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BRIEF REPORT

Short-term abstinence from cocaine self-administration, but not passive cocaine infusion, elevates αCaMKII autophosphorylation in the rat nucleus accumbens and medial prefrontal cortex

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Abstract

Increases in alpha calcium/calmodulin-dependent protein kinase type II (αCaMKII) activity in the nucleus accumbens shell has been proposed as a core component in the motivation to self-administer cocaine and in priming-induced drug-seeking. Since cocaine withdrawal promotes drug-seeking, we hypothesized that abstinence from cocaine self-administration should enhance αCaMKII as well.

We found that short-term abstinence from contingent, but not non-contingent, cocaine i.v. self-administration (2 h/d for 14 d; 0.25 mg/0.1 ml, 6s infusion) elevates αCaMKII autophosphorylation, but not the kinase expression, in a dynamic, time- and brain region-dependent manner. Increased αCaMKII autophosphorylation in the nucleus accumbens (NAc) and medial prefrontal cortex (mPFC), but not dorsolateral striatum (dlS), was found 24 h, but not immediately, after the last cocaine self-administration session. Notably, in the mPFC, but not NAc and dlS, αCaMKII autophosphorylation was still enhanced 7 d later. The persistent enhancement in the mPFC of abstinent rats may represent a previously unappreciated contribution to initial incubation of cocaine-seeking.

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Introduction

Identifying the neurobiological changes persisting into or occurring during abstinence from use of a drug of abuse may provide targets for developing medications to reduce craving and possibly the likelihood of relapse. Although in the preclinical setting there has been a growing understanding of the molecular changes induced by cocaine, few studies have examined the onset and persistence of changes contributing to the high motivation to seek drugs during abstinence, as reported by clinical and preclinical studies (Gawin, 1991; Tran-Nguyen et al., 1998).

Recent preclinical research on psychostimulant-induced changes has focused on limbic structures, particularly the nucleus accumbens (NAc), as key sites for reinforcement learning and drug-induced neuroadaptations (Everitt and Robbins, 2013). Thus, alpha calcium/calmodulin-dependent protein kinase type II (αCaMKII), a kinase critically involved in learning and memory, has been proposed as a core component

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of the long-term molecular and structural changes contributing to addiction (Li et al., 2008). This is corroborated by the observation that increased αCaMKII transcription in the NAc shell is essential for the motivation to self-administer cocaine (Wang et al., 2010) as well as for cocaine-seeking behaviour induced by drug priming in abstinent rats after extinction of self-administration (Anderson et al., 2008). Considering the increased motivation to take the drug during abstinence (Gawin, 1991; Tran-Nguyen et al., 1998) and the role of NAc αCaMKII in the motivation to self-administer cocaine (Wang et al., 2010), we hypothesized that an increase of the αCaMKII kinase in the NAc might well serve as a molecular trigger for greater motivation to relapse during abstinence.

In drug addiction, the transition from controlled to compulsive drug-seeking reflects a shift from medial prefrontal cortex (mPFC) to striatal control over drug-seeking and drug-taking behaviours, as well as progression from ventral to more dorsolateral domains of the striatum (dlS) (Everitt and Robbins, 2013). Thus, based on the role of dlS and mPFC in maintaining cocaine-seeking after abstinence (Koya et al., 2009; Reichel and Bevins, 2009) we extended the analysis of αCaMKII in these brain regions where its role is still elusive. We investigated the expression and phosphorylation of αCaMKII in the NAc, dlS and mPFC of rats at three times after the last cocaine self-administration session, i.e. immediately, 24 h or 7 d. To distinguish changes in αCaMKII due to the direct pharmacological effects of cocaine from those caused by the cognitive processes associated with active drug self-administration, we adopted the yoked-control-operant paradigm. This involves triads of rats run simultaneously, with two groups serving as yoked-controls receiving non-contingent infusion of either the same dose of cocaine or saline in exactly the same pattern as their cocaine self-administering partner (Jacobs et al., 2003; Fumagalli et al., 2013).

To date, there is no information on the involvement of αCaMKII in short-term abstinence from cocaine self-administration. In the light of recent findings of an increase in αCaMKII during withdrawal from other drugs of abuse (opiates, alcohol and benzodiazepines (Shen et al., 2010; Liu et al., 2012; Wang et al., 2012)), this study will fill this gap in knowledge, at least in the brain regions examined.

Materials and methods

Animals

Naïve male Sprague–Dawley CD®IGS rats (Charles River, Italy) weighing 250–280 g at the beginning of the experiments were housed individually at 21 ± 1 °C and 60% humidity under an inverted light/dark schedule (light on 19.30–07.30 hours) with food and water freely available. Rats were allowed to adapt to these conditions for 2 wk and handled daily during this period. After this habituation period they were fed 20 g of rat chow per animal per day in the early evening.

Procedures involving animals and their care were conducted in conformity with the institutional guidelines at the IRCCS – Institute for Pharmacological Research 'Mario Negri' in compliance with national (Decreto Legge nr 116/92, Gazzetta Ufficiale, supplement 40, February 18, 1992; Circolare nr 8, Gazzetta Ufficiale, July 14, 1994) and international laws and policies (EEC Council Directive 86/609, OJL 358, 1, Dec. 12, 1987; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council (Eighth Edition) 2011).

Self-administration

Self-administration sessions began 1 wk after the jugular catheters were implanted as previously described (Cervo et al., 2007). The 2 h self-administration sessions were conducted 7 d/wk for 14 d at approximately the same time during the dark phase of the light/dark cycle in operant chambers (MED Associates Ltd, USA) enclosed in sound-attenuating ventilated cubicles, as previously described (Fumagalli et al., 2013). Cocaine was delivered by an infusion pump, through a swivel device mounted above the operant chamber and connected to the external guide cannulae of the implanted catheter. Cocaine hydrochloride solution was freshly re-mixed every 4 d in a laminar airflow cabinet, filtered through a 20 μm filter and stored in aliquots at 4 °C.

Retractable levers, stimulus lights and syringe pumps were controlled by a computer with MED-associated software which also monitored input from the levers, recording the results of each experiment on files on the hard disk. Rats were randomly assigned to three groups of triads, cocaine self-administration (SA), yoked cocaine (YC) and yoked saline (YS), to be killed immediately, 24 h or 7 d after the last session.

Rats of each triad were trained simultaneously in individual operant chambers and the sessions started with the illumination of the house light and introduction of active and inactive levers (randomly counterbalanced as either active and inactive drug levers across experimental groups). The self-administering (SA) rat of each triad was allowed to self-administer i.v. cocaine (0.25 mg/0.1 ml, 6 s infusion; MacFarlan Smith, UK) on

a FR-1 schedule. The cocaine dose of 0.75 mg/kg (0.25 mg infusion) was chosen on the basis of previous studies reporting incubation of drug-seeking after abstinence from cocaine self-administration (Tran-Nguyen et al., 1998) as well as αCaMKII involvement in the cocaine-priming induced reinstatement (Anderson et al., 2008). Each time the SA rat earned a cocaine infusion, the other two rats of the triad, serving as yoked controls, received either a non-contingent i.v. infusion of cocaine (YC) or saline (YS). In all three cages, each infusion was followed by the house-light turned off, retraction of both levers and illumination of the stimulus-light above the active lever, all of which reverted after 20 s. Further pressure on the active lever by SA rats triggered the same sequence of events. Inactive lever responding by SA rats as well as lever presses by YC and YS rats were recorded but had no programmed consequences. After the last self-administration session, rats were returned to their colony room and left undisturbed until killing. At the scheduled times, rats were individually taken in a room different from the self-administration one and decapitated. Brains were quickly removed and the mPFC, NAc and dlS dissected on ice after coronal sectioning, according to the brain atlas, as previously described (Fumagalli et al., 2013). Briefly, the mPFC was dissected from a 2 mm section extending from approximately bregma +5.16 to +3.24. The NAc and dlS were dissected from a 2 mm section, immediately caudal to the mPFC section, approximately from bregma +2.28 mm to +1.08 mm. Tissues were immediately frozen on dry ice and stored at −80 °C until assay.

Preparation of protein extracts and Western blot analysis

The preparation of the protein extracts from mPFC, NAc and dlS and the Western blot procedure were as previously described (Fumagalli et al., 2013). Total protein content was measured by the Bradford Protein Assay (Bio-Rad Laboratories, Italy) and protein concentrations were adjusted to the same amount for all samples (10 μg/lane). The following antibodies were used: anti-phospho-αCaMKII(Thr286) (1:2500; Affinity Bioreagents, USA), anti-phospho-GluA1 (Ser831) (1:500; Affinity Bioreagents, USA), anti-total αCaMKII (1:5000; Millipore, USA), anti-total GluA1 (1:2000; Upstate, USA). Immunocomplexes were visualized by chemiluminescence using the ECL Western Blotting kit (GE Healthcare, Italy) according to the manufacturer's instructions. Results were standardized to β-actin control protein, on the basis of the band density at 43 kDa after probing with a monoclonal antibody (1:10000; Sigma-Aldrich, Italy).

Statistical analysis

The numbers of active and inactive lever presses were analysed by mixed factorial analysis of variance (ANOVA) with time of death (immediate, 24 h and 7 d) and modality of self-administration (SA, YC, YS) as between-factor and levers (active, inactive) as within-factor. The number of infusions and the amount of cocaine (mg/kg) self-administered during the 14 sessions or earned during the last self-administration session were analysed, respectively, by two-way (mixed factorial) ANOVA for repeated measures, with time of death as the between-subjects factor and days of self-administration as the within-subjects factor, or by one-way ANOVA, with time of death as the main factor. Molecular data were analysed by two-way ANOVA, with modality of cocaine exposure and time of death as main factors. When appropriate, post-hoc comparisons were done with the Newman–Keuls (NK) test.

Results

All SA rats acquired cocaine self-administration, and no group differences were observed on active and inactive lever responding during chronic cocaine self-administration ($F_{\text{self-condition}}(8, 40)=6.6$, $p<0.01$; $F_{\text{lever}}(1, 40)=69.6$, $p<0.01$; $F_{\text{interaction}}(8, 40)=13.7$, $p<0.01$). As reported in Table 1, rats in SA groups emitted more active, but not inactive, lever presses than respective YS and YC ($p<0.05$, NK test). Only SA animals pressed significantly more the active than the inactive lever ($p<0.05$, NK test).

During the 14 d of self-administration, the number of infusions ($F_{\text{time-death}}(2, 13)=0.6$, $p>0.05$, $F_{\text{days-self-administration}}(13, 169)=4.8$, $p<0.01$, $F_{\text{interaction}}(26, 169)=0.9$, $p>0.05$) and the corresponding amount of cocaine self-administered ($F_{\text{time-death}}(2, 13)=0.6$, $p>0.05$, $F_{\text{days-self-administration}}(13, 169)=4.8$, $p<0.01$, $F_{\text{interaction}}(26, 169)=0.9$, $p>0.05$) gradually increased to reach stability after nine sessions (±10% daily variation). There was no significant difference in the daily number of infusions and the corresponding amount of cocaine self-administered among the three different SA groups ($p>0.05$, NK test).

Figure 1 shows the effects of cocaine self-administration on p-αCaMKII(Thr286) and total αCaMKII levels in the NAc, mPFC and dlS. In the NAc, there was a selective increase of αCaMKII autophosphorylation in the SA rats killed 24 h after the

Table 1. Lever pressing, number of infusions and corresponding amounts of cocaine during the last session or the 14 sessions of self-administration

		Last session				Fourteen sessions	
Time after last session		Active lever	Inactive lever	Number of infusions	Cocaine mg/kg	Mean number of infusions	Average cocaine intake mg/kg
Immediately	SA	24.8±2.1	0.5±0.5	24.0±1.7	17.1±1.2	18.1±1.1	12.9±0.8
	YS	1.2±0.7[a]	1.4±0.9				
	YC	7.0±7.0[a]	2.3±1.9				
24 h	SA	24.4±2.9	1.2±1.2	23.2±2.9	16.6±2.0	20.6±1.2	14.7±0.8
	YS	4.7±1.3[a]	4.9±1.8				
	YC	1.0±1.0[a]	1.0±0.7				
7 d	SA	26.5±0.3	0.0±0.0	26.2±0.5	18.7±0.3	21.5±1.0	15.4±0.7
	YS	3.8±3.2[a]	2.6±1.7				
	YC	2.8±2.6[a]	0.6±0.4				

Data are means±S.E.M. of 5–8 rats (cocaine self-administration (SA), yoked cocaine (YC) and yoked saline (YS)) killed immediately, 24 h or 7 d after the last of 14 self-administration sessions. Self-administration sessions lasted 2 h during which cocaine was available at 0.25 mg/0.1 ml, 6s infusion under FR1TO20 s. The number of infusions and the amount of cocaine (mg/kg) self-administered during the 14 sessions or earned during the last self-administration session were analysed, respectively, by two-way (mixed factorial) ANOVA for repeated measures (with time of death as between-subjects factor and days of self-administration as within-subjects factors) or by one-way ANOVA (with time of death as main factor). For clarity, only the mean number of infusions and the average of cocaine intake are reported. See Results for further details. When appropriate, post-hoc comparisons were done with the Newman–Keuls test.

[a] $p<0.05$ *vs.* respective SA, Newman-Keuls test.

last session ($F_{\text{self-condition}}(2, 47)=2.7$, $p>0.05$, $F_{\text{time-death}}(2, 47)=10.1$, $p<0.01$; $F_{\text{interaction}}(4, 47)=4.4$, $p<0.01$; $p<0.05$ *vs.* YS and YC rats, NK test) (panel A). In the mPFC, αCaMKII autophosphorylation rose selectively in the SA rats killed 24 h and 7 d after the last self-administration session ($F_{\text{self-condition}}(2, 50)=11.9$, $p<0.01$, $F_{\text{time-death}}(2, 50)=10.1$, $p<0.01$; $F_{\text{interaction}}(4, 50)=4.4$, $p<0.05$; $p<0.05$ *vs.* YS and YC rats, NK test) (panel B). Conversely, no significant changes in αCaMKII autophosphorylation were observed in the dlS at any of the time points investigated (panel C). No change in the expression of total αCaMKII in the NAc, mPFC and dlS (panels 1D, 1E and 1F) were observed. No differences were found at any time point on the phosphorylation and expression of the AMPA receptor subunit GluA1 in the mPFC and NAc of SA, YS and YC rats (data not shown).

Discussion

We found that short-term abstinence from cocaine self-administration, but not from non-contingent exposure, elevates αCaMKII autophosphorylation in the rat brain without altering the total levels of the kinase. This effect occurs in a dynamic, time- and brain region-dependent manner. In fact, increased αCaMKII autophosphorylation in the NAc and mPFC, but not dlS, was observed 24 h, but not immediately, after the last cocaine self-administration session. Moreover, in the mPFC, but not NAc and dlS, αCaMKII autophosphorylation was still enhanced 7 d later. Notably, the lack of changes in its activation in the dlS indicates that αCaMKII may not be involved in the transition from the NAc to dlS control over drug-seeking and drug-taking behaviours or in the short-term abstinence as measured after chronic short access cocaine self-administration. This is in line with a recent finding by White et al. (2013) who, using a self-administration procedure very close to our, did not find changes in αCaMKII autophosphorylation even after cocaine priming-induced reinstatement of drug-seeking after extinction.

The three independent groups of rats self-administered similar amounts of cocaine over the 14 sessions ruling out any bias due to different psychostimulant exposure. In addition, since the rats were not subjected to behavioural tests during abstinence (such as extinction learning, drug-associated cues- or cocaine priming-induced reinstatement), the molecular changes are independent from effects elicited by any

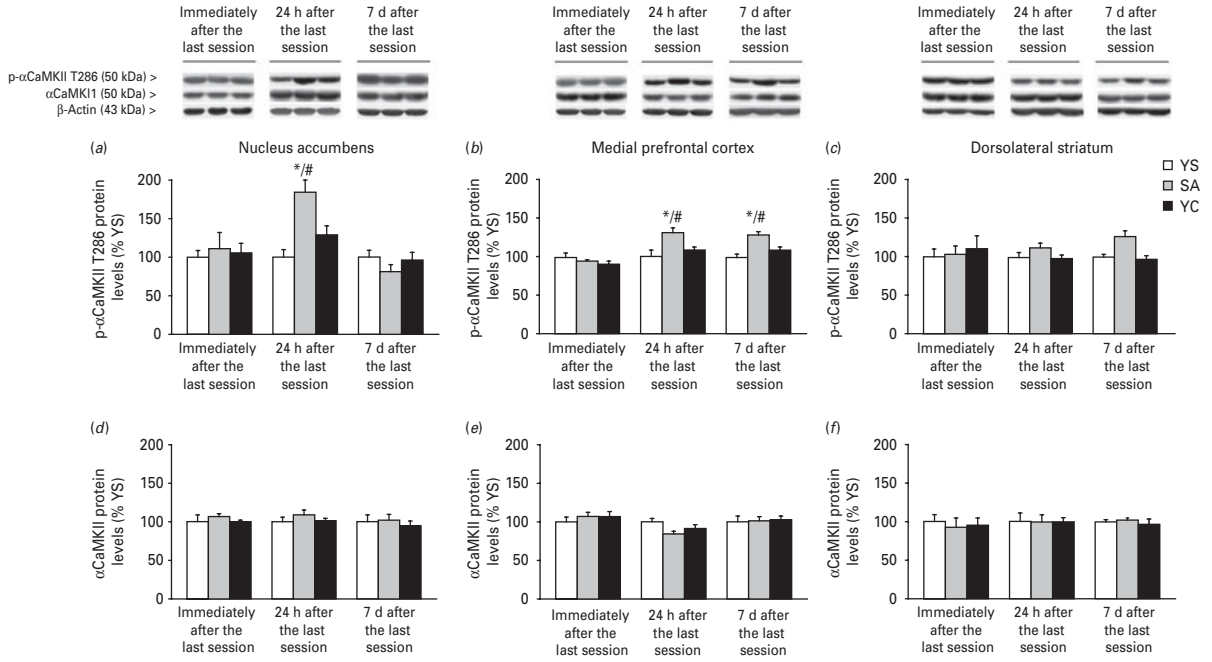

Fig. 1. Effect of cocaine self-administration on p-*α*CaMKII(Thr286) and total *α*CaMKII levels in the nucleus accumbens (NAc), medial prefrontal cortex (mPFC) and dorsolateral striatum (dlS). Rats (cocaine self-administration (SA), yoked cocaine (YC) and yoked saline (YS)) were killed immediately, 24 h or 7 d after the last cocaine session. Representative immunoblots are shown for p-*α*CaMKII(Thr286) (50 KDa) and *α*CaMKII (50 KDa) proteins in the NAc, mPFC and dlS homogenates of rats exposed to cocaine and killed at the different times. Panels (*a*), (*b*) and (*c*) show the levels of p-*α*CaMKII(Thr286) in the NAc (*a*), mPFC (*b*) and dlS (*c*) of rats killed at the different times. Panels (*d*), (*e*) and (*f*) show the total *α*CaMKII levels in the NAc (*d*), mPFC (*e*) and dlS (*f*) of rats killed at the different times. Results are expressed as percentages of YS rats. Histograms show the mean±SEM of at least 5–8 rats per group. $^{*}p<0.05$ *vs.* YS and $^{\#}p<0.05$ *vs.* YC, two-way ANOVA, with modality of cocaine exposure and time of death as main factors, followed by Newman–Keuls test.

form of new learning or memory reconsolidation (Kimura et al., 2008; Knackstedt et al., 2010).

Our experimental paradigm distinguished *α*CaMKII autophosphorylation changes associated with short-term abstinence (24 h and 7 d) from those in presence of cocaine (immediately after the last self-administration session). Accordingly, since *α*CaMKII phosphorylation was increased 24 h and 7 d after the last self-administration session in rats receiving cocaine contingently, but not non-contingently, we point to short-term abstinence from cocaine self-administration, rather than from non-contingent cocaine, as the major contributor for the changes observed in NAc and mPFC.

In the early period after withdrawal from cocaine, addicts commonly report heightened states of anxiety (Gawin, 1991). Likewise, rodents exhibit anxiety-like behaviour during short-term abstinence from chronic cocaine exposure (Erb, 2010). Since up-regulation of *α*CaMKII expression in the forebrain leads to an increase in anxiety-like behaviours (Hasegawa et al., 2009) and altered reactivity of the vmPFC has been associated with cocaine withdrawal-induced negative emotional state (El Hage et al., 2012), it cannot be excluded that, in our rats, the increase in *α*CaMKII in the mPFC may be caused by early abstinence-induced anxiety-like behaviours. Although negative emotional responses during the first 48 h of cocaine withdrawal have been extensively demonstrated in animals given the drug non-contingently (Erb, 2010), recent studies employing either long (6 h/d) or short (2 h/d) cocaine self-administration showed increased anxiety-like behaviour, but in response to a stressor rather than at baseline (Aujla et al., 2008; Buffalari et al., 2012). Considering that, in our experiments, rats were left undisturbed during the short-term abstinence and, more importantly, that the short-term abstinence from cocaine elevates *α*CaMKII autophosphorylation only in the animals that self-administered cocaine, it is unlikely that the kinase activation may be a consequence of short abstinence-induced anxiety-like behaviour.

At present we do not have a clear, unambiguous explanation of the selective enhancement of αCaMKII autophosphorylation induced by short-term abstinence from cocaine self-administration; however, its selective expression in rats self-administering cocaine indicates that it should involve an associative process acquired during self-administration (Everitt and Robbins, 2013). Abstinence from cocaine self-administration has also been associated with 'incubation of craving', in which animals exhibit enhanced responding during extinction following experimenter-forced abstinence (Tran-Nguyen et al., 1998).

Whether the moderate increase of αCaMKII autophosphorylation in the NAc and mPFC is important for the incubation of drug seeking after abstinence from cocaine self-administration is difficult to assess. In fact, no studies have tested the effect of intra-NAc or intra-mPFC injection of CaMKII inhibitors on cue- or drug priming-induced reinstatement of cocaine seeking in rats after short or prolonged 'forced abstinence' (i.e. in rats not subjected to extinction). Interestingly, CaMKII inhibition in the NAc shell attenuated cocaine and morphine priming-induced reinstatement in rats after extinction (Anderson et al., 2008; Liu et al., 2012), a procedure known to modulate neuroadaptive responses during withdrawal from chronic cocaine self-administration (Kimura et al., 2008; Knackstedt et al., 2010). Moreover, no information on αCaMKII inhibition in the mPFC is available. It should be noted that Liu et al. (2012) inhibited CaMKII in the NAc shell at 24 h from the last morphine self-administration session 15 min before an extinction test. Unfortunately, their results were inconclusive, since no evidence of morphine seeking were found either in control and treated rats.

With respect to the mechanism(s) leading to αCaMKII activation, a role for D1 dopamine (DA) receptors may be suggested. Cell surface D1 DA receptors were found increased in the NAc shell on day 1, but not on day 45, after discontinuing cocaine (long access) self-administration (Conrad et al., 2010). Since stimulation of D1-like DA receptors in the NAc shell promotes the reinstatement of cocaine seeking by serially stimulating L-type Ca^{2+} channels and CaMKII (Anderson et al., 2008), it may be reasonable to hypothesize that neurons in the NAc shell may be more responsive to D1-mediated cocaine seeking in short-term withdrawal, because of the transient D1 DA receptors up-regulation. Notably, D1 DA receptors contribute to cue-induced reinstatement of cocaine seeking also in the mPFC (Kalivas and O'Brien, 2008). However, caution must be used in extrapolating data from reinstatement after extinction to forced abstinence studies, since extinction training and home-cage withdrawal seems to be associated with different neuroadaptations in the NAc (Reichel and Bevins, 2009).

Conversely, the increased phosphorylation of αCaMKII observed in the mPFC on day 7 may represent a calcium-driven compensatory response to the withdrawal from cocaine self-administration, which reduces the glutamatergic activity in the mPFC (Kalivas and O'Brien, 2008). Thus, αCaMKII activation may occur through increased expression of L-type calcium channels that triggers increased Ca^{2+} influx (Ford et al., 2009). Such increase might be responsible for maintaining the saliency of drug-associated cues and the capacity of these cues to elicit drug craving even in protracted abstinence (Kalivas and O'Brien, 2008; Everitt and Robbins, 2013), as previously suggested for ERK phosphorylation in the vmPFC (Koya et al., 2009). However, this response is apparently insufficient to activate glutamate GluA1 receptors, at least under our experimental conditions.

In conclusion, cocaine's effects on αCaMKII autophosphorylation depend on whether the drug is delivered passively or is self-administered. The persistent enhancement of αCaMKII autophosphorylation in the mPFC of rats abstinent from cocaine self-administration may represent a previously unappreciated contribution to initial incubation of cocaine-seeking.

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Conflict of interest

None.

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