



Published in final edited form as:

Addict Biol. 2017 May ; 22(3): 629–639. doi:10.1111/adb.12354.

Cocaine-craving during protracted withdrawal requires PKC ϵ priming within vmPFC

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Abstract

In individuals with a history of drug-taking, the capacity of drug-associated cues to elicit indices of drug-craving intensifies or incubates with the passage of time during drug abstinence. This incubation of cocaine-craving, as well as difficulties with learning to suppress drug-seeking behavior during protracted withdrawal, are associated with a time-dependent deregulation of ventromedial prefrontal cortex (vmPFC) function. As the molecular bases for cocaine-related vmPFC deregulation remain elusive, the present study assayed the consequences of extended access to intravenous cocaine (6 h/d; 0.25 mg/infusion for 10 d) on the activational state of protein kinase C epsilon (PKC ϵ), an enzyme highly implicated in drug-induced neuroplasticity. The opportunity to engage in cocaine-seeking during cocaine abstinence time-dependently altered PKC ϵ phosphorylation within vmPFC, with reduced and increased p-PKC ϵ expression observed in early (3 days) and protracted (30 days) withdrawal, respectively. This effect was more robust within the ventromedial versus dorsomedial PFC, was not observed in comparable cocaine-experienced rats not tested for drug-seeking behavior and was distinct from the rise in phosphorylated extracellular signal-regulated kinase (ERK) observed in cocaine-seeking rats. Further, the impact of inhibiting PKC ϵ translocation within the vmPFC using TAT infusion proteins upon cue-elicited responding was determined and inhibition coinciding with the period of testing attenuated cocaine-seeking behavior, with an effect also apparent the next day. In contrast, inhibitor pretreatment prior to testing during early withdrawal was without effect. Thus, a history of excessive cocaine-taking influences the cue-reactivity of important intracellular signaling

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molecules within the vmPFC, with PKC ϵ playing a critical role in the manifestation of cue-elicited cocaine-seeking during protracted drug withdrawal.

Keywords

incubation; craving; cocaine; PKC epsilon; ERK; prefrontal cortex

INTRODUCTION

Cocaine-associated cues induce intense drug-craving, increasing the propensity for relapse to drug-taking in human cocaine addicts (e.g., Childress et al., 1999; Volkow et al., 1999; Garavan et al., 2000). In cocaine addicts, the medial prefrontal cortex (mPFC) exhibits baseline hypofunctionality (e.g., Volkow et al., 1993) and self-reports of cue-elicited cocaine-craving are associated with heightened activation of mPFC (e.g., Childress et al., 1999, 2008; Volkow et al., 1999; Garavan et al., 2000). Anomalous mPFC activity is theorized to underpin the great difficulty faced by addicts with controlling and inhibiting their drug-related craving and seeking (e.g., Goldstein and Volkow, 2002) and is consistent with the hypothesis that excessive cocaine consumption results in neuroadaptations within the mPFC that render the addict especially vulnerable to relapse, particularly upon presentation of drug-associated stimuli (e.g., Crunelle et al., 2012; Jasinska et al., 2014).

Studies utilizing animal models of addiction further support the hypothesis that cocaine experience produces enduring anomalies in excitatory signaling within mPFC that may be critical for cue-induced drug-seeking (e.g., Ben-Shahar et al., 2012, 2013; Rebec and Sun, 2005; Ary et al., 2013). Also, cue-induced reinstatement of extinguished cocaine-seeking behavior, as well as incubation of cue-elicited cocaine-seeking, are paralleled by increased indices of mPFC activation (e.g., Neisewander et al., 2000; Ciccocioppo et al., 2001; Koya et al., 2009). Pharmacological inactivation of this brain area attenuates cue-induced reinstatement (McLaughlin and See, 2003; Fuchs et al., 2005), as well as the incubation of cue-elicited cocaine-seeking (Koya et al., 2009), while inhibition of Group1 metabotropic glutamate receptors (mGluRs) within the more ventral aspects of the mPFC (vmPFC) impair the consolidation of inhibitory learning during cocaine abstinence to promote continued drug-seeking during long-term withdrawal (Ben-Shahar et al., 2013).

Group1 mGluRs activate a canonical intracellular signaling pathway involving phospholipase C (e.g., Neisewander and Conn, 2010), that can activate a wide variety of intracellular effectors, including: extracellular signal-regulated kinase (ERK), and various protein kinase C isozymes. These downstream effectors are highly implicated in drug-induced neuroplasticity (e.g., Olive and Newton, 2010; Zamora-Martinez and Edwards, 2014) and the activation states of ERK and several PKCs are up-regulated within mPFC of cocaine-experienced rodents (Steketee et al., 1998; Koya et al., 2009). Although, inhibition of ERK within PFC subregions does not influence the incubation of cocaine-seeking during protracted withdrawal (Koya et al., 2009), prior neuropharmacological research implicates PKCs in the reinstatement of cocaine-seeking (Ortinski et al., 2015; Schmidt et al., 2015). Further, there is substantial literature implicating the atypical PKC isozyme PKC ϵ in the

neurobiology of drug addiction (c.f., Olive and Newton, 2010; see also Cozzoli et al., 2015). However, there is a notable lack of study pertaining to PKC ϵ in cocaine intake and seeking behavior. Given the emerging relation between PKCs, mGluR function, and cocaine withdrawal in cocaine addiction neurobiology, and extant literature indicating a critical role for vmPFC activity in the incubation of cocaine-craving (Koya et al., 2009), we investigated the hypothesis that PKC ϵ plays a crucial role in the time-dependent incubation of cocaine-seeking behavior using immunoblotting and neuropharmacological approaches. We also employed correlational analyses to examine the relation of PKC ϵ in vmPFC to cocaine-seeking, as well as to other molecular mediators of incubated cocaine-seeking behavior, including mGluRs, Homers, and ERK (Ben-Shahar et al., 2013; Gould et al., 2015).

MATERIALS AND METHODS

Subjects, Surgery and Self-administration Training

Male Sprague–Dawley rats (275–325 g; Charles River Laboratories, Hollister, CA, USA) were initially trained in standard operant chambers (Med Associates, St. Albans, VT, USA) to lever press for 45 mg of food pellets (Noyes, Lancaster, NH, USA) on an FR1 reinforcement schedule (20-sec time-out) and then were surgically fitted with IV catheters, as well as bilateral guide cannulae (30-gauge, Plastics One Inc.) aimed 2 mm above the vmPFC according to the following coordinates with respect to Bregma (in mm): AP= +2.5; ML= \pm 1.0 and DV=–2.0 (Paxinos and Watson (2009)). One week later, animals were subjected to cocaine self-administration procedures (Coc6h; 0.25 mg/0.1 ml/infusion; National Institute on Drug Abuse, Bethesda, MD, USA), with control animals in our immunoblotting study trained to self-administer saline (0.1 ml/infusion) during 10 daily 1- or 6-hour sessions (respectively, Sal1h and Sal6h) and the details of the self-administration procedures employed are provided in Ben-Shahar et al. (2013).

Immunoblotting Studies

The tissue employed for this study was the same tissue as that immunoblotted for the expression of mGlu1/5 (Ben-Shahar et al., 2013) and Homer proteins (Gould et al., 2015) and was derived from gross dissections of the vmPFC (infralimbic cortex + ventral prelimbic cortex) and dmPFC (dorsal prelimbic cortex + anterior cingulate cortex; see Fig. 1B) from groups of Sal1h, Sal6h and Coc6h rats that either underwent a 2-h test for cue-reinforced lever-pressing behavior under extinction conditions, conducted at either 3 or 30 days following the end of self-administration training or were not subjected to the behavioral testing procedures upon completion of self-administration training. Rats subjected to our cue-testing procedures were sacrificed immediately upon completion of the 2-h test session to examine for differential responsiveness of ERK and PKC ϵ between saline and cocaine self-administering animals. Protein levels were then assayed by immunoblotting in two separate experiments as detailed in Ben-Shahar et al. (2013) and specific procedural details of the immunoblotting are described in provided in our prior work (e.g., Goulding et al. 2011; Cozzoli et al., 2015) and summarized in the on-line supplemental material.

Microinfusion of TAT-e-V1-2

The procedures for infusing Tat-eV1-2 (500 nM) or a Tat-scrambled control peptide (generous gifts from Dr. Robert O. Messing, Univ. Texas at Austin; Bajo et al., 2008; Cozzoli et al., 2015) were similar to those described recently for mice (Cozzoli et al., 2015) and are described in the on-line supplemental material. The data were analyzed using TAT (2 or 3 levels: Tat-scrambled, Tat-eV1-2-t=0 and/or Tat-eV1-2-t=+20 min) X Test (2 levels: Test 1 vs. Test 2) ANOVA, with repeated measures on the Test factor ($\alpha=0.05$). As the microinjection experiments conducted at 3 versus 30 days withdrawal involved distinct cohorts of rats that were tested by different research teams and in different non-colony rooms/operant chambers, separated in time by 3 years, the data for the two experiments were analyzed independently and too many procedural differences rendered the results of the experiments directly comparable.

Inter-correlations between PFC Group1 mGluRs, Homer proteins, kinase activity/priming, and cocaine-seeking

Group1 mGluR stimulation can activate ERK and PKC ϵ signaling, in a manner dependent upon Homer protein scaffolding (e.g., Liu et al., 2006; Cozzoli et al., 2009, 2015; Emery et al., 2012). While mGlu1/5 and Homer1b/c expression decreases within vmPFC during protracted cocaine withdrawal (Ben-Shahar et al., 2013; Gould et al., 2014), Homer2a/b levels remain elevated (Gould et al., 2014). As we observed changes in levels or priming of PKC ϵ priming and ERK activation within the vmPFC of Coc6h rats and relations between cue-elicited cocaine-seeking and kinase activity/priming (see below), we also determined whether or not the changes in vmPFC Group1 mGluR and Homer expression reported previously by our group (Ben-Shahar et al., 2013; Gould et al., 2014) might relate to: (1) cocaine-seeking behavior and (2) the activational state of the kinases examined herein using Pearson correlational analyses.

Results

Immunoblotting

The results pertaining to the self-administration behavior and cocaine intake of the rats employed in the immunoblotting studies are detailed in Ben-Shahar et al. (2013) and the data for the Sal6h and Coc6h rats are summarized in Fig. 1A. In short, an analysis of the change in active vs. inactive lever-presses from 3 to 30 days withdrawal indicated an incubation of cue-reinforced responding in cocaine-experienced, but not saline-experienced, rats. The lever pressing and additional immunoblotting data (for mGlu1/5 and Homer proteins) from our prior publications (Ben-Shahar et al., 2013; Gould et al., 2015) were employed here to determine the correlations between molecular and behavioral endpoints. *A priori* contrasts conducted on the data from SAL1h versus SAL6h rats failed to indicate group differences for any variable (Table S1 in the on-line supplemental materials). Thus, the data from these controls were collapsed for the analyses of cocaine-induced changes in protein expression at each withdrawal time-point.

PKC ϵ

When the tissue from each withdrawal time-point was immunoblotted in separate experiments to examine for cocaine-induced changes in protein expression, the results for PKC ϵ , p-PKC ϵ , as well as their relative expression within PFC subregions suggested time-dependent changes in PKC ϵ priming, particularly within vmPFC (see Fig. S1 in the on-line supplemental materials). A direct analysis for time-dependent changes in vmPFC PKC ϵ expression/phosphorylation in Sal6h versus Coc6h animals indicated an interaction between self-administration history and withdrawal for total PKC ϵ levels [$F(1,39)=6.84$, $p=0.01$; other p 's >0.10], which reflected, respectively, a modest time-dependent rise and reduction in total kinase levels in saline rats [$t(20)=1.89$, $p=0.08$] and cocaine animals (Fig. 1C) [$t(16)=1.81$, $p=0.09$]. p-PKC ϵ levels were higher overall at 30 days withdrawal [Withdrawal effect: $F(1,39)=8.48$, $p=0.006$] and this effect was driven primarily by the time-dependent increase in p-PKC ϵ in cocaine-experienced animals (Fig. 1C), although the results of the statistical analysis of total p-PKC ϵ levels did not detect any IV effect or IV X Withdrawal interaction for this variable (p 's >0.10). However, when the relative expression of p-PKC ϵ (ratio) was considered, an interaction emerged between self-administration and withdrawal (Fig. 1C) [$F(1,39)=9.51$, $p=0.004$]. This interaction reflected a marked, time-dependent increase in the p-PKC ϵ ratio in cocaine rats [$t(16)=3.35$, $p=0.004$], but no significant change in the p-PKC ϵ ratio in saline controls (t -test, $p=0.95$). Interestingly, a similar analysis conducted on vmPFC tissue from rats left undisturbed in the home cage revealed a pattern of changes in vmPFC PKC ϵ expression and phosphorylation that was distinct from that of animals tested for cue-reinforced behavior (Fig. 1D). For one, the total expression of both PKC ϵ and p-PKC ϵ was elevated in cocaine-experienced rats, regardless of withdrawal [for PKC ϵ , IV effect: $F(1,55)=6.30$, $p=0.02$; for p-PKC ϵ , IV effect: $F(1,55)=5.03$, $p=0.03$; all other p 's >0.30]. As such, there were no group differences in the p-PKC ϵ ratio in cocaine-experienced not tested for cocaine-seeking (Fig. 1D; 2-way ANOVA, all p 's >0.75).

ERK

The results of the initial analysis of ERK, p-ERK, as well as their relative expression, within PFC subregions are provided in the on-line supplemental materials and indicated a persistent rise in p-ERK expression in cocaine-experienced animals that was selective for the vmPFC (Fig. S2). As Koya et al. (2009) reported a time-dependent increase in vmPFC p-ERK expression by immunohistochemical approaches, our initial results (Fig. S2A,B) prompted us to directly compare the vmPFC tissue from Sal6h and Coc6h rats to directly examine for changes in total and p-ERK expression within this subregion across the 3- and 30-day withdrawal time-points. This experiment revealed a reduction in total ERK expression within the vmPFC of cocaine-experienced rats, irrespective of withdrawal (Fig. 1C) [IV effect: $F(1,39)=5.78$, $p=0.02$; other p 's >0.05], but no change in the total levels of p-ERK (p 's >0.30). Although inspection of Fig. 1C suggested that the increase in the p-ERK ratio exhibited by cocaine-experienced animals was greater at 30 vs. 3 days withdrawal, the results of the analyses did not reach statistical significance [IV effect: $F(1,39)=7.79$, $p=0.03$, other p 's >0.1]. Interestingly, the cocaine-elicited changes in ERK and relative p-ERK expression within the vmPFC were not apparent in rats that were left undisturbed in the home cage prior to tissue collection [Fig 1D; for total ERK, IV effect: $F(1,53)=3.14$, $p=0.08$; other p 's >0.40 ; for p-ERK, all p 's >0.25 ; for ratio: all p 's >0.10].

vmPFC infusion of Tat-eV1-2

The unique temporal dependence and statistical reliability of PKC ϵ priming within the vmPFC (Fig. 1C) prompted a functional investigation into the potential relevance for vmPFC PKC ϵ activation and cocaine-seeking during protracted withdrawal employing Tat infusion proteins targeted to PKC ϵ . A comparison of the average number of cocaine reinforcers earned over the last 3 days of cocaine self-administration training failed to indicate any differences between rats slated to receive an intra-vmPFC infusion of the Tat-Scrambled control and rats slated to receive a Tat-eV1-2 infusion either 20 min or immediately prior to a 30-min test for the number of cocaine reinforcers earned [Scrambled: 110.11 ± 12.71 ; Tat-eV1-2 (t=0 min): 95.44 ± 6.78 ; Tat-eV1-2 (t=20 min): 94.07 ± 4.52 ; one-way ANOVA, $p=0.49$]. As reported previously (Ben-Shahar et al., 2013), cocaine-experienced rats tested for cue-reinforced responding at 30 days withdrawal exhibited difficulty in suppressing drug-seeking behavior as manifested by no change in the number of active lever-presses emitted across the two 30-min test sessions, spaced 24 h apart (Fig. 2; Test effect for all variables, p 's>0.09). Although immediate pretreatment with an intra-vmPFC infusion of Tat-eV1-2 (which corresponds with effective suppression of PKC ϵ at about 20 min into the session) did not influence the total number of lever-presses emitted by cocaine-experienced rats on either test (Fig. 2A), Tat-eV1-2 pretreatment 20 min prior to testing (which corresponds with effective suppression of PKC ϵ throughout the session) reduced active lever responding during both tests [Tat effect: $F(2,15)=5.40$, $p=0.02$; Tat X Test interaction, $p=0.83$]. Although all groups tended to lever-press more on the inactive lever from Test 1 to Test 2 (Test effect: $p=0.09$), there was no indication that PKC ϵ inhibition altered responding on the inactive lever (Tat effect and interaction: p 's>0.75; data not shown). Further, there were no differences in the proportion of responses on the active and inactive levers on either test or across tests (data not shown), indicating no change in the allocation of behavior across tests. The microinjection sites were localized to the ventral portion of the prelimbic cortex and infralimbic cortex (Fig. 2C) and were localized to the vmPFC area sampled for immunoblotting (see Ben-Shahar et al., 2013).

To determine whether or not the attenuation of cue-elicited responding produced by an intra-vmPFC infusion of the PKC ϵ inhibitor was dependent upon the duration of withdrawal, we replicated the study in a group of rats tested during early withdrawal. The cocaine self-administration behavior was similar between rats slated to receive intracranial infusions of VEH vs. Tat-eV1-2 as indicated by the results of t-tests conducted on the average number of reinforcers earned during the last 3 self-administration sessions [VEH: 93.78 ± 7.25 vs. TAT: 95.94 ± 10.34 , $t(24)=0.18$, $p=0.86$]. When tested at 3 days withdrawal, we failed to detect a significant effect of PKC ϵ inhibitor pretreatment (20 min earlier) upon lever-pressing behavior during either of the 30-min tests of drug-seeking [Treatment effect: $F(1,24)=0.47$, $p=0.50$; Treatment X Test: $F(1,24)=0.005$, $p=0.95$], with both groups exhibiting less cue-elicited active lever-responding with subsequent testing (Fig. 2D) [Test effect: $F(1,24)=5.43$, $p=0.03$]. There were no group differences in inactive lever-presses during the 30-min cue-test conducted at the 3-day withdrawal time-point (Fig. 2E) [Treatment X Test ANOVA, all p 's>0.15]. As depicted in Fig. 2F, the localization of the microinjectors within the vmPFC of the rats tested at 3 days withdrawal were comparable to those for the rats tested at the later withdrawal time-point (Fig. 2C).

vmPFC Protein-Cocaine-seeking inter-relations

In Coc6h rats, mGlu5 expression within both PFC subregions inversely predicted cue-reinforced responding, although the relation between these variables was statistically significant only for the vmPFC (Fig. S3A,B).. In contrast, there was absolutely no relation between mGlu5 expression within either subregion and cue-reinforced responding in saline self-administering animals and, irrespective of self-administration history, there were no significant predictive relations between the expression of mGlu1, Homer1b/c or Homer2a/b within either PFC subregion and cue-reinforced responding under extinction conditions (data not shown, Pearson correlations, all p 's>0.1).

vmPFC Protein-Protein inter-relations

Within the vmPFC of Coc6h rats, low mGlu5 expression predicted low mGlu1 levels (Fig. S4A) and higher Homer2a/b levels (Fig. S4B), while low levels of mGlu5 and mGlu1 both inversely predicted p-PKCe ratio (Fig. S4C, D). There were no significant correlations between mGlu1 or mGlu5 expression and ERK activity within vmPFC of Coc6h rats (data not shown, Pearson correlations, p 's>0.09). However, vmPFC Homer1b/c expression predicted the activational state of ERK within this subregion (Figs. S4E). vmPFC Homer2a/b expression was unrelated to the activational/priming state of either kinase (data not shown, Pearson correlations, p 's>0.09). No significant predictive relations were indicated by the correlational analyses of protein expression within the vmPFC of saline self-administering rats (data not shown, Pearson tests, all p 's>0.07).

dmPFC Protein-Protein Inter-relations

Relative to the vmPFC, there were very few inter-relations between our proteins of interest within the dmPFC and the results of these analyses are provided in the on-line supplemental material.

DISCUSSION

Herein, highly cocaine-experienced rats responding for cocaine-associated cues during protracted drug withdrawal (i.e. cue-elicited cocaine-seeking) exhibit increased indices of PKCe priming and ERK activation within vmPFC. Moreover, inhibition of PKCe translocation within vmPFC blunts cue-elicited cocaine-seeking associated with protracted withdrawal. Additionally, correlational analyses suggest a unique relation between PKCe and glutamate receptor systems within the vmPFC following cue-elicited cocaine-seeking. Generally, these findings expand our understanding of how specific molecules contribute to cocaine-seeking during protracted withdrawal and begin to examine the relation between multiple proteins in drug-craving.

Heightened kinase responsiveness in cocaine-seeking rats was localized to the vmPFC (i.e., ventral prelimbic and infralimbic cortices), and to a much lesser extent, the dmPFC (dorsal prelimbic and anterior cingulate). This finding is consistent with correlations between cue/imagery-elicited metabolic activity within mPFC and self-reports of drug-craving in humans (c.f., Parvaz et al. 2011; Jasinska et al., 2014). Further, our results are consistent with basic science study of rats exhibiting incubated cocaine-seeking that indicate greater p-ERK

immunostaining within vmPFC versus dmPFC (Koya et al., 2009), as well as, vmPFC-selective changes in mGlu1/5 receptors and Homer protein expression (Ben-Shahar et al., 2013; Gould et al., 2015). Also consistent with these earlier studies, the increased kinase activity/priming within vmPFC was either distinct from, or not apparent in, test-naïve rats with an equivalent history of IV cocaine self-administration. Thus, the heightened cue-related responsiveness of PKC ϵ and ERK within the vmPFC of cocaine-seeking rats does not merely reflect pharmacodynamic effects of cocaine intake/withdrawal history, but rather an interaction between cocaine intake/withdrawal history and testing for cue-elicited drug-seeking behavior. While, the present study and those previous (Koya et al., 2009; Ben-Shahar et al., 2013; Gould et al., 2015) do not readily permit determination of the relative contribution of cocaine-associated cue/context re-exposure versus the act of lever-pressing to changes in protein expression, the mere visual exposure to cocaine-associated cues or imagery of drug-taking is sufficient to elicit an increase in mPFC metabolic activity in human cocaine addicts and does so outside of the self-administration context and in the absence of the opportunity to engage in gross motor activity (e.g., Childress et al., 1999; Volkow et al., 1999; Garavan et al., 2000; Goldstein and Volkow, 2002). These clinical observations, coupled with evidence from rodent studies that re-exposure to discrete cocaine-paired cues or contexts can induce immediate early gene expression within mPFC (e.g., Neisewander et al., 2000; Ciccocioppo et al., 2001), argues that either cue/context re-exposure and/or craving elicited by this re-exposure is sufficient to augment ERK activity and to prime PKC ϵ within vmPFC to impact cellular activity within this subregion.

The present study provides novel direct evidence for a functional role of vmPFC PKC ϵ activation in cue-elicited cocaine-seeking during protracted withdrawal, which may have relevance to understanding the incubation phenomenon. Cocaine augments the phosphorylation of several different PKC isozymes within PFC and nucleus accumbens (e.g., Xue et al., 2012; Schmidt et al., 2015). Further, PKC priming is implicated in both stimulant-induced alterations in dopamine transporter and glutamate receptor function (e.g., Cervinski et al., 2005; Bakshi et al., 2009; Kim et al., 2009), as well as cocaine-induced behavioral sensitization, conditioned place-preference and drug-primed reinstatement of cocaine-seeking (e.g., Cervo et al., 1997; Pierce et al., 1998; Schmidt et al., 2015; Howell et al., 2014; Ortinski et al., 2015). There is growing interest how atypical/novel PKCs regulate addiction-related behavior with PKC ϵ identified as critical in animal models of alcoholism (e.g., Newton and Ron, 2007; Olive and Newton, 2010; Cozzoli et al., 2015) and morphine addiction (Newton et al., 2007). Although cocaine is reported to reduce dose-dependently PKC ϵ expression *in vitro* (Onaivi et al., 1998), this study is the first to report on cocaine-PKC ϵ interactions in cocaine self-administering animals and demonstrates that total PKC ϵ expression is elevated in cocaine-experienced rats that are test-naïve. Further, this study is the first to report on cocaine-PKC ϵ interactions in relation to drug-seeking behavior, showing that cue-elicited cocaine-seeking reduced and elevated, respectively, vmPFC levels of p-PKC ϵ following early and protracted withdrawal (Fig. 1, S1). Together, these correlative findings indicate that PKC ϵ priming within the vmPFC, rather than the dmPFC, is associated with time-dependent incubation of cocaine-seeking.

The localization of incubation-associated changes in p-PKC ϵ expression to the vmPFC (Fig. 1, S1), coupled with prior neuropharmacological evidence indicating a critical role for the

vmPFC in incubated cocaine-seeking (Koya et al., 2009), prompted us to examine the cause-effect relation between vmPFC PKC ϵ activity and incubated cue-elicited responding during cocaine withdrawal. Interestingly, an intra-vmPFC infusion of the cell-penetrant Tat-eV1-2 translocation inhibitor (Bajo et al., 2009; Cozzoli et al., 2015) failed to significantly alter cue-elicited drug-seeking behavior in rats tested during early withdrawal and both Tat-eV1-2-infused and control animals exhibited a test-dependent reduction in cue-elicited lever-pressing, indicative of extinction learning (Fig. 2C). In contrast, an intra-vmPFC infusion of Tat-eV1-2 attenuated cue-elicited cocaine-seeking when assessed at the 30-day withdrawal time-point (Fig. 2A), but this effect was observed only in animals pretreated with the inhibitor 20 min prior to testing (i.e., only in animals experiencing maximal or near maximal kinase inhibition at the initiation of behavioral testing). Further, the suppressive effect of Tat-eV1-2 infusion persisted for at least 24 h and was apparent on a subsequent test for cocaine-seeking in the absence of any further kinase inhibition (Fig. 2A). In contrast, an intra-vmPFC Tat-eV1-2 infusion immediately prior to the initial test of drug-seeking conducted at 30 days withdrawal did not impact lever-responding on either test (Fig. 2A). Thus, submaximal PKC ϵ inhibition within the vmPFC at the onset of cue re-exposure is insufficient to impact drug-seeking during protracted withdrawal, arguing that the capacity of the Tat-eV1-2 inhibitor to inhibit cocaine-craving in late withdrawal is concentration-dependent and not due to the infusion manipulation. Unfortunately, the exclusion of an early withdrawal group in the experimental design of our 30-day Tat-eV1-2 inhibitor study precludes firm conclusions regarding the role for PKC ϵ priming in the incubation of cocaine-craving. Nevertheless, the fact that PKC ϵ inhibition was effective at reducing cocaine-seeking during late, but not early, cocaine withdrawal, suggests a potentially important role for this kinase in incubated cocaine-craving.

From the present immunoblotting and neuropharmacological data, we hypothesize that cue-induced PKC ϵ priming within the vmPFC may be a negative regulator of a drug-experienced animal's ability to learn to suppress cue-elicited drug-seeking in the face of null-drug outcomes (a.k.a. extinction learning). In support of this hypothesis, cocaine-experienced rats in protracted withdrawal exhibit high levels of vmPFC PKC ϵ priming (Fig. 1A), concomitant with impaired extinction learning (Fig. 2A; Ben-Shahar et al., 2013). In early withdrawal, the converse is true of cocaine-experienced rats; vmPFC PKC ϵ priming is low and extinction learning is intact (Fig. 1C,D, 2D). Thus, we rationalize that inhibition of PKC ϵ translocation via intra-vmPFC pretreatment with Tat-eV1-2 disinhibits extinction learning processes in "incubated" animals, thereby facilitating the acquisition of the new cue-null outcome association in both an immediate and persistent (at least 24 h) fashion to reduce cue-elicited drug-seeking responses. If correct, a time-dependent shift in the maturational state of PKC ϵ within the vmPFC during cocaine withdrawal might underpin, or at least contribute to, the time-dependent impairment in cognitive processing observed in cocaine-experienced animals (Fig. 2A; Ben-Shahar et al., 2013). Thus, although PKC ϵ priming within PFC does not reliably predict the magnitude of cue-elicited drug-seeking behavior (Fig. S2), our functional data support an important role for vmPFC PKC ϵ activity in cue reactivity during protracted withdrawal of potential relevance to the incubation of craving phenomenon.

The present results also expand our knowledge regarding the relation between mPFC ERK activation and cocaine-seeking. Although cue-elicited cocaine-seeking elevated ERK activity only in the vmPFC, a positive predictive relation exists between the intensity of cue-elicited cocaine-seeking and ERK activation in both the vmPFC and dmPFC during withdrawal (Fig 1, S2). The localization of elevated p-ERK expression to the vmPFC in the present immunoblotting study contrasts with a less subregionally selective increase in p-ERK expression measured by immunohistochemistry under similar conditions (Koya et al., 2009). However, it is unclear if these divergent results reflect technical approaches (i.e. activated cells versus protein levels in tissue homogenates), differences in the amount of time engaged in cocaine-seeking (i.e. 2 h in the present study versus 30 min in Koya et al.) or a marginal effect within the dmPFC subregion. Importantly, the augmentation of mPFC ERK activity may be contingent upon learning, as changes in p-ERK expression associated with drug-seeking dissipate after the first session of extinction training (Miszkiel et al., 2014). Indeed, learning to suppress drug-seeking (i.e., extinction training) can reverse certain drug-induced neuroadaptations within mesocorticolimbic structures (e.g., Self et al., 2004; Sutton et al., 2003) or elicit changes in protein expression that are distinct from those observed in drug-withdrawn animals (e.g., Fig. 1; see also Ghasemzadeh et al. 2009 vs. Ben-Shahar et al., 2013; Gould et al., 2015). Further, we have shown that the capacity of self-administered cocaine to lower extracellular glutamate within prelimbic cortex develops tolerance with more extensive cocaine-taking history (Ben-Shahar et al., 2012), arguing also drug history as an important variable in determining the direction of the effects of IV cocaine upon excitatory neurotransmission within mPFC and likely intracellular signaling stimulated by this neurotransmission. In support of a potential link between glutamate transmission and ERK activation, the level of ERK phosphorylation within vmPFC was predicted by the levels of Homer1/c (Fig. 4E), a glutamate receptor scaffolding protein that regulates extracellular glutamate levels within PFC (Lominac et al., 2005; Ary et al., 2013), as well as the psychomotor-activating, -sensitizing (Lominac et al., 2005; Szumlinski et al., 2004; 2006) and motivational properties of cocaine (Ary et al., 2013; Gould et al., 2015).

The present study also forms an initial examination of the interactions between potential protein networks with mPFC subregions in relation to cocaine-seeking behavior. In this regard, the temporally biphasic patterning of PKC ϵ priming within PFC of rats exhibiting incubated drug-seeking is very interesting and quite distinct from the patterning of withdrawal-dependent changes in mGlu1/5 (Ben-Shahar et al., 2013), Homer1/2 (Gould et al., 2015), and ERK observed in these same rats. mGlu1/5 bi-directionally regulate indices of basal PKC ϵ activation both *in vitro* and *in vivo* (c.f., Olive and Newton, 2007; Basu and Sivaprasad, 2007). Moreover, PKC ϵ priming tends to change in concert with alcohol-elicited increases in mGlu1/5 expression (at least in striatal and amygdala subregions) (e.g., Goulding et al., 2011; Cozzoli et al., 2015). However, we observed an inverse relation between total mGlu1/5 expression within the vmPFC of cocaine-seeking rats and PKC ϵ priming in this subregion (Fig. S4C,D). Moreover, a dissociation exists with respect to the effects of vmPFC manipulations of mGlu1/5 vs. PKC ϵ upon cue-elicited drug-seeking. For one, unlike Tat- ϵ V1-2 infusion (Fig. 2A), intra-vmPFC pharmacological manipulations of mGlu1/5 receptors produce no immediate effects upon cue-elicited drug-seeking behavior (Ben-Shahar et al., 2013). Together, these data do not support a direct interaction between

Group1 mGluRs and PKC ϵ in mediating cue-elicited cocaine-seeking behavior during protracted withdrawal, which is in line with prior evidence that the capacity of PKC ϵ to influence morphine intake is independent of mGlu5 activation (Newton et al., 2007).

Decades of cancer research indicate an important inter-relation between PKC ϵ and the activation of the Ras/Raf/MAP kinase signaling pathways, to include a direct interaction with ERK (c.f., Basu and Sivaprasad, 2007). Yet, we failed to detect any obvious correlations between PFC levels of p-ERK and PKC ϵ priming in the present study. However, regulation of PKC ϵ by mGluRs and ERK signaling varies across tumorigenic cell-lines or cancers (e.g., Basu and Sivaprasad, 2007) and may very well depend upon the mitotic status or differentiation fate of a cell. Indeed, convergent evidence from the alcohol literature indicates that the functional interactions between mGlu1/5, PLC, PI3K and PKC ϵ operating to regulate binge drinking are brain region-specific (Cozzoli et al., 2012, 2014, 2015; Lum et al., 2014). Thus, it is very likely that the regulation of PKC ϵ by mGluRs and/or ERK signaling is both drug- and brain region-selective and a more concerted, multi-faceted, research effort is required to determine whether or not a functional interaction exists between these signaling molecules within PFC subregions and interconnected structures (e.g., nucleus accumbens) of cocaine-seeking animals, and to determine how these interactions depend upon the presence of discrete drug-associated cues, how they vary with the passage of time in withdrawal and how they change with repeated opportunity to engage in drug-seeking behavior.

Conclusions

Time-dependent changes in the cue reactivity of PKC ϵ and ERK occurs within the vmPFC of cocaine-experienced rats during drug withdrawal. Inhibition of PKC ϵ function within the vmPFC blunts cue-elicited cocaine-seeking, posing an important role for PKC ϵ -dependent signaling in the manifestation of cue-elicited drug-craving and cognitive inflexibility in addiction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

Funding provided by NIH/NIDA grant DA024038 to KKS. The authors would like to thank NIDA for the generous donation of cocaine and Dr. Robert O. Messing (University of Texas Austin) for the generous gift of the Tat-fusion peptides. The authors wish to acknowledge the following individuals for their excellent technical assistance and assistance with data organization: Rianne R. Campbell, Amanda L. Caruana, Evan J. Gordon and Kyle L. Ploense.

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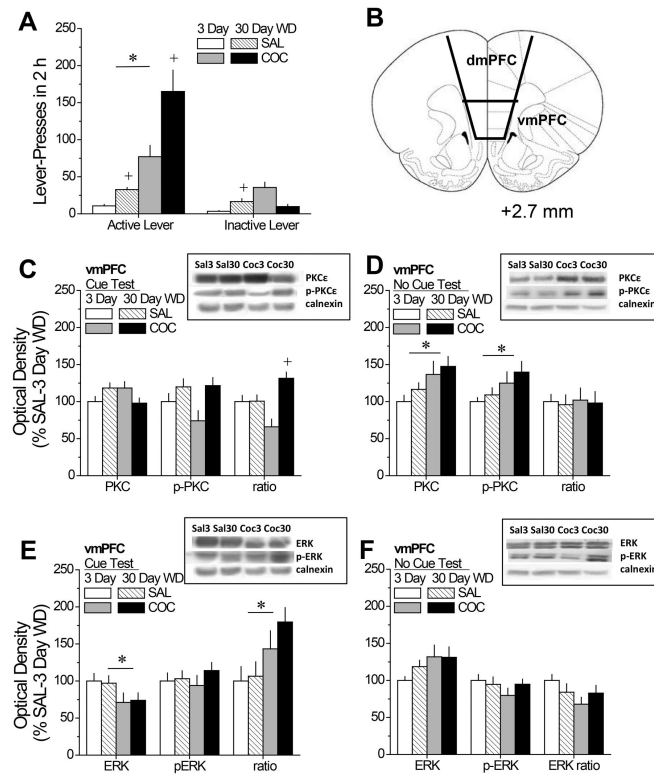


Figure 1. Time-dependent regulation of PKCε and ERK phosphorylation within vPFC subregions by the opportunity to engage in cocaine-seeking behavior during protracted withdrawal

(A) Summary of the total number of active and inactive lever-presses emitted by rats with a 10-day history of extended access (6 h/d) to either cocaine (COC; $n=6-11$) or saline (SAL; $n=11$) during 2-h tests for cue-elicited responding, conducted at either 3 or 30 days withdrawal (WD), indicating that active lever-pressing behavior incubated only in COC animals. (B) Cartoon diagramming the subdivision of the mPFC used in sampling tissue from the dorsal and ventral mPFC (dPFC and vPFC, respectively) for immunoblotting. (C-F) Total and relative protein expression of PKCε and p-PKCε (top panels), as well as ERK and p-ERK (bottom panels) observed within the vmPFC of rats with a 10-day history of extended access (6 h/d) to either cocaine (COC; $n=6-11$) or saline (SAL; $n=11$). Representative immunoblots are provided in each panel. At 3 or 30 days withdrawal (WD), rats from these self-administration groups were either assayed for cue-reinforced lever-pressing behavior during a 2-h test (Cue Test; left panels), or left undisturbed in the home cage prior to tissue collection (No Cue Test; right panels). (C) A direct comparison of tissue from Sal6h and Coc6h rats obtained immediately following cue-testing revealed a time-dependent change in relative PKCε levels only in COC rats. (D) In contrast, COC rats not tested for cocaine-seeking exhibited a time-independent increase in both PKCε and p-PKCε, but no change in their relative expression. (E) A comparable analysis for ERK and p-ERK levels in vmPFC tissue from Sal6h and Coc6h rats, obtained immediately following cue-testing, indicated a decrease in total ERK and an increase in relative p-ERK expression but both effects were time-independent. (F) No changes in ERK or p-ERK expression were observed within the

vmPFC of COC rats not tested for cocaine-seeking. * indicates a main effect of cocaine self-administration; + $p < 0.05$ COC-30 day WD vs. COC-3 day WD.

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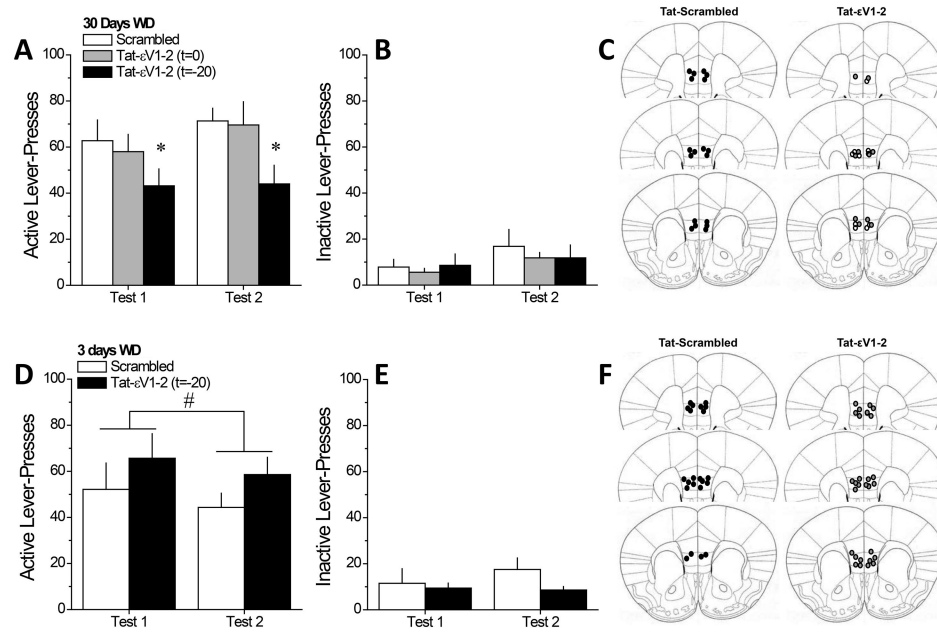


Figure 2. Inhibition of PKCε translocation within vmPFC attenuates cocaine-seeking during protracted withdrawal

(A) When assessed at 30 days withdrawal, intra-vmPFC pretreatment (20 min earlier; $t=-20$) with the Tat-εV1-2 PKCε translocation inhibitor reduced the total number of cue-reinforced active lever-presses emitted by cocaine-trained rats during an initial 30-min test of drug-seeking (Test 1) and this effect was still apparent the next day (Test 2). In contrast, Tat-εV1-2 infusion immediately prior to Test 1 ($t=0$) was without effect on responding. (B) Tat-εV1-2 infusion did not alter inactive lever-presses during testing, irrespective of pretreatment interval. (C) Summary of the locations of the microinjector tips within the ventral prelimbic and infralimbic cortices of the rats employed to generate the behavioral data in panels A and B. (D) When assessed at 3 days withdrawal, intra-vmPFC pretreatment (20 min earlier; $t=-20$) with the Tat-εV1-2 PKCε translocation inhibitor failed to influence the total number of cue-reinforced active lever-presses emitted by cocaine-trained rats during an initial 30-min test of drug-seeking (Test 1), nor were any effects apparent during a subsequent test, however, both groups exhibited significant extinction of responding of the second test relative to the first test. (E) There were no effects of the inhibitor upon the total number of inactive lever-presses emitted at the 3-day time-point. (F) Summary of the locations of the microinjector tips within the ventral prelimbic and infralimbic cortices of the rats employed to generate the behavioral data in panels D and E. * $p<0.05$ vs. Scrambled control (LSD post-hoc tests; $n=5-9$); # denotes main Test effect.