Regulation of gene expression and cocaine reward by CREB and ∆FosB

Colleen A McClung & Eric J Nestler

∆FosB (a truncated form of FosB) and CREB (cAMP response element binding protein) are transcription factors induced in the brain's reward pathways after chronic exposure to drugs of abuse. However, their mechanisms of action and the genes they regulate remain unclear. Using microarray analysis in the nucleus accumbens of inducible transgenic mice, we found that CREB and a dominant-negative CREB have opposite effects on gene expression, as do prolonged expression of ∆FosB and the activator protein-1 (AP-1) antagonist ∆cJun. However, unlike CREB, short-term and prolonged ∆FosB induction had opposing effects on gene expression. Gene expression induced by short-term ∆FosB and by CREB was strikingly similar, and both reduced the rewarding effects of cocaine, whereas prolonged ∆FosB expression increased drug reward. Gene expression after a short cocaine treatment was more dependent on CREB, whereas gene expression after a longer cocaine treatment became increasingly ∆FosB dependent. These findings help define the molecular functions of CREB and ∆FosB and identify clusters of genes that contribute to cocaine addiction.

Exposure to drugs of abuse leads to short- and long-term adaptive changes in the brain, many of which are thought to involve the regulation of gene expression[1,2]. Two transcription factors implicated in these adaptations to drugs of abuse are CREB and ∆FosB[1,2]. CREB is ubiquitously expressed and is activated through the cAMP pathway as well as several other second-messenger systems[3,4]. In the brain, CREB has been implicated in multiple phenomena including learning and memory[5–7], depression[8,9] and responses to emotional stimuli[10]. Administration of several drugs of abuse upregulates the cAMP pathway and causes CREB activation in the nucleus accumbens (also called ventral striatum)[11–14], a major reward center in the brain[1,2]. These effects are relatively short-lived, and revert to normal within days of cessation of drug exposure. Upregulation of CREB in the nucleus accumbens by cocaine and other drugs mediates tolerance to the reinforcing effects of the drugs and may mediate a state of aversion or dysphoria during early drug withdrawal[1,10,15–18]. For example, CREB overexpression in the nucleus accumbens reduces the rewarding properties of cocaine and increases depression-like behavior, whereas expression of a dominant-negative form of CREB (mCREB) in this region has the opposite effect[10,16,17]. Furthermore, mice that are partially deficient in CREB ($CREB^{\alpha\Delta}$) show an increased preference for cocaine[18].

∆FosB is a member of the Fos family of transcription factors, which dimerize with Jun family members to form activator protein-1 (AP-1) transcription factor complexes. Expression of most Fos-family proteins (c-Fos, FosB, Fra1 and Fra2) is rapidly induced in the nucleus accumbens after acute exposure to drugs of abuse[19,20]. This induction is transient, lasting only 4–12 h after drug exposure. In contrast, ∆FosB is induced in the nucleus accumbens only after chronic drug exposure. Levels of ∆FosB accumulate during chronic treatment and remain elevated even after weeks of withdrawal, due to the unusually high stability of the protein[19–23]. Thus, it has been proposed that ∆FosB is responsible for many of the longer-lived changes in gene expression that underlie addiction[1,22]. Indeed, prolonged ∆FosB expression in the nucleus accumbens increases the rewarding effects of cocaine[22–25]. Mice overexpressing ∆FosB in this region show increased place conditioning, self-administration and incentive motivation for cocaine, whereas mice that express a dominant-negative form of cJun (∆cJun), which disrupts normal AP-1 function, show less preference for cocaine[23–25]. Furthermore, drug induction of ∆FosB has been shown to be greater in adolescent animals than adults, providing a molecular mechanism for the increased vulnerability of younger individuals to addiction[26].

Previous studies[16,23,27–30] have identified a small number of target genes for CREB or ∆FosB in the nucleus accumbens: proenkephalin (Penk1), prodynorphin (Pdyn) and c-fos (Fos) for CREB, and GluR2 (Gria2), NF-κB (Nfkb1) and Cdk5 for ∆FosB. A more comprehensive examination of CREB and ∆FosB target genes in this brain region is still needed. In the present study, we used DNA microarrays from Affymetrix to characterize global patterns of gene expression in the nucleus accumbens over a time course of CREB and ∆FosB induction. These patterns were compared to those induced by mCREB and ∆cJun. We also compared CREB and ∆FosB gene expression profiles to those seen after varying periods of cocaine treatment, and we were able to examine the effects of CREB and ∆FosB on measures of cocaine reward. Together, these coordinated analyses make it possible to understand CREB- and ∆FosB-regulated genes in terms of their role in addiction.

The University of Texas Southwestern Medical Center, Department of Psychiatry and Center for Basic Neuroscience, 5323 Harry Hines Boulevard, Dallas, Texas 75390-9070, USA. Correspondence should be addressed to E.J.N. (Eric.Nestler@UTSouthwestern.edu).
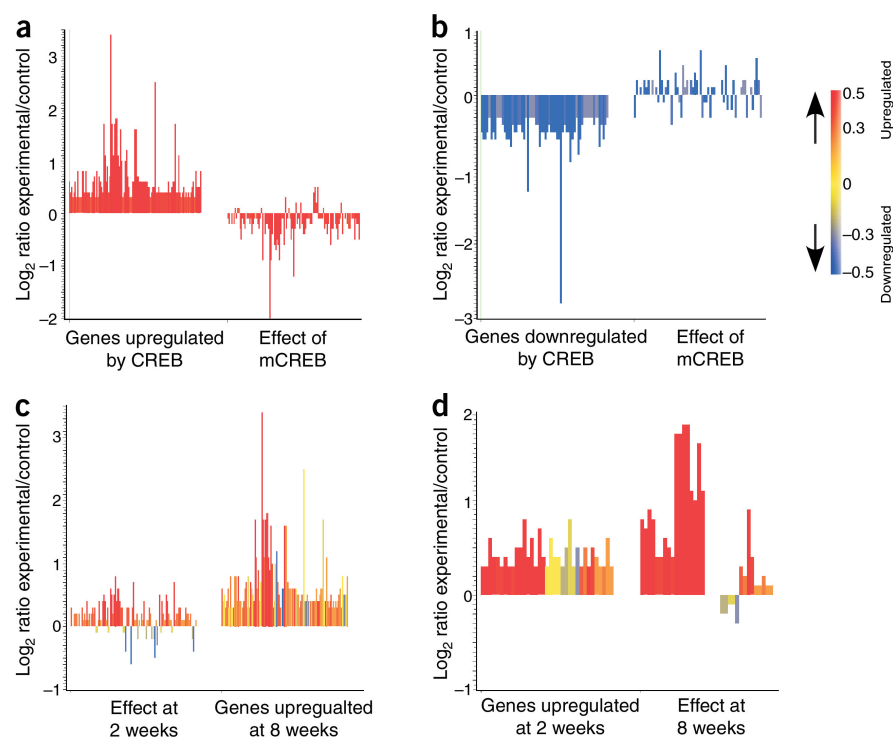
Figure 1 Regulation of gene expression by CREB. (**a**,**b**) CREB and mCREB have opposing effects on gene expression. RNA from the nucleus accumbens of mice overexpressing CREB or mCREB for 8 weeks, and their littermate controls, was subjected to microarray analysis. (**a**) CREB-upregulated genes and how they are altered in mCREB overexpressing mice vs. littermate controls (see **Supplementary Table 1** online for full list). (**b**) Genes downregulated by CREB and the effect of mCREB expression on these genes (see **Supplementary Table 2** online for full list). (**c**,**d**) Regulation by CREB is similar after 2 or 8 weeks of expression. (**c**) Genes upregulated by CREB after 8 weeks of overexpression vs. littermate controls and the effect of overexpressing CREB for 2 weeks on these genes (see **Supplementary Table 3** online for full list). (**d**) Genes upregulated in mice overexpressing CREB for 2 weeks vs. littermate controls and how these genes are regulated after 8 weeks of CREB overexpression (see **Supplementary Table 4** online for full list). The effects shown in the figure were replicated at least twice on independent groups of animals ($P < 0.01$).

We used several lines of double-transgenic mice, in which the tetracycline gene regulation system enables the inducible overexpression of CREB, mCREB, ΔFosB or ΔcJun in the brains of adult mice[23,25,31–33]. In these mice, the tetracycline transactivator (tTA) is driven with some selectivity in the nucleus accumbens and dorsal striatum by the neuron-specific enolase (NSE) promoter, under the influence of a regionally specific enhancer. tTA binds to the tetracycline responsive promoter (TetOp) on the transgene encoding CREB, mCREB, ΔFosB or ΔcJun, activating transcription in the absence of tetracycline. Therefore, mice raised with doxycycline (a tetracycline derivative) in their drinking water do not express the transgene until the doxycycline is removed. Because we used the same *NSE-tTA* line to drive expression of each of the transgenes, expression of all four transcription factors is enriched in the same brain regions—nucleus accumbens and dorsal striatum—with much less expression seen in hippocampus and frontal cortex and virtually no expression elsewhere in the brain or peripheral tissues[23,25,31–33]. Furthermore, double-labeling studies have been used to determine whether the transgenes are expressed in the two major subtypes of striatal medium spiny neuron: dynorphin[+]/substance P^{+} cells and enkephalin^{+} cells. CREB, mCREB and ΔcJun are expressed equally in both cell types[25,31–33], whereas ΔFosB is expressed solely in dynorphin^{+} cells[23]. Fortuitously, the induction of CREB in both cell types, and the induction of ΔFosB in dynorphin^{+} cells only, mimics the activation of these transcription factors seen after chronic drug administration[23,34].

RESULTS
Regulation of gene expression by CREB
Microarray analysis was performed on CREB double-transgenic mice that had been off doxycycline for 8 weeks and their littermate controls. At this time point, CREB expression is maximal and causes increases in phospho-CREB levels in the nucleus accumbens[32]. This finding indicates that increased CREB expression in the double-transgenic mice is associated with increased CREB function.

We found 111 transcripts to be significantly upregulated by CREB in the nucleus accumbens (see **Supplementary Tables** online, which correspond to the figures in this article). Importantly, 77% of these genes upregulated by CREB are downregulated by mCREB (**Fig. 1a**). The converse is true for genes downregulated by CREB, most of which are upregulated by mCREB (**Fig. 1b**). These results demonstrate that CREB and mCREB, as expected, exert generally opposite effects on gene expression. To determine if these changes require prolonged CREB overexpression, microarray analysis was performed on mice that had been off doxycycline for 2 weeks, at which point CREB overexpression is at low levels (**Fig. 1c,d**). The results indicate that 70% of genes upregulated after 8 weeks of CREB overexpression are already upregulated, but to a lesser extent, after 2 weeks of CREB overexpression (**Fig. 1c**). Furthermore, 74% of genes that are upregulated after 2 weeks of CREB expression remain upregulated after 8 weeks (**Fig. 1d**). These results indicate that the qualitative effect of CREB on gene regulation in the nucleus accumbens shows little variation with the duration of CREB expression. We have a high level of confidence in our array data for the following reasons. First, we used Affymetrix chips in which each gene is represented multiple times. Second, we used rigorous statistical analysis to identify regulated genes. Third, for each array, the RNA used was from bilateral samples pooled from 5–8 animals in each group. Fourth, all arrays were performed in duplicate or triplicate using separate groups of animals. Fifth, representative regulated genes were verified by real-time PCR (**Table 1**) on an independent (third or fourth) pool of animals. We consistently found a false positive rate of <15%.

Regulation of gene expression by ΔFosB
We next analyzed double-transgenic mice that overexpress ΔFosB in the nucleus accumbens. Microarray analysis was performed on mice expressing ΔFosB for 1, 2, 4 or 8 weeks, and their age-matched littermate controls. We found a surprisingly complex response to ΔFosB expression: most regulated genes did not simply become progressively more up- or downregulated with increasing times of ΔFosB induc-

Table 1 Examples of genes regulated by CREB and ΔFosB

Examples of genes upregulated by CREB at 8 weeks

Gene	Known CREB targets	Verified by PCR[a]	Examples of other highly regulated genes[b]
BDNF	*	*	
Cholecystokinin	*	*	
FISP12	*		
c-Fos	*		
GABA-A receptor alpha 1	*		
GIF transcription factor			*
Glutathione S-transferase 3			*
MEF2C	*		
Neurofilament medium subunit	*		
NOV		*	
PACAP	*	*	
Somatostatin	*		
Somatostatin receptor type 2	*		*
Stathmin-like protein type 2			*
Adenylate cyclase 6	*		
T-box brain 1		*	
B-FABP		*	
G-protein β1		*	

Examples of genes upregulated by ΔFosB at 8 weeks

Gene	Known ΔFosB targets	Verified by PCR[a]	Examples of other highly regulated genes[b]
Adenosine receptor 2A			*
ARNT2		*	
BAT3		*	
CaMKII		*	
CLCN4		*	
GluR2	*		
NMDA Z1			*
St gal Nac VI		*	
Cry2		*	
GAD		*	
MAP2		*	
Raly		*	

Table 1 Examples of genes regulated by CREB and ΔFosB

continued

Genes upregulated by CREB, ΔFosB (2 wks) and ΔcJun, and downregulated by ΔFosB (8 wks) and mCREB

Gene	Protein structure/function
BDNF	Neurotrophin
Cholecystokinin	Neuropeptide
leukemia-associated gene(stathmin)	Neuron-enriched phosphoprotein
NOV	Growth factor (CCN family)
Pancortin-4	Olfactomedin-related glycoprotein
T-box brain 1	Transcription factor (T-box)

[a]Examples of genes significantly and consistently regulated on the microarrays, and verified in triplicate by real-time PCR on RNA from an independent group of animals ($P < 0.05$ by t-test).
[b]Examples of additional genes significantly and consistently regulated on the microarrays multiple times in each experiment. Abbreviations: BDNF, brain-derived neurotrophic factor; FISP12, fibroblast inducible secreted protein 12; GIF transcription factor, glial cell-derived neurotrophic factor inducible transcription factor; MEF2C, myocyte enhancer factor 2C; NOV, nephroblastoma overexpressed protein; PACAP, pituitary adenylyl cyclase activating peptide; B-FABP, brain fatty acid binding protein; G-protein β1, guanine nucleotide binding protein beta 1; ARNT2, AhR receptor nuclear translocator 2; BAT3, HLA-B associated transcript 3; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; CLCN4, voltage-gated chloride channel N4; GluR2, AMPA glutamate receptor subunit 2; NMDA Z1, NMDA glutamate receptor subunit Z1; St gal Nac VI, ST6GalNAc VI,N-acetylgalactosaminide alpha2,6-sialyltransferase; GAD2, glutamate decarboxylase 2; Cry2, cryptochrome 2; MAP2, microtuble associated protein 2; Raly, hnRNP associated with lethal yellow.

tion, as found with CREB, but actually reversed their pattern of expression (**Fig. 2**). Over half (55%) of the genes that are initially upregulated after 1–2 weeks of ΔFosB overexpression are later downregulated after 8 weeks of expression. Likewise, the large majority (78%) of genes that are upregulated after 8 weeks of expression are initially downregulated after 1–2 weeks of expression. We next examined how these genes were regulated in animals expressing ΔcJun. We found that ΔcJun upregulates most (71%) of the genes upregulated by ΔFosB at earlier time points, but downregulates most (64%) of the genes that are upregulated by more prolonged ΔFosB expression (**Fig. 3**). These results suggest that initially, upon induction, ΔFosB generally exerts the same effect on gene expression as ΔcJun (that is, ΔFosB acts as a transcriptional repressor at AP-1 sites), but with more prolonged induction, ΔFosB has effects mostly opposite to those of ΔcJun (that is, ΔFosB acts as a transcriptional activator at AP-1 sites).

Comparison between CREB and ΔFosB gene regulation

When we compared the genes regulated in the nucleus accumbens by CREB to those regulated by ΔFosB, we discovered a striking similarity

(90%) in the genes upregulated both by short-term ΔFosB expression and by CREB (**Fig. 4**). Furthermore, the majority of these genes were also downregulated after 8 weeks of ΔFosB expression as well as by mCREB (**Fig. 4**). These results indicate that activation of CREB and early induction of ΔFosB in nucleus accumbens exert very similar effects on gene expression.

The altered patterns of gene expression seen in the nucleus accumbens of CREB- and ΔFosB-expressing transgenic mice could conceivably be mediated indirectly via altered gene expression induced by the transgenes in other regions, such as the hippocampus. This seems unlikely given the enrichment of transgene expression in striatal regions and the lack of hippocampal (that is, spatial learning) deficits in these mutant lines (refs. 23, 25 and unpublished observations).

Correlation of gene expression and cocaine reward

Our findings of similar genomic effects of CREB and initial ΔFosB induction on gene expression were surprising in light of previous studies, cited earlier, which report opposite effects of CREB and ΔFosB on cocaine reward. However, in these studies, the behavioral effects of ΔFosB were examined only after long-term expression. Therefore, we determined the effect of short-term ΔFosB overexpression on the rewarding properties of cocaine using the place conditioning assay. In contrast to the increased preference for cocaine seen after prolonged ΔFosB overexpression[23], 2–3 weeks of ΔFosB expression reduces the rewarding effects of cocaine (**Fig. 5a**). Thus, the opposite effects of short- versus longer-term ΔFosB overexpression on gene regulation correlate with the opposite effects on cocaine reward observed under these two conditions. Furthermore, in accordance with previous viral expression studies[16,17], CREB overexpression in our inducible double-transgenic mice reduced preference for cocaine (**Fig. 5b**). These results suggest that the genes showing shared regulation by CREB and short-term ΔFosB overexpression may be involved in reducing the rewarding effects of cocaine, and those showing opposite regulation by long-term ΔFosB overexpression may be involved in enhancing cocaine reward.

Regulation of gene expression by cocaine treatment

Since CREB and ΔFosB are both induced in the nucleus accumbens by cocaine, we investigated the gene expression changes in this brain

Figure 2 Regulation of gene expression by ΔFosB. RNA from the nucleus accumbens of mice overexpressing ΔFosB for 1, 2, 4 or 8 weeks, and littermate controls, was subjected to microarray analysis. (**a**) Genes upregulated after 2 weeks of ΔFosB overexpression and the effect at 1, 4 and 8 weeks of expression (see **Supplementary Table 5** online for full list). (**b**) Genes upregulated after 8 weeks of ΔFosB overexpression and the effect at 1, 2 and 4 weeks of expression (see **Supplementary Table 6** online for full list). The figure shows the generally reciprocal regulation of these genes after short- vs. longer-term ΔFosB expression. The effects shown in the figure were replicated at least twice on independent groups of animals (*P* < 0.01).
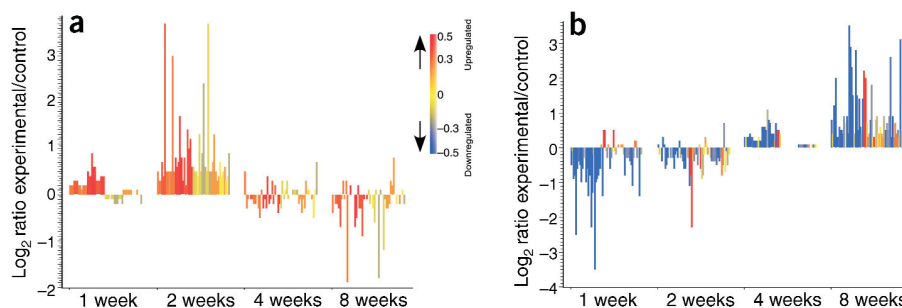
region after short- and longer-term cocaine treatments. We found that cocaine administration for 5 days leads to the induction of 89 genes, and a substantial subset of these are also induced by CREB (21%) and, to a lesser extent, short-term ΔFosB (9%) (**Fig. 6a**). This demonstrates that gene expression after a short cocaine treatment is highly dependent on the actions of CREB and less dependent on ΔFosB. In contrast, 4 weeks of cocaine administration resulted in a gene expression profile that is largely different from that regulated by short-term cocaine exposure: fewer than 10% of the same genes were regulated under these two conditions. Moreover, 4 weeks of cocaine administration resulted in a gene expression profile that is highly dependent on ΔFosB (27%) but far less dependent on CREB (6%) (**Fig. 6b**). These observations support the likely relevance of shifting patterns of gene expression over a sustained period of cocaine administration, and further implicate a role for CREB and ΔFosB in mediating particular aspects of these drug-induced adaptations over time.

DISCUSSION

In this study, we identified changes in gene expression in the nucleus accumbens induced by CREB or ΔFosB, and correlated the opposing patterns of gene regulation with modulation of the behavioral effects of cocaine. Our data indicate that CREB induces relatively straightforward alterations in gene expression. Overexpression of CREB in the nucleus accumbens leads to the induction of a specific subset of target genes, and expression of a dominant-negative mutant form of CREB downregulates the large majority of these genes. These results suggest that CREB in the nucleus accumbens acts primarily as a transcriptional activator. A smaller number of genes are downregulated by CREB, and upregulated by mCREB; CREB may regulate these

genes via the induction of transcriptional repressors. Furthermore, 2 and 8 weeks of CREB overexpression led to the induction of many of the same genes in a time-dependent manner. This could be due to the longer period of CREB expression or the higher levels of CREB expression seen at this longer time point. The fact that several of the genes identified in this study have previously been implicated as target genes of CREB or contain CRE sites in their promoter (**Table 1**) further substantiates the success of our approach in identifying target genes of CREB in the nucleus accumbens. One caveat is that transgene expression in the tetracycline-responsive system occurs relatively slowly (days or weeks) after doxycycline removal, such that the system is not ideal for studies of some immediate early genes. For example, the dynorphin gene, a known CREB target[14,16], is not upregulated in the CREB mice, consistent with findings that sustained activation of CREB causes desensitization of dynorphin gene expression[32].

Surprisingly, short-term ΔFosB induction leads to the upregulation of many of the same genes in the nucleus accumbens that are upregulated by CREB. This could be due to direct activation at the promoters of these genes by both CREB and ΔFosB. However, this action by ΔFosB is unlikely to be at AP-1 sites, since many of these same genes are also upregulated by ΔcJun, a protein known to act solely as a transcriptional repressor of AP-1 complexes[35,36]. Thus, our data suggest that at this short time point, or at relatively lower levels, ΔFosB activates these genes indirectly through its actions as a transcriptional repressor. One conceivable mechanism would be for ΔFosB to enhance CREB expression via repression of some other regulatory gene. However, we did not see an increase in CREB mRNA levels after ΔFosB induction in our microarray experiments or by real-time PCR (data not shown). Alternatively, ΔFosB could enhance CREB protein levels or activity through the regulation of any of several signaling proteins, a possibility that now warrants further investigation.

After longer periods or higher levels of overexpression, ΔFosB generally produces opposite effects on gene expression. Most of the genes
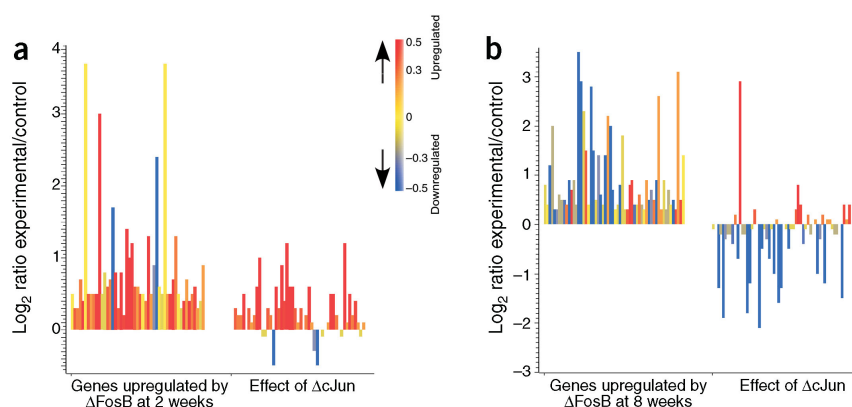

Figure 3 Comparison of the regulation of gene expression by ΔFosB and ΔcJun. RNA was extracted from the nucleus accumbens of mice overexpressing ΔcJun for 8 weeks, ΔFosB for 2 weeks, ΔFosB for 8 weeks, and littermate controls, and was subjected to microarray analysis. Genes upregulated by ΔFosB at 2 weeks (**a**; see **Supplementary Table 7** online for full list) or 8 weeks (**b**; see **Supplementary Table 8** online for full list) and the effect of ΔcJun are shown. The figure shows similar regulation by ΔFosB at 2 weeks compared to ΔcJun, but reciprocal regulation by ΔFosB at 8 weeks. The effects shown in the figure were replicated at least twice on independent groups of animals (*P* < 0.01).

Figure 4 Comparison of the regulation of gene expression by CREB and ΔFosB. (**a**) Genes upregulated by CREB after 8 weeks of overexpression, showing generally similar regulation by 2 weeks of ΔFosB overexpression and opposite effects by 8 weeks of ΔFosB and by mCREB (see **Supplementary Table 9** online for full list). (**b**) Genes upregulated by ΔFosB after 2 weeks of overexpression, showing generally similar regulation by CREB and opposite effects by 8 weeks of ΔFosB and by mCREB (see **Supplementary Table 10** online for full list). The effects shown in the figure were replicated at least twice on independent groups of animals ($P < 0.01$).
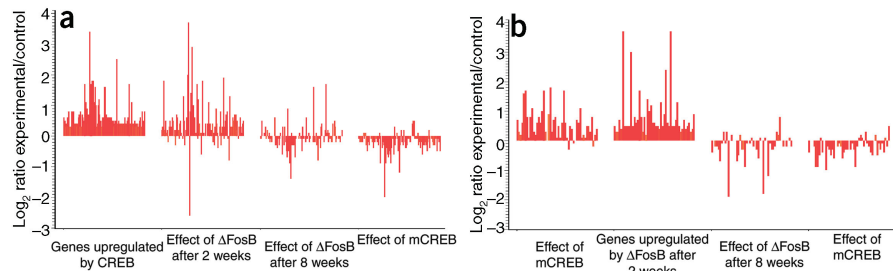
that were initially downregulated by short-term ΔFosB overexpression (or by CREB) are later upregulated by ΔFosB. ΔcJun expression down-regulates most of these genes, suggesting that, as ΔFosB accumulates, it begins to act as a transcriptional activator at AP-1 sites. Though ΔFosB lacks most of the transactivation domain found in FosB and other Fos family members, and studies have shown that ΔFosB can act as an AP-1 repressor *in vitro*[37,38], higher levels of ΔFosB can clearly activate transcription at AP-1 sites[21,39,40]. In addition, ΔFosB may produce some of its prolonged effects via interactions with CREB. Genes that are upregulated by CREB are generally downregulated by prolonged expression of ΔFosB. It is possible that higher levels of ΔFosB compete with CREB at CRE sites and block CREB-mediated transcriptional activation. ΔFosB can bind CRE sites and, in fact, ΔFosB/JunD dimers can bypass CREB to become the dominant proteins bound to CRE sites in the striatum after induction of high levels of ΔFosB (that is, after dopamine denervation and L-dopa treatment)[28]. Alternatively, one or more genes that are upregulated by prolonged ΔFosB expression may reduce the expression or activity of CREB. One possible candidate for such regulation is CaM-kinase II. We found that CaM-kinase II is upregulated with prolonged ΔFosB overexpression (**Table 1**). CaM-kinase II can phosphorylate CREB and prevent its dimerization with CBP (CREB binding protein), thereby inhibiting

CREB action[41]. Thus, CaM-kinase II may act to reduce the activity of CREB and reduce the expression of CREB target genes.

When we studied the regulation of gene expression by cocaine, we found that subsets of the genes upregulated by a short (5-day) cocaine treatment were also upregulated by CREB (21% of the genes) and to a lesser extent by ΔFosB. In contrast, 4 weeks of cocaine treatment induced a gene expression profile that became more dependent on prolonged ΔFosB overexpression, accounting for 27% of the cocaine-regulated genes, whereas very few genes regulated after 4 weeks of cocaine are dependent on CREB. These findings suggest that the consequences of shorter cocaine treatments are partly mediated via CREB- and ΔFosB-dependent mechanisms, while the effects of more chronic cocaine treatments become increasingly dependent on the actions of accumulating ΔFosB. Indeed, the findings support a dominant role for ΔFosB in the long-term genomic actions of cocaine[22]: this single transcription factor accounts for more than one-quarter of the effects of chronic cocaine on gene expression. Still, cocaine upregulates large numbers of genes that do not appear to be regulated by ΔFosB or CREB. There are several other cocaine-regulated transcription factors that likely contribute to the regulation of these genes, including Egr1-3, Nac1, Nurr1 and NF-κB[29,42–44].

Other laboratories have carried out microarray studies using various cocaine treatment protocols, in multiple organisms, and in a vari-
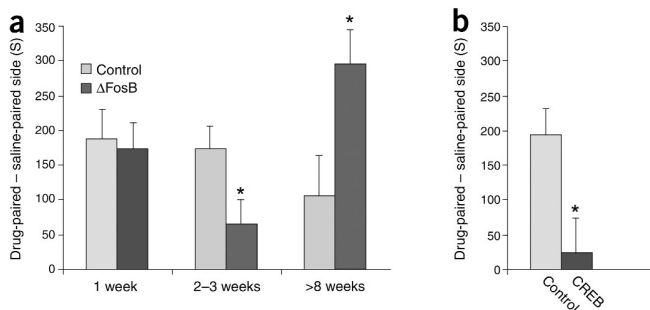

Figure 5 Regulation of cocaine reward by ΔFosB and CREB. (**a**) Mice overexpressing ΔFosB for 1 week or 2–3 weeks were tested in the conditioned place preference paradigm with 5 mg/kg cocaine. Mice off doxycycline for 1 week (at which point very little ΔFosB is expressed) showed no difference in cocaine preference compared to control littermates, whereas mice off doxycycline for 2–3 weeks (at which point low levels of ΔFosB are expressed) showed a significant reduction in preference for cocaine ($P < 0.05$, $n = 21$–28). Removal of doxycycline for 8–12 weeks (at which point ΔFosB expression is maximal) enhances cocaine preference[23]. (**b**) Mice overexpressing CREB for 2–3 weeks were tested for conditioned place preference to cocaine (2.5 mg/kg). CREB-overexpressing mice showed a significant reduction in cocaine preference ($P < 0.05$, $n = 13$–15).
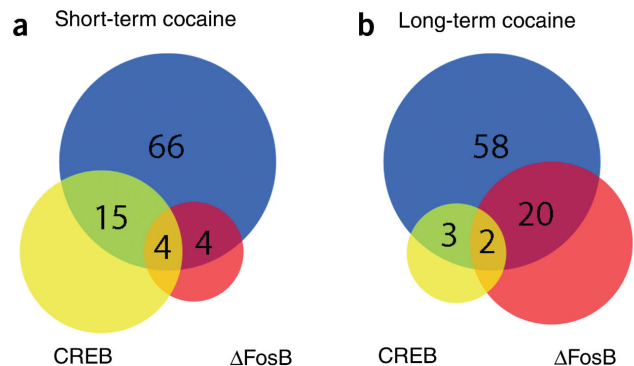

Figure 6 Regulation of gene expression by cocaine: comparison to effects of CREB and ΔFosB. Mice carrying the *NSE-tTA* transgene alone were given daily i.p. injections of cocaine (10 mg/kg) or saline for 5 d (**a**), or cocaine (15 mg/kg) or saline for 5 d/week for four consecutive weeks (**b**), and were used 24 h after the last injection. RNA was isolated from the nucleus accumbens and subjected to microarray analysis. (**a**) Venn diagram showing the number of genes with shared upregulation among short-term cocaine treatment, short-term ΔFosB expression (1–2 weeks) and CREB expression (8 weeks) (see **Supplementary Table 11** online for full list). (**b**) Venn diagram showing the number of genes with shared upregulation among long-term cocaine treatment, long-term ΔFosB (4–8 weeks) and CREB expression (see **Supplementary Table 12** online for full list).

Table 2 Functional clusters of genes upregulated by CREB and ΔFosB

CREB	ΔFosB
Vesicle transport/synaptic transmission	
Syntaxin 1A	Synaptotagmin 6
Complexin 1	Synaptogyrin 1
Synaptophysin	
Oxysterol binding protein-like 1A	
Ceramide kinase	
Neurotransmitter biochemistry/receptors	
Branched chain aminotransferase 1	Glutamate receptor NMDA zeta 1
GABA-A receptor alpha 1	Glutamate receptor AMPA 2 alpha 2
	Glutamic acid decarboxylase 2
Ion channels	
Excitatory channels	*Inhibitory channels*
Sodium channel voltage gated 1 beta	Potassium channel shab-related
Transient receptor potential cation channel C 4	Potassium channel eag-related
	Hyperpolarization cyclic nucleotide gated potassium 2
	Chloride channel 4-2
Cell signaling	
Adenylate cyclase 6	Adenylate cyclase activating peptide receptor
Adenylate cyclase activating polypeptide 1	Adenosine A2a receptor
Adenylate cyclase associated protein	Cholinergic receptor c1
Ras related simian leukemia viral oncogene A	Calmodulin 3
Neuropeptide Y	Calcium/calmodulin dependent. Protein kinase II alpha
Cholecystokinin	Protein kinase C beta
ADP ribosylation factor 6	Lymphoid blast crisis-like 1
Chemokine orphan receptor 1	G-protein alpha o
Protein tyrosine phosphatase receptor type D	Protein tyrosine phosphatase IF2P
G-protein beta 1	ADP ribosylation factor 3
Thioredoxin-like2	
PFTAIRE protein kinase 1	
Cell growth/differentiation	
Growth promoting	*Growth inhibiting/apoptosis*
Nephroblastoma overexpressed	Max dimerization protein 4
Brain derived neurotrophic factor	CDP-diacylglycerol synthase 2
Connective tissue growth factor	HLA-B associated transcript 3
Fibroblast growth factor 12	Ubiquitin-specific protease 41
Deiodinase iodothyronine II	

continued

CREB	ΔFosB
Cell growth/differentiation	
Atonal homolog 2	
Neurogenic differentiation 2	
Isopentenyl-diphosphate isomerase	
T-box brain gene 1	
Myocyte enhancer factor 2C	
cFos	
TGFB inducible early growth response 1	
Neuroserpin	
Pancortin-4	
Brain abundant membrane attached signal protein	
Cell adhesion and motility	
CD59a antigen	Cadherin 2
Cadherin 13	Actin related protein 2 homolog
Protocadherin alpha 4	Kinesin family 1B
Leukemia associated gene (stathmin)	Kinesin family 5C
Stathmin-like 1	Microtubule associated protein 2
Stathmin-like2	Kinase interacting with stathmin
Stathmin like 3	Nonmuscle myosin heavy chain IIB
Tubulin beta 3	
Tubulin alpha 4	
Neurofilament 3, medium	
SH3-domain GRB2-like	
Multiple PDZ domain protein	
Somatostatin	
Somatostatin receptor 2	
Olfactomedin 1	
Lamin A	
Matrix metalloproteinase 16	
Stress and immune response	
Heat shock protein 1B	Immunoglobulin kappa chain V8
T-cell receptor alpha	DNAJ (heat shock protein 40) homolog B5
	Aryl hydrocarbon receptor nuclear translocator 2
	Heat shock protein 40

Genes that were upregulated by CREB or ΔFosB after 8 weeks of expression were clustered into functional categories based on Affymetrix gene ontology (www.affymetrix.com), KEGG (www.genome.ad.jp/kegg/), GenMAPP (www.GenMAPP.org) and sequence and literature searches. Abbreviations: GABA, gamma aminobutyric acid; NMDA, *N*-methyl-D-aspartate; AMPA, alpha-amino-3-hydroxyl-5-methyl-4 isoxazole propionic acid; ADP, adenosine diphosphate; PFTAIRE, the amino acid sequence present in the conserved PSTAIRE region of all Cdc2 kinase family members; CDP, cytidine diphosphate; HLA, histocompatibility loci; FISP, fibroblast inducible secreted protein; TGF, transforming growth factor; SH, Src homology; GRB, growth factor receptor bound protein 2; PDZ, postsynaptic density/disc large/zona occludens-1; cFos, FBJ osteosarcoma oncogene.

ety of brain regions, to identify genes that are regulated by cocaine. The study most similar to ours examined the effects of 'binge' cocaine administration in the rat caudate-putamen[45]. Though the experimental design of this study differs from ours in several ways, we find some commonalities in the changes in gene expression observed in response to cocaine, including upregulation of *Fos*, protein tyrosine phosphatase (*Ptpn5*), period 1 (*Per1*) and period 2 (*Per2*). Furthermore, a previous study examining gene expression changes after chronic cocaine in the nucleus accumbens of nonhuman primates reports an upregulation of genes that are reflective of those found in our study, including genes associated with the cAMP and map kinase pathways, as well as cell adhesion and structural proteins[46]. Our study differs significantly from previous studies in that we sought to identify genes that are regulated by cocaine downstream of specific transcription factors and to correlate these changes in gene expression with measures of cocaine reward.

Indeed, we found that the similar effects of CREB, short-term ΔFosB and ΔcJun overexpression on gene regulation correlate with

the fact that all decrease the rewarding effects of cocaine. Conversely, the opposite effect of long-term ΔFosB overexpression on activity of many of these same genes is consistent with the observation that ΔFosB, under these conditions, increases the rewarding effects of cocaine. Together, these data strongly implicate these clusters of genes in the regulation of cocaine reward (**Table 1**). One such gene identified in this study is cholecystokinin (*Cck*). CCK is a neural and gut peptide upregulated in our studies by CREB, short-term ΔFosB overexpression, ΔcJun and a short cocaine treatment, but downregulated by prolonged expression of ΔFosB and by mCREB. Previous studies have shown that CCK levels are increased in the nucleus accumbens after short-term cocaine treatment, and that CCK negatively modu-

lates dopaminergic and GABAergic function in this region[47,48]. Furthermore, infusion of a CCKB receptor antagonist into the nucleus accumbens increases the ability of amphetamine to enhance conditioned reward[49], suggesting that CCK's actions on CCKB receptors may inhibit reward circuitry, a scheme consistent with our data. Similar types of schemes can be proposed for several of the other genes identified in this study, including many that have not been previously implicated in cocaine action (**Table 1** and **Table 2**). The influence of each of these various genes on behavioral responses to cocaine now requires direct investigation.

Overall, prolonged CREB and ΔFosB expression induces very different functional clusters of gene expression (**Table 2**). One striking example is that CREB generally upregulates excitatory ion channels, whereas ΔFosB upregulates inhibitory channels. This is consistent with the view that activation of nucleus accumbens neurons is associated, like CREB, with reduced drug reward, and inhibition of the neurons, like ΔFosB, with increased drug reward[50]. Another clear difference in gene clusters regulated by CREB and ΔFosB is that CREB upregulates several genes that promote cell growth, differentiation and the extension of neuritic processes, whereas ΔFosB upregulates genes involved in cell growth inhibition, cellular stress responses and even apoptosis. These findings provide new leads that will help us understand the mechanisms controlling changes in neuronal morphology and viability as a consequence of chronic drug administration.

Taken together, our results support a model in which short-term regulation of gene expression by CREB and ΔFosB, during early phases of cocaine treatment, contributes to a feedback loop mediating reduced cocaine reward, whereas accumulation of ΔFosB during more chronic cocaine treatment mediates increasing responsiveness to cocaine. We have identified several genes in the nucleus accumbens that could be involved in this biphasic modulation of cocaine's rewarding effects. An increased appreciation of the individual contributions of these CREB and ΔFosB target genes to cocaine reward will help direct future efforts toward the development of more effective treatments for cocaine addiction.

METHODS

Mice. Male mice carrying either the *NSE-tTA* transgene and a *TetOp-CREB*, *mCREB* or *ΔcJun* transgene, or the *NSE-tTA* transgene alone, were raised on 100 μg/ml doxycyline (Sigma) in the drinking water until 3–6 weeks of age. Animals were then switched to water to induce expression of the transgene. Double-transgenic animals (*NSE-tTA; TetOp-ΔFosB*) were either taken off doxycycline (experimentals) or left on doxycycline (controls) at 3–6 weeks of age. Mice were maintained in a 12:12 h light/dark cycle and housed in groups of 2–5 with food and water *ad libitum.* All animal protocols were in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by our Institutional Animal Care and Use Committee.

RNA isolation. Bilateral nucleus accumbens punches, pooled from 5–8 male mice (~8 weeks old), were dissected and frozen immediately on dry ice. RNA was isolated using the TriZol reagent (Invitrogen) according to the manufacturer's instructions. After RNA extraction, any remaining genomic DNA was digested for 30 min using the DNA Free system (Ambion) according to the manufacturer's instructions.

Microarray analysis. cDNA synthesis, cRNA labeling, hybridization and scanning were accomplished according to manufacturer's instructions (Affymetrix). All studies used the Murine Genome U74AV2 oligonucleotide microarray (Affymetrix) containing ~12,423 genes and ESTs. The raw data were initially analyzed using Microarray Suite version 5.0 (Affymetrix), which calculates normalized expression levels and generates ratios of experimental/control signals with *P* values based on the 8–20 different probe pairs

that represent each gene on the array. Then data sets from comparison files were imported into excel (Microsoft) and Genespring (Silicon Genetics) for further comparative analysis. Experimental groups were all compared to littermate controls harboring either the *NSE-tTA* transgene alone, or left on doxycycline, that were treated and used at the same time. Control arrays were run on *NSE-tTA* animals left on or taken off doxycycline to assure that gene expression changes were not due to doxycycline alone. Arrays were chosen for statistical analysis based on quality control measures that include 3′/5′ ratios, the percentage of genes present, overall scaling factors, and the regulation and expression of various control genes. Genes that did not share an up- or downregulation in the duplicate, or in some cases triplicate, array (hybridized with RNA from separate groups of animals) similar to the first were discarded. We used a moderately stringent cut-off for significance. Genes were considered to be regulated if the experimental or control signal (in the case of downregulated genes) was ≥40 and the $\log_2$ ratio of experimental vs. control was 0.3–0.5 (or –0.3 to –0.5) (inclusive; $P < 0.003$) or the $\log_2$ ratio was ≥0.6 or ≤–0.6 (inclusive; $P < 0.01$). As further confirmation of gene regulation in the ΔFosB time course, genes were not considered upregulated at 2 weeks if they were downregulated at 1 week. Similarly, genes were not considered upregulated at 8 weeks if they were downregulated at 4 weeks. For further discussion of the experimental design and other details of the methods (MIAME report), see **Supplementary Note** online.

Conditioned place preference. An unbiased conditioning protocol was used as described previously[18]. Briefly, mice were habituated in the testing room for 30 min to 1 h prior to testing or conditioning. Mice were tested for 20 min in the boxes containing three chambers with distinct flooring and walls before conditioning on day 1 to ensure that there was no bias toward any chamber of the apparatus. Mice that spent more then 10 min in any one compartment were discarded from the study. On days 2, 3 and 4, mice were given a saline injection in the morning paired with one side of the apparatus, and then 4 h later they were given a cocaine injection paired with the other side of the apparatus. Each conditioning session lasted 20 min. On day 5, mice were assayed for the time spent in either side of the apparatus.

Real-time PCR. We confirmed gene expression changes under multiple conditions by real-time PCR in approximately 25 genes that were regulated on our arrays (see **Table 1** for abbreviated list). RNA was converted to cDNA using Superscript II reverse transcriptase according to the manufacturer's instructions (Invitrogen). PCR reaction cocktails (25 μl) contained cDNA, Fast-start LC SYBR green master mix (Roche), MgCl₂, water and custom primers designed for each gene of interest (Operon). The PCR reactions were performed in the Cepheid Light Cycler (Cepheid). Each reaction was performed in triplicate and threshold cycles (C_T) were calculated using the second derivative of the reaction. The C_T of each gene was normalized against that of GAPDH. RNA used in the PCR analysis was from 3–6 pooled bilateral nucleus accumbens punches, isolated from a separate pool of animals from those used in microarray analysis. RNA controls were used to insure that amplification of products did not come from genomic DNA.

Note: Supplementary information is available on the Nature Neuroscience website.

1. Nestler, E.J. Molecular basis of long-term plasticity underlying addiction. *Nat. Rev. Neurosci.* **2**, 119–128 (2001).
2. Berke, J.D. & Hyman, S.E. Addiction, dopamine, and the molecular mechanisms of memory. *Neuron* **25**, 515–532 (2000).
3. Mayr, B. & Montminy, M. Transcriptional regulation by the phosphorylation-dependent factor CREB. *Nat. Rev. Mol. Cell Biol.* **2**, 599–609 (2001).

4. Lonze, B.E. & Ginty, D.D. Function and regulation of CREB family transcription factors in the nervous system. *Neuron* **35**, 605–623 (2002).
5. Yin, J.C. & Tully, T. CREB and the formation of long-term memory. *Curr. Opin. Neurobiol.* **6**, 264–268 (1996).
6. Mayford, M. & Kandel, E.R. Genetic approaches to memory storage. *Trends Genet.* **15**, 463–470 (1999).
7. Impey, S. *et al.* Stimulation of cAMP response element (CRE)-mediated transcription during contextual learning. *Nat. Neurosci.* **1**, 595–601 (1998).
8. Duman, R.S. Synaptic plasticity and mood disorders. *Mol. Psychiatry* **7** (Suppl.) S29–34 (2002).
9. Nestler, E.J. *et al.* Neurobiology of depression. *Neuron* **34**, 13–25 (2002).
10. Barrot, M. *et al.* CREB activity in the nucleus accumbens shell controls gating of behavioral responses to emotional stimuli. *Proc. Natl. Acad. Sci. USA* **99**, 11435–11440 (2002).
11. Terwilliger, R.Z., Beitner-Johnson, D., Sevarino, K.A., Crain, S.M. & Nestler, E.J. A general role for adaptations in G-proteins and the cyclic AMP system in mediating the chronic actions of morphine and cocaine on neuronal function. *Brain Res.* **548**, 100–110 (1991).
12. Unterwald, E.M., Cox, B.M., Kreek, M.J., Cote, T.E. & Izenwasser, S. Chronic repeated cocaine administration alters basal and opioid-regulated adenylyl cyclase activity. *Synapse* **15**, 33–38 (1993).
13. Turgeon, S.M., Pollack, A.E. & Fink, J.S. Enhanced CREB phosphorylation and changes in c-Fos and FRA expression in striatum accompany amphetamine sensitization. *Brain Res.* **749**, 120–126 (1997).
14. Cole, R.L., Konradi, C., Douglass, J. & Hyman, S.E. Neuronal adaptation to amphetamine and dopamine: molecular mechanisms of prodynorphin gene regulation in rat striatum. *Neuron* **14**, 813–823 (1995).
15. Self, D.W. *et al.* Involvement of cAMP-dependent protein kinase in the nucleus accumbens in cocaine self-administration and relapse of cocaine-seeking behavior. *J. Neurosci.* **18**, 1848–1859 (1998).
16. Carlezon, W.A. *et al.* Regulation of cocaine reward by CREB. *Science* **282**, 2272–2275 (1998).
17. Pliakas, A.M. *et al.* Altered responsiveness to cocaine and increased immobility in the forced swim test associated with elevated cAMP response element binding protein expression in the nucleus accumbens. *J. Neurosci.* **21**, 7397–7403 (2001).
18. Walters, C.L. & Blendy, J.A. Different requirements for cAMP response element binding protein in positive and negative reinforcing properties of drugs of abuse. *J. Neurosci.* **21**, 9438–9444 (2001).
19. Hope, B.T. *et al.* Induction of a long-lasting AP-1 complex composed of altered Fos-like proteins in brain by chronic cocaine and other chronic treatments. *Neuron* **13**, 1235–1244 (1994).
20. Moratalla, R., Elibol, B., Vallejo, M. & Graybiel, A.M. Network-level changes in expression of inducible Fos-Jun proteins in the striatum during chronic cocaine treatment and withdrawal. *Neuron* **17**, 147–156 (1996).
21. Chen, J., Kelz, M.B., Hope, B.T., Nakabeppu, Y. & Nestler, E.J. Chronic Fos-related antigens: stable variants of deltaFosB induced in brain by chronic treatments. *J. Neurosci.* **17**, 4933–4491 (1997).
22. Nestler, E.J., Barrot, M. & Self, D.W. DeltaFosB: a sustained molecular switch for addiction. *Proc. Natl. Acad. Sci. USA* **98**, 11042–11046 (2001).
23. Kelz, M.B. *et al.* Expression of the transcription factor ΔFosB in the brain controls sensitivity to cocaine. *Nature* **401**, 272–276 (1999).
24. Colby, C.R., Whisler, K., Steffen, C., Nestler, E.J. & Self, D.W. Striatal cell type-specific overexpression of DeltaFosB enhances incentive for cocaine. *J. Neurosci.* **23**, 2488–2493 (2003).
25. Peakman, M.C. *et al.* 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 (2003).
26. Ehrlich, M.E., Sommer, J., Canas, E. & Unterwald, E.M. Periadolescent mice show enhanced DeltaFosB upregulation in response to cocaine and amphetamine. *J. Neurosci.* **22**, 9155–9159 (2002).
27. Simpson, J.N. & McGinty, J.F. Forskolin induces proenkephalin and preprodynorphin mRNA in rat striatum as demonstrated by in situ hybridization histochemistry. *Synapse* **19**, 151–159 (1995).
28. Andersson, M., Konradi, C. & Cenci, M.A. cAMP response element-binding protein is required for dopamine-dependent gene expression in the intact but not the dopamine-denervated striatum. *J. Neurosci.* **21**, 9930–9943 (2001).
29. Ang, E. *et al.* Induction of nuclear factor-κB in nucleus accumbens by chronic cocaine administration. *J. Neurochem.* **79**, 221–224 (2001).
30. Bibb, J.A. *et al.* Effects of chronic exposure to cocaine are regulated by the neuronal protein Cdk5. *Nature* **410**, 376–380 (2001).
31. Chen, J. *et al.* Transgenic animals with inducible, targeted gene expression in the brain. *Mol. Pharmacol.* **54**, 495–503 (1998).
32. Sakai, N. *et al.* Inducible and brain region-specific CREB transgenic mice. *Mol. Pharmacol.* **61**, 1453–1464 (2002).
33. Newton, S.S. *et al.* Inhibition of cAMP response element-binding protein or dynorphin in the nucleus accumbens produces an antidepressant-like effect. *J. Neurosci.* **22**, 10883–10890 (2002).
34. Shaw-Lutchman, S.Z., Impey, S., Storm, D. & Nestler, E.J. Regulation of CRE-mediated transcription in mouse brain by amphetamine. *Synapse* **8**, 10–17 (2003).
35. Lloyd, A., Yancheva, N. & Wasylyk, B. Transformation suppressor activity of a Jun transcription factor lacking its activation domain. *Nature* **352**, 635–638 (1991).
36. Brown, P.H., Kim, S.H., Wise, S.C., Sabichi, A.L. & Birrer, M..J. Dominant-negative mutants of cJun inhibit AP-1 activity through multiple mechanisms and with different potencies. *Cell Growth Differ.* **7**, 1013–1021 (1996).
37. Nakabeppu, Y. & Nathans, D. A naturally occurring truncated form of FosB that inhibits Fos/Jun transcriptional activity. *Cell* **64**, 751–759 (1991).
38. Yen, J., Wisdom, R.M., Tratner, I. & Verma, I.M. An alternative spliced form of FosB is a negative regulator of transcriptional activation and transformation by Fos proteins. *Proc. Natl. Acad. Sci. USA* **88**, 5077–5081 (1991).
39. Dobrazanski, P. *et al.* Both products of the fosB gene, FosB and its short form, FosB/SF, are transcriptional activators in fibroblasts. *Mol. Cell Biol.* **11**, 5470–5478 (1991).
40. Chen, J. *et al.* Induction of cyclin-dependent kinase 5 in the hippocampus by chronic electroconvulsive seizures: role of deltaFosB. *J. Neurosci.* **20**, 8965–8971 (2000).
41. Wu, X. & McMurray, C.T. Calmodulin kinase II attenuation of gene transcription by preventing cAMP response-element binding protein (CREB) dimerization and binding of the CREB-binding protein. *J. Biol. Chem.* **276**, 1735–1741 (2001).
42. Korutla, L. *et al.* Differences in expression, actions and cocaine regulation of two isoforms for the brain transcriptional regulator NAC1. *Neuroscience* **110**, 421–429 (2002).
43. Bannon, M.J. *et al.* Decreased expression of the transcription factor NURR1 in dopamine neurons of cocaine abusers. *Proc. Natl. Acad. Sci. USA* **99**, 6382–6385 (2002).
44. O'Donovan, K.J., Tourtellotte, W.G., Millbrandt, J. & Baraban J.M. The EGR family of transcription-regulatory factors: progress at the interface of molecular and systems neuroscience. *Trends Neurosci.* **22**, 167–173 (1999).
45. Yuferov, V. *et al.* Differential gene expression in the rat caudate putamen after "binge" cocaine administration: advantage of triplicate microarray analysis. *Synapse* **48**, 157–169 (2003).
46. Freeman, W.M. *et al.* Chronic cocaine-mediated changes in non-human primate nucleus accumbens gene expression. *J. Neurochem.* **77**, 542–549 (2001).
47. Beinfeld, M.C., Connolly, K.J. & Pierce, R.C. Cocaine treatment increase extracellular cholecystokinin (CCK) in the nucleus accumbens shell of awake, freely moving rats, an effect that is enhanced in rats that are behaviorally sensitized to cocaine. *J. Neurochem.* **81**, 1021–1027 (2002).
48. Tanganelli, S., Fuxe, K., Antonelli, T., O'Connor, W.T. & Ferraro, L. Cholecystokinin/dopamine/GABA interactions in the nucleus accumbens: biochemical and functional correlates. *Peptides* **22**, 1229–1234 (2001).
49. Josselyn, S.A., Franco, V.P. & Vaccarino, F.J. PD-135158, A CCKB receptor antagonist, microinjected into the nucleus accumbens and the expression of conditioned rewarded behavior. *Neurosci. Lett.* **209**, 85–88 (1996).
50. Beurrier C. & Malenka R.C. Enhanced inhibition of synaptic transmission by dopamine in the nucleus accumbens during behavioral sensitization to cocaine. *J. Neurosci.* **22**, 5817–5822 (2002).