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Cocaine craving during protracted withdrawal requires PKCε 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 hours/day; 0.25 mg/infusion for 10 day) on the activational state of protein kinase C epsilon (PKCε), an enzyme highly implicated in drug-induced neuroplasticity. The opportunity to engage in cocaine seeking during cocaine abstinence time-dependently altered PKCε phosphorylation within vmPFC, with reduced and increased p-PKCε 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 observed in cocaine-seeking rats. Further, the impact of inhibiting PKCε 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 molecules within the vmPFC, with PKCε playing a critical role in the manifestation of cue-elicited cocaine seeking during protracted drug withdrawal.

Keywords Cocaine, craving, ERK, incubation, PKC epsilon, prefrontal cortex.

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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, 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 & 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. Sun & Rebec 2006; Ben-Shahar *et al.* 2012, 2013; 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, Sanna, & Weiss 2001; Koya *et al.* 2009). Pharmacological inactivation of this brain area attenuates cue-induced reinstatement (McLaughlin & 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. Niswender & Conn 2010), which 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 & Newton 2010; Zamora-Martinez & Edwards 2014), and the activational states of ERK and several PKCs are up-regulated within mPFC of cocaine-experienced rodents (Steketee, Rowe, & Chandler 1998; Koya *et al.* 2009). Although the 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, Kimmey, & Arreola 2015). Further, there is substantial literature implicating the atypical PKC isozyme PKCε in the neurobiology of drug addiction (c.f., Olive & Newton 2010; see also Cozzoli *et al.* 2015). However, there is a notable lack of study pertaining to PKCε 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 PKCε 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 PKCε 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-second time-out) and then were surgically fitted with IV catheters, as well as bilateral guide cannulae (30-guage, Plastics One Inc.) aimed 2 mm above the vmPFC according to the following coordinates with respect to Bregma (in mm): AP = +2.5; ML = +/− 1.0 and DV = −2.0 (Paxinos & Watson (2007). 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-hour 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; Fig. 1B) from groups of Sal1h, Sal6h and Coc6h rats that either underwent a 2-hour 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-hour test session to examine for differential responsiveness of ERK and PKCε 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-ε-V1-2

The procedures for infusing Tat-εV1-2 (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-εV1-2-*t* = 0 and/or Tat-εV1-2-*t* = +20 minute) X Test (2 levels: Test 1 versus Test 2) ANOVA, with repeated measures on the

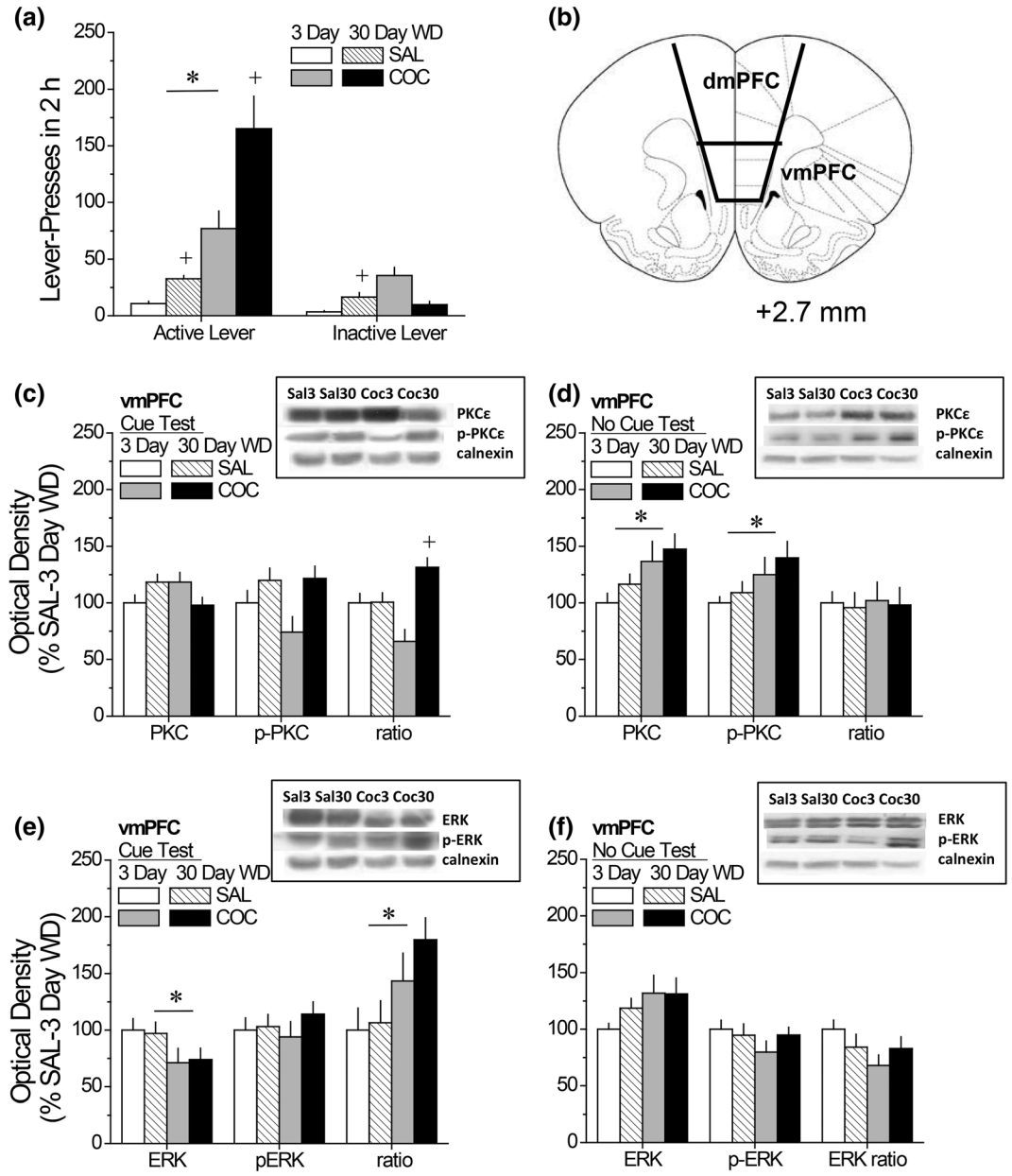

Figure 1 *Time-dependent regulation of PKCε and ERK phosphorylation within vPFC subregions by the opportunity to engage in cocaine-seeking behavior during protracted withdrawal.* (a) Summary of the total number of active and inactive lever-presses emitted by rats with a 10-day history of extended access (6 hours/day) to either cocaine (COC; $n = 6–11$) or saline (SAL; $n = 11$) during 2-hour 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 diagraming 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 hours/day) 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-hour 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 versus COC-3 day WD

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 PKCε signaling, in a manner dependent upon Homer protein scaffolding (e.g. Liu *et al.* 2006; Cozzoli *et al.* 2009, Emery *et al.* 2012; Cozzoli *et al.* 2015). While mGlu1/5 and Homer1b/c expression decreases within vmPFC during protracted cocaine withdrawal (Ben-Shahar *et al.* 2013; Gould *et al.* 2015), Homer2a/b levels remain elevated (Gould *et al.* 2015). As we observed changes in levels or priming of PKCε 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 xexpression reported previously by our group (Ben-Shahar *et al.* 2013; Gould *et al.* 2015) 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 versus 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 (Supporting Information 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 timepoint.

PKCε

When the tissue from each withdrawal timepoint was immunoblotted in separate experiments to examine for cocaine-induced changes in protein expression, the results for PKCε, p-PKCε, as well as their relative expression within PFC subregions suggested time-dependent changes in PKCε priming, particularly within vmPFC (Supporting Information Fig. S1 in the on-line supplemental materials). A direct analysis for time-dependent changes in vmPFC PKCε expression/phosphorylation in Sal6h versus Coc6h animals indicated an interaction between self-administration history and withdrawal for total PKCε 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-PKCε 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-PKCε in cocaine-experienced animals (Fig. 1C), although the results of the statistical analysis of total p-PKCε 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-PKCε (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-PKCε ratio in cocaine rats [$t(16) = 3.35$, $p = 0.004$], but no significant change in the p-PKCε 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 PKCε expression and phosphorylation that was distinct from that of animals tested for cue-reinforced behavior (Fig. 1D). For one, the total expression of both PKCε and p-PKCε was elevated in cocaine-experienced rats, regardless of withdrawal [for PKCε, IV effect: $F(1, 55) = 6.30$, $p = 0.02$; for p-PKCε, 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-PKCε ratio in cocaine experienced not tested for cocaine seeking (Fig. 1D; 2-way ANOVA, all p's > 0.75).

Signal-regulated kinase

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 (Supporting Information 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-day 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 versus 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-εV1-2

The unique temporal dependence and statistical reliability of PKCε priming within the vmPFC (Fig. 1C) prompted a functional investigation into the potential relevance for vmPFC PKCε activation and cocaine seeking during protracted withdrawal employing Tat infusion proteins targeted to PKCε. 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-εV1-2 infusion either 20 minute or immediately prior to a 30-minute test for the number of cocaine reinforcers earned [Scrambled: 110.11 ± 12.71; Tat-εV1-2 ($t = 0$ minutes): 95.44 ± 6.78; Tat-εV1-2 ($t = 20$ minutes): 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-minute test sessions, spaced 24 hours apart (Fig. 2; Test effect for all variables, p's > 0.09). Although immediate pretreatment with an intra-vmPFC infusion of Tat-εV1-2 (which corresponds with effective suppression of PKCε 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-εV1-2 pretreatment 20 minutes prior to testing (which corresponds with effective suppression of PKCε 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 PKCε 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 (Ben-Shahar *et al.* 2013).

To determine whether or not the attenuation of cue-elicited responding produced by an intra-vmPFC infusion of the PKCe 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 versus Tat-εV1-2 as indicated by the results of t-tests conducted on the average number of reinforcers earned during the last three self-administration sessions [VEH: 93.78 ± 7.25 versus 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 PKCε inhibitor pretreatment (20 minutes earlier) upon lever-pressing behavior during either of the 30-minute 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-minute cue-test conducted at the 3-day withdrawal timepoint (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 timepoint (Fig. 2C).

vmPFC protein–cocaine-seeking inter-relations

In Coc6h rats, mGlu5 expression within both PFC xsubregions inversely predicted cue-reinforced responding, although the relation between these variables was statistically significant only for the vmPFC (Supporting Information 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,

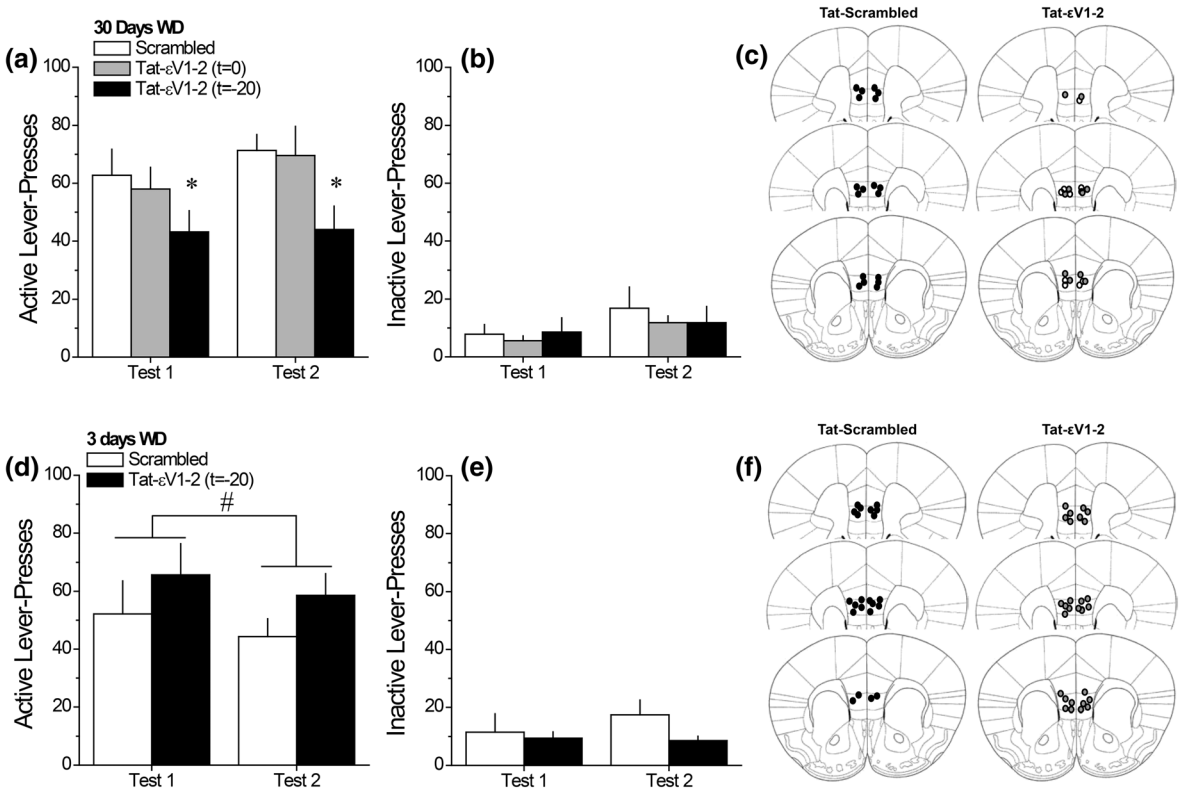

Figure 2 *Inhibition of PKCε translocation within vmPFC attenuates cocaine-seeking during protracted withdrawal.* (a) When assessed at 30 days withdrawal, intra-vmPFC pretreatment (20 minutes earlier; $t = -20$) with the Tat-εV1-2 PKCε translocation inhibitor reduced the total number of cue-reinforced active lever-presses emitted by cocaine-trained rats during an initial 30-minute 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 microinjectors 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 minutes 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-minute 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 microinjectors tips within the ventral prelimbic and infralimbic cortices of the rats employed to generate the behavioral data in panels D and E. *$p < 0.05$ versus Scrambled control (LSD *post hoc* tests; $n = 5$–9); # denotes main Test effect

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 (Supporting Information Fig. S4A) and higher Homer2a/b levels (Supporting Information Fig. S4B), while low levels of mGlu5 and mGlu1 both inversely predicted p-PKCε ratio (Supporting Information 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 (Supporting Information 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 PKCε priming and ERK activation within vmPFC. Moreover, inhibition of PKCε translocation within vmPFC blunts cue-elicited cocaine seeking associated with protracted withdrawal. Additionally, correlational analyses suggest a unique relation between PKCε 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 the 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 PKCε 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 & 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 PKCε within vmPFC to impact cellular activity within this subregion.

The present study provides novel direct evidence for a functional role of vmPFC PKCε 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, Foster, & Vaughan 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; Howell *et al.* 2014; Ortinski *et al.* 2015; Schmidt *et al.* 2015). There is growing interest how atypical/novel PKCs regulate addiction-related behavior with PKCε identified as critical in animal models of alcoholism (e.g. Newton & Ron 2007; Olive & Newton 2010; Cozzoli *et al.* 2015) and morphine addiction (Newton *et al.* 2007). Although cocaine is reported to reduce dose-dependently PKCε expression *in vitro* (Onaivi, Ali, & Chakrabarti 1998), this study is the first to report on cocaine-PKCε interactions in cocaine self-administering animals and demonstrates that total PKCε expression is elevated in cocaine-experienced rats that are test-naïve. Further, this study is the first to report on cocaine-PKCε interactions in relation to drug-seeking behavior, showing that cue-elicited cocaine-seeking reduced and elevated, respectively, vmPFC levels of p-PKCε following early and protracted withdrawal (Supporting Information Fig. 1, S1). Together, these correlative findings indicate that PKCε 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-PKCε expression to the vmPFC (Fig. 1, Supporting Information 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 PKCε activity and incubated cue-elicited responding during cocaine withdrawal. Interestingly, an intra-vmPFC infusion of the cell-penetrant Tat-εV1-2 translocation inhibitor (Bajo *et al.* 2008; Cozzoli *et al.* 2015) failed to significantly alter cue-elicited drug-seeking behavior in rats tested during early withdrawal and both Tat-εV1-2-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-εV1-2 attenuated cue-elicited cocaine-seeking when assessed at the 30-day withdrawal timepoint (Fig. 2A), but this effect was observed only in animals pretreated with the inhibitor 20 minutes 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-εV1-2 infusion persisted for at least 24 hours 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-εV1-2 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 PKCε 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-εV1-2 inhibitor to inhibit cocaine craving in late withdrawal is concentration-dependent and not because of the infusion manipulation. Unfortunately, the exclusion of an early withdrawal group in the experimental design of our 30-day Tat-εV1-2 inhibitor study precludes firm conclusions regarding the role for PKCε priming in the incubation of cocaine craving. Nevertheless, the fact that PKCε 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 PKCε 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 PKCε 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 PKCε priming is low and extinction learning is intact (Fig.1C,D, 2D). Thus, we rationalize that inhibition of PKCε translocation via intra-vmPFC pretreatment with Tat-εV1-2 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 hours) fashion to reduce cue-elicited drug-seeking responses. If correct, a time-dependent shift in the maturational state of PKCε 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 PKCε priming within PFC does not reliably predict the magnitude of cue-elicited drug-seeking behavior (Supporting Information Fig. S2), our functional data support an important role for vmPFC PKCε 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, Supporting Information 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 hours in the present study versus 30 minutes 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. Sutton *et al.* 2003; Self *et al.* 2004) 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 the 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, psychomotor-sensitizing (Szumlinski *et al.* 2004, 2006; Lominac *et al.* 2005) 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 PKCε 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 PKCε activation both *in vitro* and *in vivo* (c.f., Olive & Newton 2010; Basu & Sivaprasad 2007). Moreover, PKCε 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 PKCε priming in this subregion (Supporting Information Fig. S4C, D). Moreover, a dissociation exists with respect to the effects of vmPFC manipulations of mGlu1/5 versus PKCε upon cue-elicited drug seeking. For one, unlike Tat-εV1-2 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 to not support a direct interaction between Group1 mGluRs and PKCε in mediating cue-elicited cocaine-seeking behavior during protracted withdrawal, which is in line with prior evidence that the capacity of PKCε to influence morphine intake is independent of mGlu5 activation (Newton *et al.* 2007).

Decades of cancer research indicate an important inter-relation between PKCε and the activation of the Ras/Raf/MAP kinase signaling pathways, to include a direct interaction with ERK (c.f., Basu & Sivaprasad 2007). Yet, we failed to detect any obvious correlations between PFC levels of p-ERK and PKCε priming in the present study. However, regulation of PKCε by mGluRs and ERK signaling varies across tumorogenic cell-lines or cancers (e.g. Basu & 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 PKCε 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 PKCε by mGluRs and/or ERK signaling is both drug selective 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 PKCε and ERK occurs within the vmPFC of cocaine-experienced rats during drug withdrawal. Inhibition of PKCε function within the vmPFC blunts cue-elicited cocaine-seeking, posing an important role for PKCε-dependent signaling in the manifestation of cue-elicited drug craving and cognitive inflexibility in addiction.

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Authors' Contributions

Bailey W. Miller conducted the immunoblotting studies and edited the manuscript. Melissa G. Wroten conducted the immunoblotting studies and edited the manuscript. Arianne D. Sacramento conducted the behavioral studies and edited the manuscript. Hannah E. Silva conducted the behavioral studies and edited the manuscript. Christina B. Shin conducted the behavioral studies and edited the manuscript. Philip A. Vieira conducted the behavioral studies and edited the manuscript. Osnat Ben-Shahar conducted and supervised all aspects of behavioral studies, contributed to experimental design and edited the manuscript Tod E. Kippin contributed to experimental design and composed the manuscript. Karen K. Szumlinski supervised aspects of the behavioral and immunoblotting studies, contributed to experimental design, conducted all data analyses and composed the manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1 Regulation of PKCε activity within PFC subregions by the opportunity to engage in cocaine-seeking behavior during protracted withdrawal.

Figure S2 Regulation of ERK activity within PFC subregions by the opportunity to engage in cocaine-seeking behavior during protracted withdrawal.

Figure S3 Inter-relations between mGlu5 expression and cue-reinforced lever-pressing behavior.

Figure S4 Significant inter-relations between kinase activity/priming and Group1 mGluR or Homer protein expression within the vmPFC of Coc6h rats.

Table S1 Comparison of the changes in vmPFC and dmPFC protein expression of SAL1h and SAL6h rats at 3 and 30days withdrawal from saline self-administration. The data for the SAL6h rats are expressed as a percent of the optical density of the SAL1h animals. Analysis of the data using independent t-tests failed to indicate any significant group differences with α=0.05. Sample sizes ranged from 11-12/group/analysis.