔFosB Thr[149] but not Thr[180] in mouse brain is increased by chronic cocaine. ***A***, MRM analysis peak for synthetic peptide mimicking phospho-Thr[149]. MRM analysis of striatal extracts from saline-treated (***B***) and cocaine-treated (***C***) mice reveals a peak corresponding to the phospho-Thr[149] peptide. ***D***, Quantitation of MRM analysis demonstrating a significant increase in the phospho-Thr[149] peptide in the cocaine sample compared with the control sample. ***E***, MRM analysis peak for synthetic peptide mimicking phospho-Thr[180]. MRM analysis of striatal extracts from saline-treated (***F***) and cocaine-treated (***G***) mice reveals a peak corresponding to the phospho-Thr[180] peptide. ***H***, Quantitation of MRM analysis demonstrating no significant difference in the phospho-Thr[180] peptide in the cocaine sample compared with the control sample. ($n = 3$ measurements per group; error bars represent SEM; $^{*}p < 0.05$ compared with saline phosphopeptide).

ΔFosB Thr[149] phosphorylation mediates the psychomotor effects of cocaine

Viral and transgenic ΔFosB overexpression enhances the locomotor-activating effects of cocaine, whereas viral blockade of endogenous FosB transcriptional activity reduces cocaine's locomotor effects (Kelz et al., 1999; Grueter et al., 2013; Robison et al., 2013). We used HSV-mediated overexpression of WT or mutant ΔFosB to determine whether Thr[149] phosphorylation affects the ability of ΔFosB to regulate locomotor responses to cocaine. None of the ΔFosB vectors had a significant effect on baseline locomotor activity (Fig. 7*A*). We used a low dose of cocaine (3.75 mg/kg) over 5 d that does not normally elicit locomotor sensitization (Grueter et al., 2013) to maximize chances of seeing increased behavioral responses. We found a significant effect of virus ($F_{(3,113)} = 3.373$; $p < 0.0005$) and day ($F_{(2,113)} = 19.08$; $p < 0.0001$) on locomotor activity. As expected, animals overexpressing GFP alone showed no locomotor activation to initial or repeated low doses of cocaine, while animals expressing WT ΔFosB displayed increased locomotor activity only after repeated cocaine administration (*post hoc* analysis, day 5 vs day 1; $t_{(17)} = 3.098$; $p = 0.0065$; Fig. 7*B*). Animals expressing T149D ΔFosB exhibited increased locomotor activity to cocaine following the first administration (*post hoc* analysis, day 1 vs day 0; $t_{(24)} = 4.137$; $p < 0.0005$; Fig. 7*B*), which did not increase further with continued exposure (*post hoc* analysis, day 1 vs day 5; $t_{(22)} = 0.384$; $p = 0.705$; Fig. 7*B*). In contrast, animals expressing T149A ΔFosB did not sensitize to cocaine at all, thus appearing phenotypically similar to GFP-alone controls. These data indicate that ΔFosB Thr[149] phosphorylation can confer an increased initial sensitivity to the locomotor-activating effects of low-dose cocaine, which mimics that seen after repeated administration of a low dose, and is necessary for ΔFosB-mediated increases in locomotor sensitization during repeated administration.

To determine whether Thr[149] phosphorylation is also necessary for the locomotor sensitization that typically occurs in response to a higher dose of cocaine, we administered 5 d of 7.5 mg/kg cocaine to mice with HSV-mediated NAc overexpression of GFP alone, WT ΔFosB, or T149A ΔFosB (Fig. 8). As before, these mice had no difference in baseline locomotor response to a saline injection (Fig. 8*A*), but with cocaine we found a significant effect of both virus ($F_{(2,69)} = 4.092$; $p < 0.05$) and day ($F_{(2,69)} = 48.88$; $p < 0.0001$). Control (GFP-alone) mice exhibited a locomotor response to acute cocaine that was greater than the saline response (*post hoc* analysis, day 1 vs day 0; $t_{(16)} = 2.123$; $p < 0.05$; Fig. 8*B*) and exhibited locomotor sensitization over time (*post hoc* analysis, day 1 vs day 5; $t_{(16)} = 2.445$; $p < 0.05$; Fig. 8*B*). Animals expressing WT ΔFosB in NAc also exhibited a significant acute response to cocaine (*post hoc* analysis, day 1 vs day 0; $t_{(18)} = 5.097$; $p < 0.0001$; Fig. 8*B*) and exhibited locomotor sensitization over time (*post*

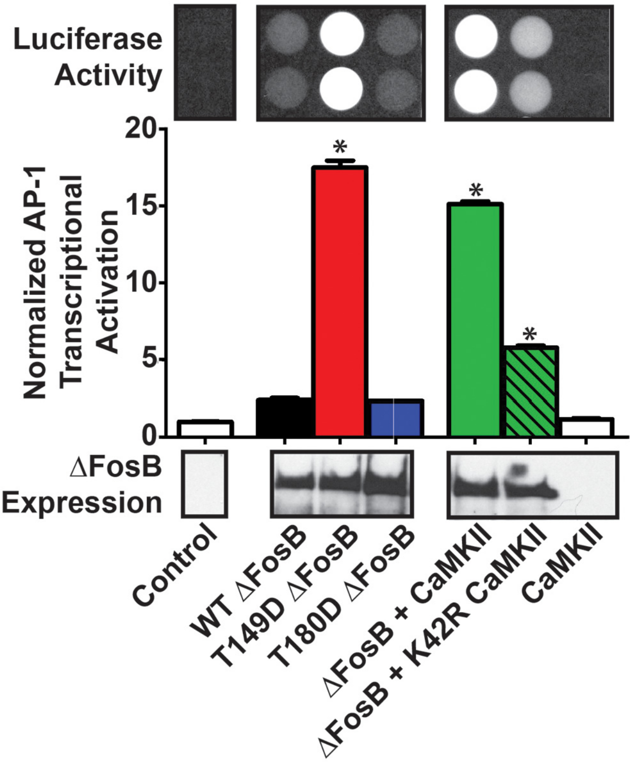


Figure 4. Thr[149] controls ΔFosB-mediated transcriptional activity. Luciferase reporter assay (top) in Neuro2A cells demonstrates that Thr[149] phosphomimetic mutation of ΔFosB increases transcriptional activation of a 4xAP-1 reporter construct >7-fold compared with WT ΔFosB or ΔFosB-T180D, both of which induce AP-1 activity ~2-fold. Cotransfection of WT CaMKII with WT ΔFosB results in a similar increase as ΔFosB-T149D, while the catalytically dead K42R CaMKII mutant, caused only a twofold change. Western blot (bottom) shows equal expression of all ΔFosB constructs. ($n = 3$ wells per group; error bars represent SEM; $*p < 0.0001$ compared with WT ΔFosB).

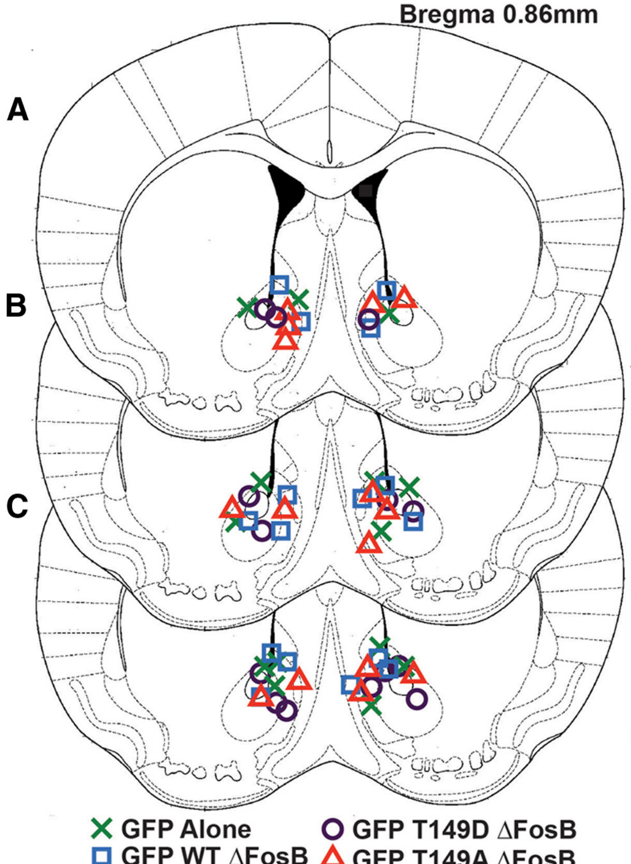


Figure 5. Injection sites for HSV-mediated ΔFosB mutant overexpression studies. Adult male mice were stereotactically injected bilaterally in the NAc with HSVs expressing GFP alone or GFP plus WT ΔFosB, T149D ΔFosB, or T149A ΔFosB. Animals were allowed to recover and then analyzed 3 d (***A***), 7 d (***B***), or 14 d (***C***) after HSV injection. Symbols mark center mass for each injection; viral spread was typically 1 mm^3 as observed previously (Robison et al., 2013).

hoc analysis, day 1 vs day 5; $t_{(16)} = 2.977$; $p < 0.01$; Fig. 8*B*). However, although animals expressing T149A ΔFosB in NAc had an acute response to cocaine (*post hoc* analysis, day 1 vs day 0; $t_{(13)} = 4.249$; $p < 0.001$; Fig. 8*B*), they exhibited no sensitization of locomotor response with repeated administration (*post hoc* analysis, day 1 vs day 5; $t_{(13)} = 0.0091$; $p = 0.99$; Fig. 8*B*). Although this lack of sensitization in the T149A ΔFosB animals appears to be driven by the acute response to cocaine on day 1, *post hoc* test reveals no significant difference between GFP alone and T149A ΔFosB in day 1 response to cocaine ($t_{(14)} = 1.965$; $p = 0.069$). Thus, the data suggest that ΔFosB Thr[149] phosphorylation is necessary for the locomotor sensitization to repeated cocaine observed in control animals.

Discussion

Here, we identify novel sites of CaMKII-mediated phosphorylation of ΔFosB *in vitro*; demonstrate that phosphorylation of one of these sites, Thr[149], is increased in striatum *in vivo* by chronic cocaine; and show that this site regulates ΔFosB-induced transcriptional activity and locomotor activation to cocaine. This novel mechanism further solidifies the NAc-specific connection between CaMKII and ΔFosB in regulating drug responses (Robison et al., 2013, 2014), and suggests that exploration of possible roles for this molecular pathway in other brain regions and in regulation of other cellular and behavioral functions is an important focus for future studies.

Although a role for NAc CaMKII expression and activity has been established in several contexts, including behavioral responses to cocaine (Pierce et al., 1998; Wang et al., 2010; Robison et al., 2013), amphetamine (Loweth et al., 2008, 2010, 2013), and antidepressants (Robison et al., 2014), the mechanism of its action in NAc has not been completely delineated. CaMKII drives surface expression of AMPA receptors (Hayashi et al., 2000), a phenomenon associated in NAc with behavioral sensitization to cocaine (Boudreau and Wolf, 2005). More recently, a detailed mechanism for CaMKII regulation of AMPA receptor surface expression has emerged involving CaMKII phosphorylation of stargazin (Stg), which modulates the ability of Stg to mediate recruitment of AMPA receptors to the postsynaptic density (PSD) by the structural proteins PSD-95 and PSD-93 (Hell, 2014). Because locomotor sensitization is dependent on CaMKII activity and AMPA receptor function (Pierce et al., 1996, 1998), and because behavioral responses to AMPA receptor activation in NAc are enhanced by CaMKIIα overexpression (Singer et al., 2010), it seems likely that the behavioral effects of CaMKII on cocaine responses are due at least in part to modulation of AMPA receptor function. Moreover, CaMKII activity in the NAc is required for reinstatement of cocaine seeking in self-administration assays, and this process results in increased phosphorylation of the AMPA receptor GluA1 at Ser[831] and is blocked by a viral vector that impairs the transport of GluA1-containing AMPA receptors to the synaptic membrane (Anderson et al.,

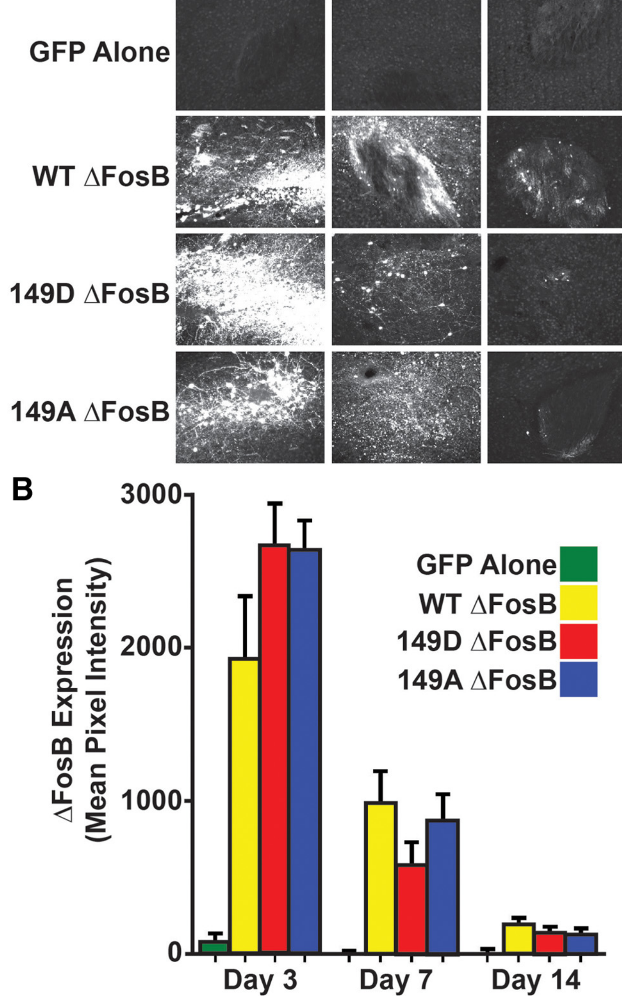


Figure 6. Thr ^{149} phosphorylation does not affect ΔFosB stability *in vivo*. ***A***, Immunohistochemistry reveals ΔFosB expression in the mouse NAc 3, 7, and 14 d after injection of HSV-GFP or HSV-GFP-WT ΔFosB, -T149D ΔFosB, or -T149A ΔFosB. ***B***, Quantitative image analysis shows that all constructs express ΔFosB to a similar extent compared with GFP alone control, and that there is no difference in the persistence of ΔFosB expression over time between any of the constructs ($n = 4-8$ injections per group error bars represent SEM).

2008). Since ΔFosB regulates AMPA receptor subunit expression in multiple contexts including chronic cocaine exposure (Kelz et al., 1999; Vialou et al., 2010), we hypothesize that CaMKII mediates complex changes in AMPA receptor function at NAc synapses both by direct modulation of receptor conductance and incorporation at PSDs and by phosphorylating ΔFosB to control receptor expression and subunit composition. However, AMPA receptor plasticity in NAc following cocaine self-administration is complicated and differs depending on route of administration, time of abstinence, and re-exposure (Wolf and Ferrario, 2010; Pierce and Wolf, 2013), and integrating these changes with the amount and location of ΔFosB expression will be a challenge going forward.

ΔFosB Ser ^{27} phosphorylation regulates protein stability (Ulery-Reynolds et al., 2009), and CaMKII phosphorylates ΔFosB at Ser ^{27} and regulates ΔFosB stability in the brain (Robison et al., 2013). However, Ser ^{27} phosphorylation also regulates ΔFosB transcriptional activity, as mutation of Ser ^{27} to Ala reduces ΔFosB-mediated AP-1-luciferase activity (Ulery and Nestler, 2007). In those earlier studies, we found that mutation of

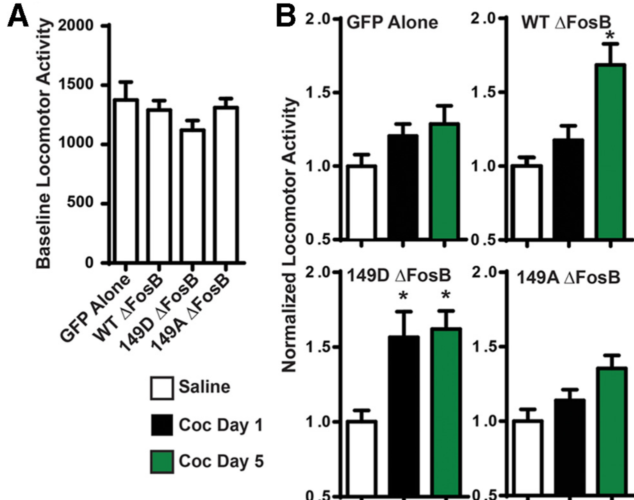


Figure 7. ΔFosB Thr ^{149} phosphorylation drives cocaine-induced psychomotor activity. ***A***, Baseline locomotor activity is unaffected by HSV-GFP-WT ΔFosB, -T149D ΔFosB, or -T149A ΔFosB in mouse NAc. ***B***, Normalized locomotor activity after intraperitoneal injection of saline or on day 1 or day 5 of five daily cocaine (3.5 mg/kg) injections in mice from ***A***. Cocaine elicited significant locomotor activity compared with saline only on day 5 in animals expressing WT ΔFosB, but on both days 1 and 5 in animals expressing T149D ΔFosB. ($n = 10$ animals per group; error bars represent SEM; *$p < 0.05$ compared with saline).

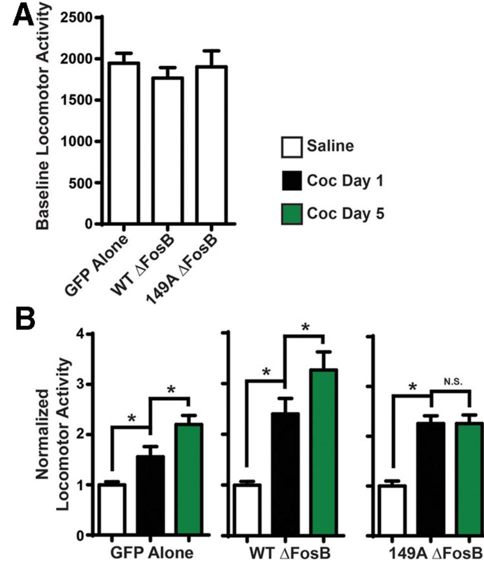


Figure 8. ΔFosB Thr ^{149} phosphorylation is required for cocaine-induced psychomotor sensitization. ***A***, Baseline locomotor activity is unaffected by HSV-GFP-WT ΔFosB or -T149A ΔFosB in mouse NAc. ***B***, Normalized locomotor activity after intraperitoneal injection of saline or on day 1 or day 5 of five daily cocaine (7.5 mg/kg) injections in mice from ***A***. Cocaine elicited significant locomotor activity compared with saline on day 1 in all animals, but only animals expressing GFP or WT ΔFosB showed a sensitized response to cocaine on day 5 compared with day 1. ($n = 7-10$ animals per group; error bars represent SEM; *$p < 0.05$ compared with saline).

Ser ^{27} to Asp has no effect on ΔFosB's transactivation potential. Moreover, the Ser ^{27} effect is specific to ΔFosB, as the same S27A mutation in the context of full-length FosB has no significant effect. Because the transactivation potential of WT ΔFosB is less than that of full-length FosB under the same conditions (Ulery and Nestler, 2007), specific regulation of ΔFosB's transactivation potential by Ser ^{27} and Thr ^{149} phosphorylation may add a level of control required for long-lasting ΔFosB to function properly, but

not necessary for the proper functioning of full-length FosB, whose transient expression may provide all of the required temporal specificity. Future studies will determine whether Thr 149 phosphorylation regulates function of full-length FosB.

The location of Thr 149, adjacent to the DNA-binding domain and very close to the transactivation domain (Fig. 2*A*; for review, see Morgan and Curran, 1995), suggests that it might regulate DNA binding or dimerization, either with Jun proteins or homodimerization (Jorissen et al., 2007), to directly alter affinity for DNA or the specificity of DNA binding sites. However, because ΔFosB is missing much of the transactivation (and degron) domains present in full-length FosB (Carle et al., 2007), the exact mechanisms of ΔFosB transactivation are unknown. Thus, it is also possible that Thr 149 phosphorylation could affect transactivation potential directly, by allosteric alteration of protein–protein interactions, or indirectly by alteration of secondary or tertiary protein structure to affect the conformation of other regions of ΔFosB important for protein–protein interactions. Because T149D mutation enhances the ability of ΔFosB to regulate the locomotor-activating effects of cocaine (Fig. 7), it is clear that Thr 149 phosphorylation must regulate the extent of ΔFosB-mediated transactivation of target genes or the specific subset of genes targeted *in vivo*. Understanding the specific genes transcriptionally altered by ΔFosB Thr 149 phosphorylation, and the extent of their induction, will require the generation of novel tools, including transgenic mice with point mutations at Thr 149. Such an understanding may uncover previously unstudied genes important for the effects of cocaine, and thus provide novel targets for therapeutic intervention in addiction.

References

Anderson SM, Famous KR, Sadri-Vakili G, Kumaresan V, Schmidt HD, Bass CE, Terwilliger EF, Cha JH, Pierce RC (2008) CaMKII: a biochemical bridge linking accumbens dopamine and glutamate systems in cocaine seeking. Nat Neurosci 11:344–353. CrossRef Medline

Been LE, Hedges VL, Vialou V, Nestler EJ, Meisel RL (2013) DeltaJunD overexpression in the nucleus accumbens prevents sexual reward in female Syrian hamsters. Genes Brain Behav 12:666–672. CrossRef Medline

Boudreau AC, Wolf ME (2005) Behavioral sensitization to cocaine is associated with increased AMPA receptor surface expression in the nucleus accumbens. J Neurosci 25:9144–9151. CrossRef Medline

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 DeltaFosB stability. Eur J Neurosci 25:3009–3019. CrossRef Medline

Colby CR, Whisler K, Steffen C, Nestler EJ, Self DW (2003) Striatal cell type-specific overexpression of DeltaFosB enhances incentive for cocaine. J Neurosci 23:2488–2493. Medline

Glover JN, Harrison SC (1995) Crystal structure of the heterodimeric bZIP transcription factor c-Fos-c-Jun bound to DNA. Nature 373:257–261. CrossRef Medline

Grueter BA, Robison AJ, Neve RL, Nestler EJ, Malenka RC (2013) FosB differentially modulates nucleus accumbens direct and indirect pathway function. Proc Natl Acad Sci U S A 110:1923–1928. CrossRef Medline

Hayashi Y, Shi SH, Esteban JA, Piccini A, Poncer JC, Malinow R (2000) Driving AMPA receptors into synapses by LTP and CaMKII: requirement for GluR1 and PDZ domain interaction. Science 287:2262–2267. CrossRef Medline

Hell JW (2014) CaMKII: claiming center stage in postsynaptic function and organization. Neuron 81:249–265. CrossRef Medline

Jorissen HJ, Ulery PG, Henry L, Gourneni S, Nestler EJ, Rudenko G (2007) Dimerization and DNA-binding properties of the transcription factor DeltaFosB. Biochemistry 46:8360–8372. CrossRef Medline

Kelz MB, Chen J, Carlezon WA Jr, Whisler K, Gilden L, Beckmann AM, Steffen C, Zhang YJ, Marotti L, Self DW, Tkatch T, Baranauskas G, Surmeier DJ, Neve RL, Duman RS, Picciotto MR, Nestler EJ (1999) Expression of the transcription factor deltaFosB in the brain controls sensitivity to cocaine. Nature 401:272–276. CrossRef Medline

Lobo MK, Covington HE 3rd, Chaudhury D, Friedman AK, Sun H, Damez-Werno D, Dietz DM, Zaman S, Koo JW, Kennedy PJ, Mouzon E, Mogri M, Neve RL, Deisseroth K, Han MH, Nestler EJ (2010) Cell type-specific loss of BDNF signaling mimics optogenetic control of cocaine reward. Science 330:385–390. CrossRef Medline

Loweth JA, Baker LK, Guptaa T, Guillory AM, Vezina P (2008) Inhibition of CaMKII in the nucleus accumbens shell decreases enhanced amphetamine intake in sensitized rats. Neurosci Lett 444:157–160. CrossRef Medline

Loweth JA, Singer BF, Baker LK, Wilke G, Inamine H, Bubula N, Alexander JK, Carlezon WA Jr, Neve RL, Vezina P (2010) Transient overexpression of alpha-Ca2+/calmodulin-dependent protein kinase II in the nucleus accumbens shell enhances behavioral responding to amphetamine. J Neurosci 30:939–949. CrossRef Medline

Loweth JA, Li D, Cortright JJ, Wilke G, Jeyifous O, Neve RL, Bayer KU, Vezina P (2013) Persistent reversal of enhanced amphetamine intake by transient CaMKII inhibition. J Neurosci 33:1411–1416. CrossRef Medline

Maze I, Schaefer A, Dietz D, Covington HE 3rd, Sampath SC, Tarakhovsky A, Greengard P, Nestler EJ (2010) G9a regulates cocaine-induced behavioral and transcriptional plasticity in a cell-type specific manner. Soc Neurosci Abstr 36:574.7.

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

Morgan JI, Curran T (1995) Immediate-early genes: ten years on. Trends Neurosci 18:66–67. CrossRef Medline

Nestler EJ (2008) Review. Transcriptional mechanisms of addiction: role of DeltaFosB. Philos Trans R Soc Lond B Biol Sci 363:3245–3255. CrossRef Medline

Peakman MC, Colby C, Perrotti LI, Tekumalla P, Carle T, Ulery P, Chao J, Duman C, Steffen C, Monteggia L, Allen MR, Stock JL, Duman RS, McNeish JD, Barrot M, Self DW, Nestler EJ, Schaeffer E (2003) Inducible, brain region-specific expression of a dominant negative mutant of c-Jun in transgenic mice decreases sensitivity to cocaine. Brain Res 970:73–86. CrossRef Medline

Perrotti LI, Weaver RR, Robison B, Renthal W, Maze I, Yazdani S, Elmore RG, Knapp DJ, Selley DE, Martin BR, Sim-Selley L, Bachtell RK, Self DW, Nestler EJ (2008) Distinct patterns of DeltaFosB induction in brain by drugs of abuse. Synapse 62:358–369. CrossRef Medline

Pierce RC, Wolf ME (2013) Psychostimulant-induced neuroadaptations in nucleus accumbens AMPA receptor transmission. Cold Spring Harb Perspect Med 3:a012021. CrossRef Medline

Pierce RC, Bell K, Duffy P, Kalivas PW (1996) Repeated cocaine augments excitatory amino acid transmission in the nucleus accumbens only in rats having developed behavioral sensitization. J Neurosci 16:1550–1560. Medline

Pierce RC, Quick EA, Reeder DC, Morgan ZR, Kalivas PW (1998) Calcium-mediated second messengers modulate the expression of behavioral sensitization to cocaine. J Pharmacol Exp Ther 286:1171–1176. Medline

Pitchers KK, Frohmader KS, Vialou V, Mouzon E, Nestler EJ, Lehman MN, Coolen LM (2010) DeltaFosB in the nucleus accumbens is critical for reinforcing effects of sexual reward. Genes Brain Behav 9:831–840. CrossRef Medline

Pitchers KK, Vialou V, Nestler EJ, Laviolette SR, Lehman MN, Coolen LM (2013) Natural and drug rewards act on common neural plasticity mechanisms with DeltaFosB as a key mediator. J Neurosci 33:3434–3442. CrossRef Medline

Robison AJ, Nestler EJ (2011) Transcriptional and epigenetic mechanisms of addiction. Nat Rev Neurosci 12:623–637. CrossRef Medline

Robison AJ, Vialou V, Sun HS, Labonte B, A Golden S, Dias C, Turecki G, Tamminga C, Russo S, Mazei-Robison M, Nestler EJ (2014) Fluoxetine epigenetically alters the CaMKIIalpha promoter in nucleus accumbens to regulate DeltaFosB binding and antidepressant effects. Neuropsychopharmacology 39:1178–1186. Medline

Robison AJ, Vialou V, Mazei-Robison M, Feng J, Kourrich S, Collins M, Wee S, Koob G, Turecki G, Neve R, Thomas M, Nestler EJ (2013) Behavioral and structural responses to chronic cocaine require a feedforward loop involving DeltaFosB and calcium/calmodulin-dependent protein kinase ii in the nucleus accumbens shell. J Neurosci 33:4295–4307. CrossRef Medline

Singer BF, Loweth JA, Neve RL, Vezina P (2010) Transient viral-mediated overexpression of alpha-calcium/calmodulin-dependent protein kinase II in the nucleus accumbens shell leads to long-lasting functional upregulation of alpha-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptors: dopamine type-1 receptor and protein kinase A dependence. Eur J Neurosci 31:1243–1251. CrossRef Medline

Ulery PG, Nestler EJ (2007) Regulation of DeltaFosB transcriptional activity by Ser27 phosphorylation. Eur J Neurosci 25:224–230. CrossRef Medline

Ulery PG, Rudenko G, Nestler EJ (2006) Regulation of DeltaFosB stability by phosphorylation. J Neurosci 26:5131–5142. CrossRef Medline

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

Vialou V Robison AJ, Laplant QC, Covington HE 3rd, Dietz DM, Ohnishi YN, Mouzon E, Rush AJ 3rd, Watts EL, Wallace DL, Iñiguez SD, Ohnishi YH, Steiner MA, Warren BL, Krishnan V, Bolaños CA, Neve RL, Ghose S, Berton O, Tamminga CA, et al. (2010) DeltaFosB in brain reward circuits mediates resilience to stress and antidepressant responses. Nat Neurosci 13:745–752. CrossRef Medline

Wang L, Lv Z, Hu Z, Sheng J, Hui B, Sun J, Ma L (2010) Chronic cocaine-induced H3 acetylation and transcriptional activation of CaMKIIalpha in the nucleus accumbens is critical for motivation for drug reinforcement. Neuropsychopharmacology 35:913–928. CrossRef Medline

Wolf ME, Ferrario CR (2010) AMPA receptor plasticity in the nucleus accumbens after repeated exposure to cocaine. Neurosci Biobehav Rev 35: 185–211. CrossRef Medline

Zachariou V, Bolanos CA, Selley DE, Theobald D, Cassidy MP, Kelz MB, Shaw-Lutchman T, Berton O, Sim-Selley LJ, Dileone RJ, Kumar A, Nestler EJ (2006) An essential role for DeltaFosB in the nucleus accumbens in morphine action. Nat Neurosci 9:205–211. CrossRef Medline