Loss of the Ca^{2+}/calmodulin-dependent protein kinase type IV in dopaminoceptive neurons enhances behavioral effects of cocaine

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The persistent nature of addiction has been associated with activity-induced plasticity of neurons within the striatum and nucleus accumbens (NAc). To identify the molecular processes leading to these adaptations, we performed Cre/loxP-mediated genetic ablations of two key regulators of gene expression in response to activity, the Ca^{2+}/calmodulin-dependent protein kinase IV (CaMKIV) and its postulated main target, the cAMP-responsive element binding protein (CREB). We found that acute cocaine-induced gene expression in the striatum was largely unaffected by the loss of CaMKIV. On the behavioral level, mice lacking CaMKIV in dopaminoceptive neurons displayed increased sensitivity to cocaine as evidenced by augmented expression of locomotor sensitization and enhanced conditioned place preference and reinstatement after extinction. However, the loss of CREB in the forebrain had no effect on either of these behaviors, even though it robustly blunted acute cocaine-induced transcription. To test the relevance of these observations for addiction in humans, we performed an association study of CAMK4 and CREB promoter polymorphisms with cocaine addiction in a large sample of addicts. We found that a single nucleotide polymorphism in the CAMK4 promoter was significantly associated with cocaine addiction, whereas variations in the CREB promoter regions did not correlate with drug abuse. These findings reveal a critical role for CaMKIV in the development and persistence of cocaine-induced behaviors, through mechanisms dissociated from acute effects on gene expression and CREB-dependent transcription.

addiction | CaMKIV | CREB | striatum

Accumulating evidence indicates that long term-neuronal plasticity is dependent on specific patterns of gene expression evoked in response to stimuli. A pivotal role in this process has been ascribed to the immediate-early genes (IEGs) (1). Therefore, establishing the pathways regulating these patterns could allow linking gene activities with specific outcomes in behavior. The cAMP-responsive element binding protein (CREB) and the related cAMP response element modulator (CREM) have been shown to be the main transcription factors regulating IEG expression (2, 3). They are activated through multiple signal pathways, including by Ca^{2+}/calmodulin-dependent kinase IV (CaMKIV) and also cAMP-dependent signaling and the MAPK pathway, although other signaling cascades have also been implicated (2, 4). The role of CaMKIV appears to be of particular interest, because it is not ubiquitously expressed, with highest levels in neurons (5–7). CaMKIV has a predominantly nuclear localization and has been suggested to be the main CREB activator in hippocampal neurons in response to electrical stimulation (4). Indeed, ablation or inactivation of the *Camk4* gene has been shown to decrease CREB phosphorylation, reduce *Fos* induction in the hippocampus, and impair formation of long-term potentiation (8–10), although the exact mechanism is still a matter of dispute (11). Although the main role of CaMKIV might be activation of CREB, it is also reported to phosphorylate the CREB-binding protein (12, 13) and to regulate histone deacetylase (HDAC) trafficking (14, 15). Finally, CaMKIV regulates splicing for pre-mRNAs from several target genes such as the BK channel and NMDA receptor subunit NR1 (16) through CaMKIV-responsive RNA elements (CaRREs).

CREB and IEGs have been implicated in cocaine-induced plasticity (17–19). Expression of a dominant-negative variant of CREB in the nucleus accumbens (NAc) enhanced cocaine-induced conditioned place preference (CPP) (20), which is used to measure drug reinforcement (21). This suggests that CREB activation may reduce the sensitivity to cocaine. In line with this notion, mice with deletion of the major CREB isoforms show enhanced cocaine-induced CPP when compared with control mice (22). Moreover, transgenic mice overexpressing CREB exhibit reduced CPP responses following cocaine treatment (23). Together, these studies imply a role of CREB in cocaine reinforcement; however, the conclusions remain limited because the applied genetic interventions either fail to block CREB activity completely or induce compensation of CREM.

Given the proposed role of CaMKIV in CREB activation, we hypothesized that CaMKIV might be a crucial molecular component in the development of cocaine addiction. To test this hypothesis, we used transgenic mice with targeted gene deletions in dopaminoceptive neurons to elucidate the contribution of CaMKIV, CREB, and CREM to cocaine-induced regulation of gene expression and its behavioral significance.

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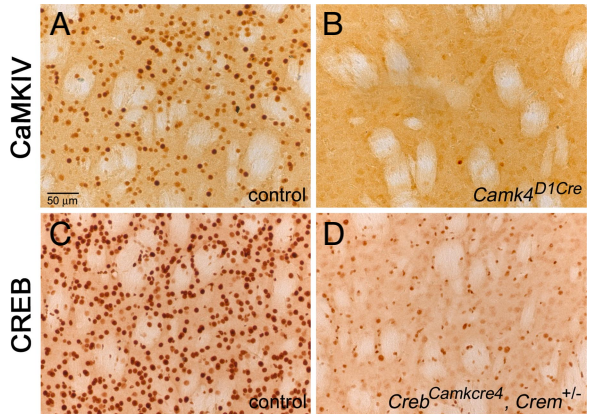

Fig. 1. Targeted inactivation of *Camk4* and *Creb1* genes. Immunostaining for CaMKIV (*A* and *B*) or CREB (*C* and *D*) was performed following the protocol described in *Methods*. Striatum from control animal (*A*), $Camk4^{D1Cre}$ mouse (*B*), $Creb1^{loxP/loxP}$, $Crem^{-/-}$ mouse (*C*), and $Creb1^{Camkcre4}$, $Crem^{+/-}$ mouse (*D*).

Results

Generation of Mice with Targeted Ablations of the *Camk4* or *Creb1* Gene. Inactivation of the *Camk4* gene was achieved using the Cre/loxP system (24). The third exon, containing the ATP binding site, was flanked with loxP sites, and its deletion produces a shift in the reading frame that prevents further translation. The Cre recombinase was expressed under the control of the D1 dopamine receptor gene (*Drd1a*) promoter from a YAC construct (25, 26). This resulted in ablation of the *Camk4* gene in the striatum, NAc, and other D1-expressing cells in the cortex and other brain areas (26). Loss of CaMKIV is observed in the great majority of striatal neurons [Fig. 1 *A* and *B*, supporting information (SI) Fig. S1], indicating that expression of the transgene is not limited to neurons of the direct pathway, which constitute roughly half of the medium spiny neurons. We suggest that this could result from transient periods of Cre expression during development as previously shown (26).

We have also used the previously described $Creb1^{Camkcre4}$ mice with additional $Crem^{-/-}$ or $Crem^{+/-}$ mutation (25). In these lines, the *Creb1* gene is inactivated in the forebrain neurons, including the striatum (Fig. 1 *C* and *D*, Fig. S1). We have previously shown that the loss of CREB is readily compensated for by overexpression of CREM (27, 28). Loss of both CREB and CREM is associated with progressive neurodegeneration of the striatum and hippocampus but is prevented by the presence of a single *Crem* allele in the $Creb1^{Camkcre4}$, $Crem^{+/-}$ animals (25).

Loss of CaMKIV Has Minor Effects on Induction of IEGs by a Single Injection of Cocaine. As anticipated, the abundance of activated CREB, phosphorylated on Ser 133, was reduced in $Camk4^{D1Cre}$ animals (Fig. S2). To assess the impact of the observed changes in phosphorylation on gene expression, we performed array gene expression profiling on the striatum (including the NAc) from $Camk4^{D1Cre}$ animals and littermate controls. As illustrated on the heat map in Fig. 2*A*, cocaine increased the transcription of several IEGs both in control and $Camk4^{D1Cre}$ animals. There were 36 transcripts induced by cocaine more than 1.5-fold with $P < 0.001$ (*t* test) as compared with saline-injected controls (Table S1). Most induced were *Fos* and *Egr2*, with a 20–30-fold increase compared with saline-injected controls, followed by *Arc*, *Fosb*, *Junb* (Table S1), and other previously reported IEGs (17–19, 29) (Fig. 2*A*, Table S1). Thus, from the 36 transcripts identified as cocaine induced in controls, none had a significantly different induction between control and $Camk4^{D1Cre}$ animals (Table S1). In line with these observations, no major differences between the genotypes were seen when we performed unbiased ontological analysis of the gene expression data, even though it hints at possible mild adaptations in transcription after CaMKIV loss (Table S2). In contrast to

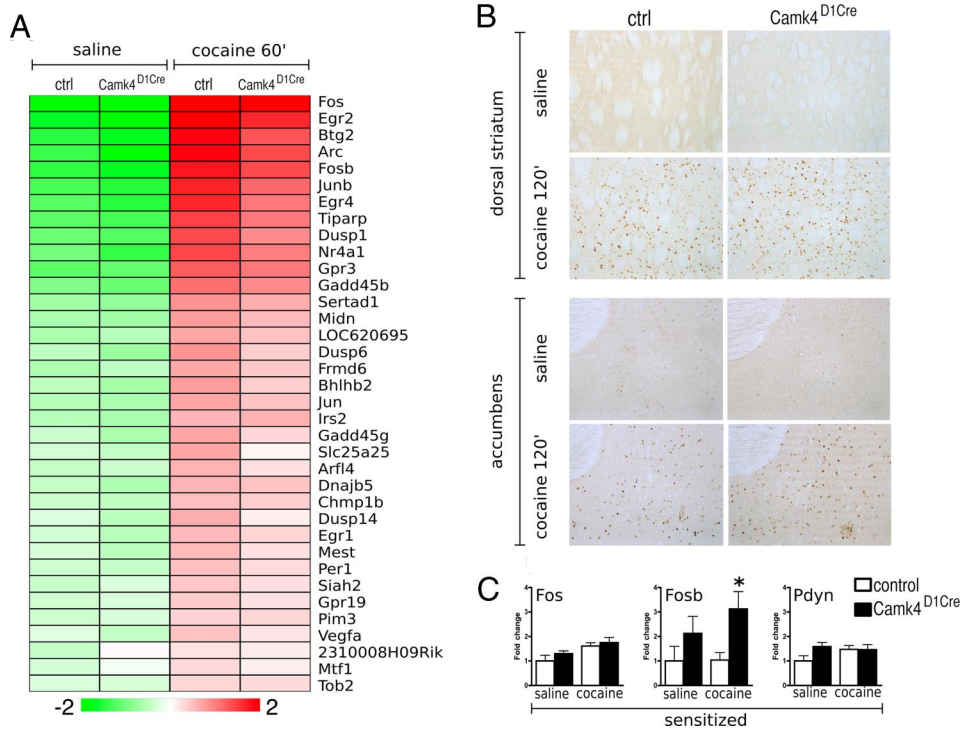

Fig. 2. Loss of CaMKIV does not impair induction of IEGs. (*A*) Profiling of gene transcription in the striatum 1 h after cocaine treatment. Gene expression profiling was performed using Affymetrix 420A 2.0 arrays on RNA samples derived from striatum from the $Camk4^{D1Cre}$ animals and littermate controls 1 h after i.p. injection with 25 mg/kg cocaine or saline. On the heat map shown in the figure, each column represents the average (3 for saline treated groups, 6 for cocaine treated groups) $\log_2$ expression values corresponding to significantly induced transcripts as indicated on the right. The color intensity is proportional to the normalized expression value as shown in the legend below. Transcripts are ordered by fold of induction in the controls treated with cocaine vs. saline. (*B*) Induction of Fos protein in the striatum 2 h after cocaine injection. Coronal sections from cocaine-injected $Camk4^{D1Cre}$ and control mice were immunostained for Fos. The upper four panels correspond to representative fragments of the dorsal striatum, and the lower four panels show a fragment of the NAc on the border of the shell and core divisions. The corresponding genotypes and treatments are indicate above and left of the images. (*C*) Expression of *Fos*, *Fosb*, and *Pdyn* after a saline or cocaine (10 mg/kg) challenge 7 days after a drug-free period in cocaine-sensitized mice. The bars represent transcript abundance normalized to the levels observed in saline-treated control animals with the SEM shown ($n = 4$–7). Empty bars correspond to control animals, and black bars correspond to $Camk4^{D1Cre}$ mice treated as indicated below the graphs. A significant difference ($P < 0.05$) between cocaine-treated $Camk4^{D1Cre}$ vs. controls is indicated by an asterisk.

Camk4^{D1Cre} mice, only 1 IEG transcript (*Nr4a1*) was significantly induced by cocaine in the striatum and NAc of *Creb1*^{Camkcre4}, *Crem*$^{-/-}$ mice and 12 in *Creb1*^{Camkcre4}, *Crem*$^{+/-}$ animals (Table S1) (30). The case of *Fos* makes a good example because its sharp increase in transcription after cocaine treatment (~30-fold compared with saline) is not affected by CaMKIV loss but is completely lost after ablation of *Creb1* and *Crem* (<2-fold), and only partially rescued by presence of a *Crem* allele (~8-fold). Thus, the ability of a single allele of *Crem* to compensate for *Creb1* is remarkable and substantiates the importance of the CREB family of transcription factors in control of activity-regulated transcription. Additionally, we performed immunostaining of Fos in *Camk4*^{D1Cre} and control animals after cocaine injection to examine if the loss of CaMKIV produced a change in the regional pattern of IEG expression (Fig. 2*B*, Fig. S3). There was a robust increase in Fos protein levels in the striatum and NAc (Fig. 2*B*), discrete areas of the cortex, and the amygdala in both mutant and control animals treated with cocaine. No appreciable changes in the regional pattern of Fos abundance in the cortex, hippocampus, or midbrain were found (Fig. S3).

CaMKIV also regulates splicing for pre-mRNAs from several target genes, such as the BK channel and NMDA receptor subunit NR1 (16), through CaRRE sites. Therefore, we assessed whether the splicing of the NR1 subunit (*Grin1*) or the BK potassium channel (*Kcnma1*) was affected by the loss of CaMKIV. No differences in the ratio of the "long" variants to total abundance of the transcripts in the striatum and NAc could be detected (Fig. S4). In our further screen, we found a putative CaRRE motif (Fig. S5) within the mouse *Fosb* gene around the 5′ border of exon V that may regulate splicing in a CaMKIV-dependent manner (16). However, no appreciable differences in the ratio of large to small isoforms could be detected in *Camk4*^{D1Cre} animals (Fig. S5). Altogether, these results indicate that CaMKIV has minor effects on the induction of IEGs after acute cocaine treatment.

Therefore, we considered the possibility that CaMKIV is more important in the regulation of gene expression after repeated cocaine treatment. We analyzed gene expression in *Camk4*^{D1Cre} and control mice after they had been subjected to our behavioral sensitization protocol (five chronic injections of cocaine) and then challenged with cocaine (10 mg/kg, i.p.) or saline after a drug-free period. No differences between genotypes in induction of *Fos* were observed (Fig. 2*C*) [two-way ANOVA: genotype F (1, 16) = 1.45; not significant]. However, *Camk4*^{D1Cre} mice displayed increased levels of *Fosb* transcript following a challenge injection of cocaine [two-way ANOVA: genotype F (1, 16) = 8.48; $P = 0.01$] and a trend toward change of *Pdyn* mRNA [two-way ANOVA: genotype F (1, 16) = 2.25; not significant] (Fig. 2*C*). Therefore, although the loss of CaMKIV does not seem to affect expression of IEGs, it may cause long-term adaptations in abundance of transcripts previously reported to represent hallmarks of addictive behavior (23, 29, 31). Because the activity of the *Fosb* promoter after chronic cocaine administration has been shown to be regulated by histone acetylation and CaMKIV is involved in HDAC trafficking, we also investigated if cocaine affected phosphorylation of HDAC4 and HDAC5 differently in the striatum of *Camk4*^{D1Cre} mice and controls. Although Ser-498 phosphorylation of HDAC5 was not affected, interestingly, HDAC4 was phosphorylated on Ser-632 in *Camk4*^{D1Cre} mice but not in controls in response to cocaine (Fig. S2).

Loss of CaMKIV in Dopaminoceptive Neurons Leads to Enhanced Psychomotor and Reinforcing Effects of Cocaine. In a series of experiments, we studied the behavioral effects of cocaine in *Camk4*^{D1Cre} mice. In baseline activity and following saline injection, these mutants did not differ from control littermates (Fig. S6). However, *Camk4*^{D1Cre} transgenic mice showed an increased initial response to cocaine (10 mg/kg, i.p.) during the first 10 min after administration (Fig. 3*A*), whereas no differences were found in mice with targeted *Creb1* ablation (Fig. 3 *B* and *C*). These results indicate the existence of an altered psychomotor responsiveness to cocaine

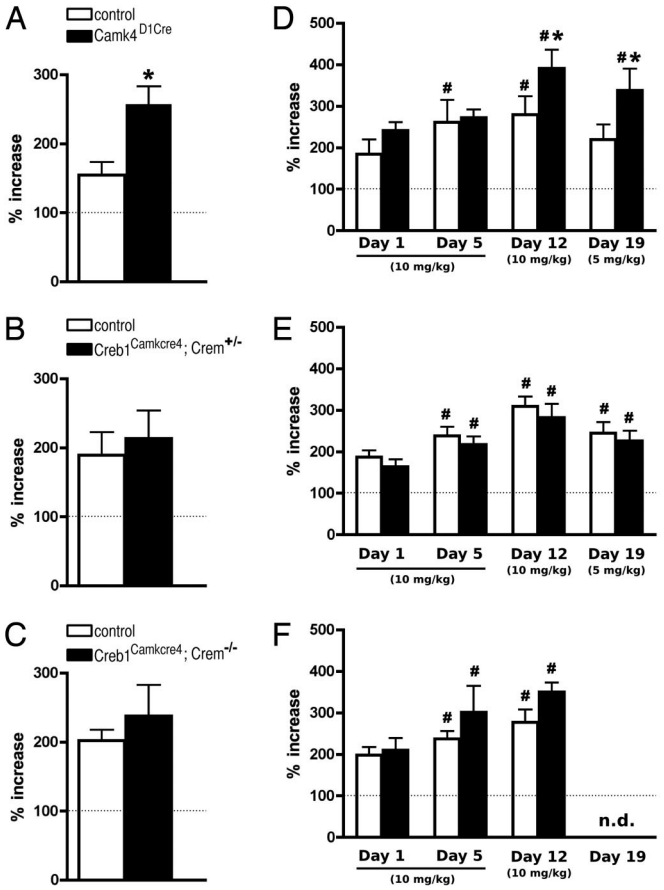

Fig. 3. Locomotor effects of cocaine in *Camk4*^{D1Cre} and *Creb1*^{Camkcre4} mice. Initial locomotor response (*A–C*) and development of cocaine sensitization (*d–f*) (10 mg/kg, i.p.) in *Camk4*^{D1Cre} ($n = 9$); *Creb1*^{Camkcre4}, *Crem*$^{+/-}$ ($n = 6$); *Creb1*^{Camkcre4}, *Crem*$^{-/-}$ ($n = 4$); and control mice for each genotype ($n = 8$, $n = 7$, and $n = 8$, respectively). (*a–c*) Cocaine induced a higher increase in locomotor activity during the first 10 min in *Camk4*^{D1Cre} mutant mice (t (15) = −3.33, $P = 0.004$) but in none of the *Creb1*^{Camkcre4} genotypes or control groups (*Creb1*^{Camkcre4}, *Crem*$^{+/-}$: t (11) = −0.4, $P = 0.6$; *Creb1*^{Camkcre4}, *Crem*$^{-/-}$: t (10) = −1.12, $P = 0.3$). (*D–F*) Control and both *Creb1*^{Camkcre4} genotypes showed intact development and expression of cocaine sensitization (*Creb1*^{CamKCre4}, *Crem*$^{+/-}$ [two-way ANOVA cocaine effect: F (3, 30) = 21.33, $P < 0.001$; *Creb1*^{Camkcre4}, *Crem*$^{-/-}$ F (2, 18) = 13.76, $P < 0.001$]. Development of sensitization was absent in *Camk4*^{D1Cre} mutant mice, but they expressed a significantly higher response to cocaine after drug-free intervals than their control littermates [two-way ANOVA day × genotype effect for *Camk4*^{D1Cre}: F (3, 45) = 10.52, $P < 0.001$; Newman-Keuls *posthoc* test: *CamKIV*^{D1Cre} vs. control, *$P < 0.01$ for coc-12 and coc-19]. Data represent the mean increase in percentage in activity in respect to saline over a 10-min (*A–C*) or 30 min (*D–F*) recording period after injection of cocaine. # represents $P < 0.05$ compared with day 1 and *$P < 0.01$ compared with control group. Because of the progressive neurodegeneration (25), *Creb1*^{Camkcre4}, *Crem*$^{-/-}$ mice were not tested on day 19.

in *Camk4*^{D1Cre} mice and suggest that CaMKIV is modulating a cocaine-induced immediate response through CREB-independent mechanisms.

Mice were further injected daily with cocaine (10 mg/kg, i.p.) for an additional 4 days, and we found a significant increase in the locomotor response to cocaine between the first and fifth sessions in all control groups of mice as well as in the *Creb1* transgenic animals, indicating development of sensitization (Fig. 3 *D–F*). In contrast, there was no significant difference between locomotor activity after the first and fifth cocaine injections in *Camk4*^{D1Cre} mice (Fig. 3*D*; $P = 0.76$) probably because of their augmented initial sensitivity to cocaine.

Following a cocaine challenge on day 12 (10 mg/kg) and day 19

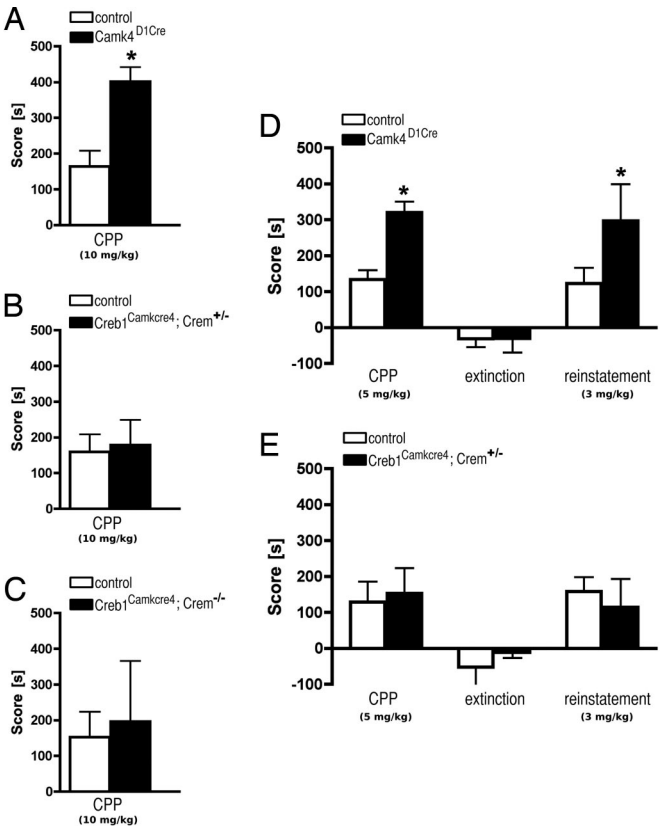

Fig. 4. Cocaine-induced reinforcement and drug-seeking behavior in *Camk4*^{D1Cre} and *Creb1*^{Camkcre4} mice. Cocaine-induced CPP (*A*–*C*), extinction and reinstatement (*D* and *E*) in *Camk4*^{D1Cre} ($n = 8$); *Creb1*^{Camkcre4}, *Crem*$^{+/-}$ ($n = 8$); *Creb1*^{Camkcre4}, *Crem*$^{-/-}$ ($n = 4$); and control mice for each genotype ($n = 10$, $n = 13$, and $n = 13$, respectively). (*A*–*C*) *Camk4*^{D1Cre} mutant mice showed higher preference for the cocaine-paired compartment [t (16) = −3.96. $P = 0.001$], whereas both *Creb1*^{Camkcre4} genotypes and control mice showed similar scores [*Creb1*^{CamKCre4}, *Crem*$^{+/-}$: t (19) = −0.23, $P = 0.6$; *Creb1*^{CamKCre4}, *Crem*$^{-/-}$: t (15) = −0.3, $P = 0.7$]. (*D* and *F*) *Camk4*^{D1Cre} mutant mice showed a more robust CPP at a dose of 5 mg/kg when compared with the control group ($n = 5$ per genotype). After extinction, a challenge injection of cocaine (3 mg/kg, i.p.) induced a similar reinstatement of the CPP in *Creb1*^{Camkcre4}, *Crem*$^{+/-}$ and control mice ($n = 8$ and $n = 13$, respectively) [two-way ANOVA conditioning × genotype effect: F (2, 18) = 0.3, $P = 0.7$], except for the *Camk4*^{D1Cre} animals, which displayed stronger preference than controls [two-way ANOVA conditioning × genotype effect: *Camk4*^{D1Cre}: F (2, 30) = 2.91, $P < 0.05$; *post hoc* for CPP, $P < 0.05$]. Results are presented as the means + SEM. CPP scores shown correspond to induction, followed by extinction and reinstatement of CPP. Statistical significance of $P < 0.05$ compared with control group is indicated by an asterisk.

(5 mg/kg), all control and *Creb1* mutant mice exhibited a robust sensitized response to cocaine, confirming the persistence of sensitization (*posthoc* test, for all $P < 0.05$). *Camk4*^{D1Cre} mice even had an augmented response when compared with their controls on both challenge days (Fig. 3*D*) (*posthoc* analysis in control vs. *Camk4*^{D1Cre} on days 12 and 19, both $P < 0.01$). In summary, despite the fact that *Camk4*^{D1Cre} mutants initially show a slower onset in the development of behavioral sensitization, they finally show an augmented sensitized behavioral response to cocaine when compared with control littermates.

We further studied the reinforcing properties of cocaine by using the CPP paradigm and found that the *Camk4*^{D1Cre}mice displayed an augmented CPP response (10 mg/kg, i.p.) (Fig. 4*A*). Conversely, ablation of *Creb1* had no effect on cocaine-induced CPP, and transgenic animals reached similar levels of preference as controls (Fig. 4 *B* and *C*). The *Camk4*^{D1Cre}animals also displayed behavioral

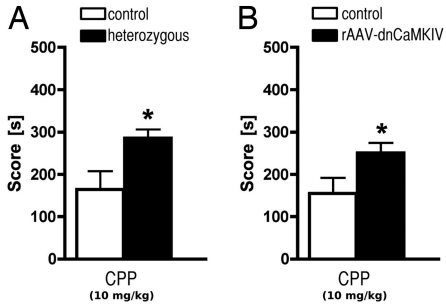

Fig. 5. Cocaine-induced reinforcement in heterozygous and virus-treated mice. Cocaine-induced CPP (10 mg/kg) in *Camk4*^{D1Cre} heterozygous ($n = 6$) and recombinant adenoassociated virus (rAAV)–dnCaMKIV ($n = 9$) mice and their respective control littermates (controls [$n = 10$] or empty virus-treated mice [$n = 8$]). Both heterozygous mice for *Camk4* (*A*) and rAAV-dnCaMKIV mice (*B*) showed a more robust CPP compared with controls [for *Camk4* ^{D1Cre} heterozygous mice: t (14) = −2.06, $P = 0.05$; for rAAV-dnCaMKIV mice: t (15) = −2.1, $P = 0.05$].

alterations in a model of cocaine seeking. We modeled cocaine-seeking behavior as the reinduction of CPP by re-exposure to the drug after an extinction period (32, 33). In this procedure, we lowered the dose of cocaine used for the conditioning to 5 mg/kg. Consistently with the higher dose, *Camk4*^{D1Cre} but not *Creb1*^{Camkcre4}, *Crem*$^{+/-}$ mice showed stronger CPP when compared with controls ($P < 0.01$). Extinction of CPP was performed by pairing saline injections with the compartment previously associated with cocaine. After eight extinction sessions, mice of all genotypes displayed no preference between the compartments anymore (Fig. 4 *D* and *E*). One day later, a challenge injection of cocaine (3 mg/kg, i.p.) induced a similar reinstatement of CPP in all genotypes, except for the *Camk4*^{D1Cre} animals, which again displayed a stronger preference than controls ($P < 0.05$).

As additional controls, we tested heterozygous mice, which express the Cre recombinase but have one WT allele of the *Camk4*, as well as mice that had recombinant adenoassociated virus expressing a dominant-negative variant of the CaMKIV injected into the NAc. The heterozygous mice exhibited a significantly higher preference for the cocaine-paired compartment than controls (Fig. 5*A*) but still lower than *Camk4*^{D1Cre} animals. This indicates that even partial loss of CaMKIV was sufficient to produce enhanced responses to cocaine and further shows a gene dosage effect. In addition, a rAAV vector expressing Flag-tagged dominant-negative CaMKIV was injected bilaterally into the NAc of adult mice. Three weeks after surgery, the transduction efficiency was assessed by Flag-immunohistochemistry and robust transgene expression was found in NAc neurons in all rAAV-dnCaMKIV–treated animals (Fig. S7) (34). Similar to our results in *Camk4*^{D1Cre} mice, cocaine-induced CPP scores in rAAV-dnCaMKIV–treated mice were significantly higher as compared with the "empty" virus-treated group (Fig. 5*B*), thus confirming the role of the NAc in the observed phenotype and excluding developmental adaptations as a confounding factor in the *Camk4*^{D1Cre} mice. Furthermore, we tested the response of dnCaMKIV-expressing animals on cocaine-induced behavioral sensitization (data not shown). Two-way ANOVA indicated a treatment × genotype effect [F (3, 67) = 5.7; $P = 0.002$]. These animals showed enhanced locomotor response to an acute cocaine challenge (empty virus-treated mice: 8359 ± 579 vs. rAAV-dnCaMKIV–treated mice: 10912 ± 972, *posthoc* $P < 0.05$) and also augmented sensitization (empty virus-treated mice: 15294 ± 907 vs. rAAV-dnCaMKIV–treated mice: 17554 ± 1079, *posthoc* $P < 0.05$).

Genetic Association Studies in Humans Indicate a Link Between CAMK4 and Addiction. Prompted by the results from animal studies, we performed an analysis of the possible association of polymor-

Table 1. SNPs genotyped in CAMK4* and CREB† with allele frequencies: odds ratio (OR) for common homozygote with 95% confidence interval (CI)

SNP	Alleles	Cases	Controls	Associated Genotype	OR	95% CI	*P* value
rs919334*	A/G	70%	64%	AA (50% vs. 40%)	1.47	1.18–1.83	0.0005
rs1457115*	C/T	55%	54%	CC	1.04	0.83–1.32	0.71
rs9285875*	C/T	76%	79%	CC	0.86	0.7–1.07	0.19
rs10876469†	T/G	65%	69%	TT	1.12	0.9–1.39	0.3
rs2177000†	A/G	69%	58%	AA	1.11	0.89–1.38	0.36

Statistics are corrected for the effects of gender, age, and population stratification.

phisms in human CAMK4 and CREB genes with cocaine dependence. Using a sample of 670 cocaine abusers and 726 controls from São Paulo, Brazil (35), we genotyped a restricted set of SNPs in the promoter regions of CAMK4 (rs919334, rs1457115, and rs9285875) and CREB (rs10876469 and rs2177000). To control for differential ethnic admixture in the heterogenous Brazilian sample, we corrected the association tests for the presence of population stratification using the program ADMIXMAP. We selected a total of 71 (64 SNPs and seven microsatellites) ancestry-informative markers (e.g., markers that exhibit large allele frequency differences between the three main Brazilian ancestral populations [Europeans, Africans, and Native Americans); details of marker set are available on request). Haplotype and association analysis was carried out with HAPLOVIEW software to examine haplotypes across all markers, with additional analyses being carried out in SPSS version 13. Of the markers rs1457115 and rs9285875, neither was associated with cocaine addiction ($P = 0.63$ and $P = 0.11$, respectively). However, rs919334 was strongly associated both allele- and genotype-wise ($P = 0.001$, allele-wise). The effect was recessive with a significant ($P = 0.006$) odds ratio of 1.47 (95% confidence interval: 1.18–1.83) for AA homozygotes after adjustment for stratification and gender effects (Table 1). Further, none of the CREB markers showed an association with cocaine addiction. Haplotype analysis failed to show any further effects and was nonsignificant (data not shown).

Discussion

The major finding of the present study is the demonstration that ablation of *Camk4* in dopaminoceptive neurons results in increased psychomotor and reinforcing effects of cocaine. These effects are independent from acutely induced IEG expression or CREB-dependent transcription but rather involve mechanisms leading to long-term alterations in *Fosb* expression. Importantly, we show that the CAMK4 gene affects development of cocaine dependence in humans, because genetic variation in its promoter is significantly associated with cocaine addiction.

The apparent dichotomy between phenotypes associated with targeted *Camk4* or *Creb1*/*Crem* deletions is intriguing. Although it has been reported that CREB activity in the NAc affects excitability of the medium spiny neurons and directly regulates locomotor responses to cocaine (36), we found that neither psychomotor sensitization to cocaine nor CPP was altered in the $Creb1^{Camkcre4}$, $Crem^{+/-}$, or $Creb1^{Camkcre4}$, $Crem^{-/-}$ animals. This observation is in agreement with previous studies on behavioral effects of morphine in mice with *Creb1* deletion in neurons (27) and the reported lack of impact of loss of major CREB isoforms on cocaine-induced reinstatement to CPP (37). Nevertheless, our observations differ from studies showing that CPP was enhanced in transgenic mice with deletion of the major CREB isoforms (22) and also in rats or mice injected with engineered herpesvirus expressing dominant-negative mCREB protein (20, 38). These discrepant results could be attributed to differences in the doses of cocaine used, mouse strain backgrounds, or other procedural differences such as biased vs. unbiased CPP procedures (33). However, we suggest that the critical difference could be the use of expression of engineered CREB variants vs. targeted *Creb1* deletions. First of all, the combined deletion of *Creb1* and *Crem* leads to progressive neuronal degeneration (25), which was a main reason for including the $Creb1^{Camkcre4}$, $Crem^{+/-}$ line in this study. The neurodegeneration was not reported with any of the other genetic approaches (20, 22, 37); hence, CREB activity was not abolished completely. Furthermore, the dominant negative CREB proteins will not only act on the CRE sequences in gene promoters but also compete with endogenous CREB for protein-protein interactions. This may lead to phenotypes resulting from interference with activity of CREB interacting proteins and not necessarily CREB-dependent transcription. In conclusion, we think that elucidating the molecular differences between these approaches may actually lead to clarifying the role of CREB in neuronal plasticity in general.

Targeted ablation of *Camk4* in dopaminoceptive neurons resulted in an augmented cocaine-induced acute response and long-term sensitization as well as reinstatement. Moreover, the $Camk4^{D1Cre}$ mice spent significantly more time in the cocaine-conditioned compartment in the CPP test compared with control littermates, demonstrating enhanced reinforcement. This effect was dependent on gene dosage, and the confirmation in rAAV-treated animals expressing a dominant-negative variant of the CaMKIV in the NAc argues strongly for a CaMKIV-dependent mechanism. It is unlikely that the stronger CPP observed in $Camk4^{D1Cre}$ mice was associated with enhanced learning ability because it did not affect the subsequent extinction of the cocaine-conditioned behavior or habituation to novelty. Furthermore, these observations have relevance to effects of cocaine in humans, because a nucleotide polymorphism in the CAMK4 promoter, rs919334, was significantly associated with cocaine addiction. The caveat applies that the effect size of the observed association is low, and this finding will require replication in similarly large, independent, case-control samples. However, the rs919334 association is robust to conservative multiple testing correction.

On the cellular level, we observed that levels of Ser 133 phosphorylated CREB were decreased in the striatum of $Camk4^{D1Cre}$ mice. Loss of CaMKIV also led to increased phosphorylation of HDAC4, which should enhance export from the cell nucleus and inhibit its function. Virally mediated expression of HDAC4 in the NAc is reported to decrease the rewarding properties of cocaine dramatically (39). Thus, CaMKIV-dependent regulation of HDAC4 activity could be involved in calibrating the response to cocaine. This is interesting in the context of observed changes in expression of *Fosb* and *Pdyn* in striatum and NAc of cocaine-sensitized mice, particularly because the phenotype observed in $Camk4^{D1Cre}$ closely resembles the one observed in case of overexpression of ΔFosB (31). We discovered a putative CaMKIV-dependent splicing site (CaRRE) surrounding the 5′ side of the exon V, which is alternatively spliced to produce the ΔFosB or FosB protein. Nevertheless, we found no significant change in the ΔFosB/FosB ratio after CaMKIV loss. In summary, although our results argue against a simplistic link between acute gene expression and behavioral outcome, they support a proposed role of epigenetic mechanisms in the development of addiction (39, 40).

In conclusion, we demonstrate that the activity of CaMKIV regulates susceptibility to cocaine in laboratory animals and in

humans. Furthermore, we find that this phenomenon is dissociated from CREB-dependent transcription and IEG induction.

Materials and Methods

Animals. Mice with ablation of the *Camk4* gene in neurons expressing the dopamine receptor 1 (*Drd1a*) were generated by the crossing the strain carrying the loxP-flanked *Camk4* gene (24) and mice harboring the Cre recombinase under the control of the D1 promoter (25, 26). We also used 5–6-week-old $Creb1^{Camkcre4}$, $Crem^{-/-}$ mice (before onset of neurodegeneration) and 5–10-week-old $Creb1^{Camkcre4}$, $Crem^{+/-}$ mice with littermate controls ($Creb1^{loxP/loxP}$, $Crem^{+/-}$ and $Creb1^{loxP/loxP}$, $Crem^{-/-}$) (25). The intra-accumbal injections of the recombinant adenoassociated virus expressing a kinase-dead mutant of CaMKIV were performed as described previously (34). The experiments were approved by the Committee on Animal Care and Use of the relevant local governmental body and were carried out following the German Law on the Protection of Animals.

Immunohistochemistry. The dissected brains were fixed in 4% paraformaldehyde, cut at 50 μm, and then processed for immunohistochemical detection with diaminobenzidine (Sigma–Aldrich Chemie GmbH). Staining of CREB was performed as described previously (25). Please see *SI Methods* for additional information.

Expression Profiling. Profiling of acutely induced gene expression was performed on $Camk4^{D1Cre}$ animals and controls that were injected once with 25 mg/kg cocaine (Sigma–Aldrich Chemie GmbH) or saline. One hour later, animals were killed and the brains were dissected and fixed overnight into RNALater solution (Sigma-Aldrich Chemie GmbHH). Then, 125-μm-thick vibratome sections were prepared, and the striatum, including the NAc, was microdissected under a binocular. RNA was prepared with the Rneasy Mini Kit (Qiagen). Microarray experiments were carried out using Mouse Genome 430A 2.0 arrays (Affymetrix) according to manufacturer's instruction. There were six chips hybridized for each cocaine group and three for saline-treated groups. Each chip corresponds to a single animal.

Behavioral Studies. Behavioral sensitization was tested in activity chambers by injecting cocaine (10 mg/kg, i.p.) for 5 consecutive days. Animals were challenged again with cocaine on days 12 and 19 after the first injection. CPP was induced by eight alternating injections of cocaine (5 or 10 mg/kg, i.p.) or saline into the corresponding compartment of the apparatus. Then, CPP was extinguished, and mice received a priming injection of cocaine (3 mg/kg, i.p.). The CPP score represents the difference between the time spent (seconds) in the cocaine or saline-paired floor during the test day (test duration: 900 sec).

Human Genetic Association Studies. Six hundred seventy cocaine abusers, 643 male and 27 female (mean age = 26.8 years, SD = 7.2), were ascertained. The study group consisted of drug users who were in treatment from August 1997 to October 1998 in one outpatient and six inpatient units located in the city of São Paulo, Brazil. Inclusion criteria were age 18 years or older, a history of cocaine abuse, and under drug treatment at the selected centers. Complete description of the methodology is provided in SI *Methods*.

Data Analysis and Statistics. Results were analyzed as appropriate with *t* tests or two-way ANOVAs, followed by the Newman-Keuls *posthoc* test. The data are presented as mean ± SEM, and in all the cases, $P < 0.05$ was considered statistically significant.

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