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Not all stress is equal: CREB is not necessary for restraint stress reinstatement of cocaine-conditioned reward

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Abstract

Stress elicits relapse to cocaine seeking in humans and in animal models. Cyclic AMP response element binding protein (CREB) is required for swim stress-induced reinstatement of cocaine conditioned place preference. However, the role of CREB in other stress-induced reinstatement models has not been examined. To determine whether CREB is required across different stressors we examined the ability of restraint to elicit reinstatement of cocaine-conditioned place preference in wild type and CREB $\alpha\Delta$ mutant mice. In contrast to previously published differences in swim stress-induced reinstatement, both wildtype and CREB $\alpha\Delta$ mutant mice demonstrated restraint stress elicited reinstatement of cocaine-conditioned reward. While CREB is necessary for swim stress-elicited zif268 expression within the nucleus accumbens (NAc) shell & prefrontal cortex (PrL), restraint-stress-elicited comparable increases in zif268 expression within these regions in both wildtype and CREB $\alpha\Delta$ mutant mice. Our findings suggest that not all stressors engage the same circuits or molecular mechanisms to elicit reinstatement behavior.

Keywords

cocaine; stress; CREB; restraint; immunoreactivity; zif268

Introduction

Clinical research has demonstrated that exposure to stress not only increases vulnerability to addiction, but can also trigger relapse to drug use (Brown et al., 1990, McFall et al., 1992, Brown et al., 1995, Dewart et al., 2006, Ouimette et al., 2007). Preclinical studies utilizing reinstatement of both cocaine conditioned place preference and cocaine self-administration models have demonstrated that stress, along with cocaine and conditioned cues, reinstates cocaine-seeking behavior in animals that have undergone extinction, suggesting that these diverse stimuli engage circuits that converge onto a final common pathway that mediates drug seeking behavior (de Wit and Stewart, 1981, Erb et al., 1996, Meil and See, 1996, Ahmed and Koob, 1997, Mueller and Stewart, 2000, Kreibich and Blendy, 2004). We have previously demonstrated that the transcription factor, cAMP response element binding protein (CREB), is necessary for forced swim stress-induced reinstatement of conditioned place preference (Kreibich and Blendy, 2004). Furthermore, we found a circuit-wide

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decrease in swim stress-induced immediate early gene expression in mice with a global deletion of CREB (CREB $\alpha\Delta$ mice), particularly in brain regions that converge on the ventral tegmental area (Briand et al., 2010). It is unclear, however, whether the ability of other stressors to elicit reinstatement is CREB dependent.

Both restraint and swim stress are thought to be psychological (or processive) stressors rather than physiological (or systemic) stressors, indicating that they require higher-order sensory processing and do not directly affect physiologic homeostasis (Herman and Cullinan, 1997, Sawchenko et al., 2000, Dayas et al., 2001). As with other psychological stressors, acute restraint and swim stress both recruit limbic nuclei, such as the amygdala, bed nucleus of the stria terminalis (BNST) and prefrontal cortex (Jaskiw and Weinberger, 1992, Figueiredo et al., 2003, Choi et al., 2007, Castro et al., 2010, Crestani et al., 2010). For example, both acute swim stress and acute restraint increase serotonin release within limbic structures, such as the hippocampus, amygdala and cortex (Linthorst et al., 2002, Mo et al., 2008). At the level of the hypothalamic-pituitary-adrenal (HPA) axis, both stressors acutely elicit similar levels of corticosterone release (Mercier et al., 2003, Andersen et al., 2004, Rademacher et al., 2008, Roberts et al., 2012) as well as c-Fos activation within the HPA axis (Dayas et al., 2001). However, along with these similarities, there are also differences between the responses to these two stressors. Acute swim stress leads to an increase in phosphorylated c-Jun-N-terminal kinase (JNK) in the prefrontal cortex, striatum, hippocampus and amygdala, whereas acute restraint stress either does not show an increase (Meller et al., 2003, Shen et al., 2004b) or exhibits a more modest change in this signaling cascade compared to swim stress (Liu et al., 2004). Additionally, acute swim stress elicits significantly greater c-Fos activation within the ventrolateral medulla, while greater c-Fos activation is seen following acute restraint stress within the medial amygdala (Dayas et al., 2001). Behavioral responses to these two stressors also differ, as swim stress elicits greater stress-induced analgesia than restraint stress (Heidari-Oranjaghi et al., 2012).

Importantly, both acute restraint and forced swim stress lead to an increase in phosphorylated CREB in a variety of brain regions, including the hippocampus, amygdala, nucleus accumbens, and cortex (Bilang-Bleuel et al., 2002, Kwon et al., 2007, Miller et al., 2007, Reagan et al., 2007, Chartoff et al., 2009). However, there are distinct differences within the activation of downstream targets of CREB following these two stressors. For example, an acute restraint stress leads to a decrease in brain-derived neurotrophic factor (BDNF) mRNA in the hippocampus, whereas acute swim has the opposite effect (Murakami et al., 2005, Shi et al., 2010). Therefore, it is not known whether the increase in CREB seen following these two stressors is equally necessary for their behavioral and neurobiological consequences. We hypothesize that these differences in downstream targets may result in differential involvement of CREB within stressor-induced behavioral responses and these differences will be reflected in differences in neuronal activation.

The present study utilized CREB $\alpha\Delta$ mutant mice, which are lacking the alpha and delta two isoforms of CREB within the brain, resulting in greater than a 90% reduction in CREB expression (Hummler et al., 1994, Blendy et al., 1996). By examining the ability of restraint stress to induce reinstatement of cocaine conditioned place preference in these mice with a dramatic reduction in CREB protein we are able to determine the necessity of CREB for this behavioral response. Furthermore, we examined the ability of both restraint and swim stress to elicit immediate early gene (IEG) expression. In CREB $\alpha\Delta$ mutant mice, the expression of these IEGs, products that are rapidly transcribed and translated, has been used as a marker of neuronal activation (Herrera and Robertson, 1996). Zif268 was chosen because of evidence that Fos activation may not be an appropriate marker for stress-induced neuronal activation within the amygdala (Rosen et al., 2005). As we did not see an increase in Fos activation in the amygdala in our previous study, the use of zif268 allowed us to examine activation

within this brain region along with those implicated in the previous study (Briand et al., 2010). In order to make a comparison to stressors that have previously been shown to depend upon CREB to elicit reinstatement, we compared zif268 expression following both restraint and forced swim stress in control animals.

Materials & Methods

Subjects

CREB α Δ mutant mice and wild-type littermates, bred and maintained on a F1 hybrid background (129SvEvTac/C57BL/6NTac) as described previously (Walters and Blendy, 2001), were group-housed with food and water available *ad libitum*. All animals (2–4 months of age) were housed in a temperature and humidity controlled animal care facility with a 12 hr light/dark cycle (lights on a 7:00 A.M.). All procedures were approved by the University of Pennsylvania Animal Care and Use Committee.

Conditioned Place Preference (CPP)

On day 1, mice were allowed to explore both sides of an unbiased 2 chambered CPP apparatus (20×20×20 cm) for 900 sec and time spent in each side was recorded. These data were used to separate animals into groups with approximately equal biases for each side. Beginning on day 2, animals were paired for 8 days, with the saline group receiving injections (0.9% sodium chloride) on both sides of the boxes, while the drug paired group received cocaine (20mg/kg) on one side and saline on the other side (n=14–15 mice per group). Drug-paired sides were randomized among all groups. Following conditioning, all animals were given a saline injection and allowed to explore freely between the two sides and time spent on each side was recorded. The Preference Score (time spent in drug paired side minus time in saline paired side) was calculated for each mouse. After the preference test, animals underwent extinction training during which saline was paired with both sides of the box for a total of 12 days. On the extinction test day, the time spent on each side was recorded. Twenty-four hours after the extinction test day, mice were exposed to a 15-minute immobilization stress during which time they were placed within a plastic Decapicone restrainer (Braintree Scientific, Braintree, MA) with holes to allow for adequate airflow. Following the restraint, the mice were immediately placed in the conditioning chamber and their time spent on each side was recorded. The length of restraint stress was chosen because the levels of corticosterone elicited by this procedure is similar to that of the 6-min swim stress that has been previously demonstrated to reinstate cocaine conditioned place preference (Mercier et al., 2003, Andersen et al., 2004, Kreibich and Blendy, 2004, Rademacher et al., 2008, Roberts et al., 2012).

Acute Stress Exposure

Naïve mice were exposed to a 15-minute immobilization stress identical to that used above in the reinstatement procedure, placed within a plastic cylinder (23 cm tall × 14 cm diameter) containing 10cm deep of 23–25 °C water for 6 minutes or taken from their home cage for transcardial perfusion (non-stressed controls; N=5–7 per group).

Tissue Collection

Mice were deeply anaesthetized with sodium pentobarbital (10mg/kg) and transcardially perfused with 30 mL of phosphate buffered saline (PBS) followed by 40 mL of 4% paraformaldehyde in PBS. The brains were removed and placed in the same fixative overnight at 4°C. The brains were then placed in a solution of 30% sucrose in PBS containing 0.1% sodium azide at 4 °C for at least 48 h. Brains were frozen on dry ice and 40

µm sections were placed in PBS with 0.1% sodium azide and stored at 4 °C until further processing.

Immunohistochemistry

The immunohistochemistry procedures are similar to those outlined previously (Briand et al., 2010). Before immunohistochemical labeling, sections were incubated for 20 min in 0.75% H₂O₂ in PBS followed by several rinses in PBS. Sections were then rinsed several times with PBS containing 0.3% Triton X-100 (PBST), 0.04% bovine serum albumin (BSA) prior to incubation in rabbit anti-EGR1 (Cell Signalling Technologies, Belmont, MA). Sections were incubated for 3 days at 4°C in EGR1 primary antisera (1:1000) diluted in PBST+BSA containing 0.1% sodium azide. Sections were rinsed several times in PBST+BSA prior to 90 min incubation in secondary antisera (1:200, biotinylated donkey anti-rabbit; Jackson ImmunoResearch, West Grove, PA). Following additional rinses, sections were incubated in avidin-biotin complex (ABC elite kit, Vector Laboratories, Burlingame, CA) for 90 min. Following PBS rinses, sections were incubated for 5 min in 0.04% 3,3'-diaminobenzidine-4HCl (DAB; Sigma, St. Louis, MO) containing 0.01% H₂O₂ and 0.06% nickel sulfate in Tris Buffer for a black reaction product that was terminated by rinses in PBS. Following processing, sections were mounted on glass slides, dehydrated, and coverslipped. Immunoreactivity was visualized using a Nikon Eclipse E600 microscope (Melville, NY) and images were captured with a QImaging Retiga 1300 (Surrey, British Columbia, Canada) using Image-Pro Plus software (MediaCybernetics, Bethesda, MD).

Data Analysis

Zif268 (EGR1)-immunolabeled profiles from each brain region were quantified bilaterally from at least 2 sections per mouse and averaged. The person quantifying was blind to group assignments. Anatomical regions were identified according to the stereotaxic atlas of Franklin and Paxinos (2007). Single-labeled zif268 images were quantified using ImageJ software.

Statistical Analyses

The ability of cocaine to induce place preference behavior, the ability of extinction training to eliminate this preference, and the ability of restraint to elicit reinstatement of place preference was assessed using two-way ANOVA with drug (cocaine vs. saline) and genotype (mutant vs. wildtype) as the independent variables and preference score as the dependent variable. The number of Zif268 positive cells in each brain region was determined for each animal. Group differences in zif268 immunoreactivity were assessed using two-way ANOVAs with condition (restraint, FST, or control) and genotype as the independent variables and number of cells as the dependent variable. Tukey's post hoc comparisons were conducted when main effects or interactions were present. For all data, statistics completed using GraphPad Prism 5.0 Software.

Results

The ability of restraint stress to elicit reinstatement of CPP is not CREB dependent

We first tested whether a 15-minute restraint stress would elicit reinstatement of cocaine-conditioned place preference in wild-type mice in an F1 hybrid background (129SvEv/C57BL/6). Preconditioning day data revealed no initial bias to either side. On test day, wild-type mice showed a significant place preference to the cocaine-paired side (compared to saline-administered controls, $p=0.0004$). On the extinction test day none of the groups demonstrated a significant place preference. Wild-type animals previously demonstrating CPP and exposed to the restraint stress show a significant preference for the side previously

paired with cocaine, whereas no change was seen in saline-treated mice exposed to the restraint (Fig. 1; *compared to saline-stressed controls*, $p=.04$). CREB $\alpha\Delta$ mutant mice injected with cocaine (20mg/kg) also exhibited a preference for the drug-paired side and this was significantly greater than the preference seen in wildtype mice (Fig. 1; interaction, $F(1,54)=4.585$, $p=.037$; post-hoc test, cocaine wild type vs. cocaine mutant, $p=.028$). These data are comparable with previous experiments (Walters and Blendy, 2001, Kreibich and Blendy, 2004). This preference was no longer present after 12 days of extinction sessions. On the reinstatement test day, in contrast to what is seen following FST (Kreibich and Blendy, 2004), restraint stress elicited reinstatement of cocaine CPP in CREB $\alpha\Delta$ mutant mice (Fig. 1; main effect of drug, $F(1,54)=7.104$, $p=.010$; no interaction).

The ability of restraint stress to elicit zif268 in key brain regions is not CREB dependent

Figure 2 shows representative photomicrographs of stress-elicited zif268 labeling in forebrain regions of wildtype and CREB deficit mice. Quantification of zif268 labeling revealed an increase in the number of zif268-immunoreactive profiles following both swim and restraint stress in wildtype mice within brain regions that have been implicated in stress responsivity and regions that have been implicated in addiction. These included the prelimbic cortex (PrL), and infralimbic cortex (IL), the nucleus accumbens core and shell, lateral septum (LS) and central amygdala (CeA). Similar results have previously been shown with Fos expression (Briand et al., 2010), CREB $\alpha\Delta$ mutant mice exhibited blunted zif268 expression in response to swim stress within the prelimbic cortex (interaction, $F(2,26)=9.104$, $p=.001$, Tukey's post-hoc test, FST wild type vs. FST mutant, $p=.001$) and NAc shell (interaction, $F(2,26)=3.937$, $p=.005$, Tukey's post-hoc test, FST wildtype vs. FST mutant, $p=.042$; Fig. 3). In contrast, wildtype and CREB $\alpha\Delta$ mutant mice exhibited a similar increase in zif268 expression in these brain regions in response to restraint stress (Tukey's post-hoc test, restraint wildtype vs. restraint mutant, NS). Within the NAc core, IL, LS and the CeA, both restraint and swim stress elicited increases in zif268 expression independent of genotype (main effect of stress, NAc core: $F(2,26)=179.9$, $p<.0001$; IL: $F(2,26)=42.23$, $p<.0001$; LS: $F(2,26)=97.71$, $p<.0001$; CeA: $F(2,26)=40.46$, $p<.0001$; no significant interactions).

Discussion

Stress has been implicated as a risk factor in vulnerability to substance abuse. Despite substantial evidence associating stress and addiction (Kalivas and Duffy, 1989, Sorg, 1992, Sorg and Kalivas, 1993, Prasad et al., 1995, Erb and Stewart, 1999, Erb et al., 2001, Bland et al., 2004, McFarland et al., 2004), the cellular and molecular substrates linking the two phenomena have yet to be elucidated. The transcription factor CREB is considered central in transducing responses to drugs of abuse as well as stressors. CREB $\alpha\Delta$ mutant mice show deficits in swim stress-induced reinstatement of conditioned place preference (Kreibich and Blendy, 2004). The present study examined the role of CREB in restraint stress-induced reinstatement of conditioned place preference as well as the ability of swim and restraint stress to initiate zif268 activity. In contrast to the previous findings with swim stress reinstatement, we found that a global deletion of CREB did not alter the ability of restraint stress to elicit reinstatement of cocaine conditioned place preference. Furthermore, restraint stress was able to elicit zif268 activity within stress- and drug-related brain regions also in a CREB-independent manner. This is in distinct contrast to the lack of swim-stress-elicited zif268 expression in CREB $\alpha\Delta$ mutant mice seen in the current study as well as the previously published results examining Fos expression following swim stress (Briand et al., 2010). These data suggest that CREB plays a distinct role in stress reactivity and stress-induced reinstatement of conditioned reward for some, but not all, stressors.

Restraint stress elicited reinstatement of cocaine-conditioned reward in wildtype mice

The current study is the first to demonstrate that immobilization stress can elicit reinstatement of cocaine-conditioned place preference in mice. This finding expands upon work demonstrating immobilization stress elicited reinstatement of cocaine CPP in rats (Sanchez et al., 2003). It should be noted that in the Sanchez et al. (2003) study the immobilization stress was performed in the conditioning environment, whereas the present study demonstrated that immobilization stress outside of the conditioning context elicited reinstatement. This is particularly relevant because the ability of stress to elicit craving in humans is not dependent upon the drug-paired environment (McFall et al., 1992, Sinha et al., 2000).

Restraint stress-induced reinstatement of cocaine-conditioned reward and zif268 expression is CREB independent

In contrast to our published findings on the role of CREB in swim stress-induced reinstatement of cocaine conditioned place preference (Kreibich and Blendy, 2004), the current study found that the ability of restraint stress to elicit reinstatement was not CREB dependent. Furthermore, we found that CREB mutants and wildtype mice exhibited similar zif268 protein expression following restraint stress. This is contrary to previous findings demonstrating the CREB mutant mice exhibit lower IEG activation following swim stress in select brain regions involved in stress-induced reinstatement (Briand et al., 2010). Our findings are somewhat surprising given that both restraint and swim stress have been shown to elicit CREB phosphorylation, a marker of CREB activation (Pliakas et al., 2001, Bilang-Bleuel et al., 2002, Sabban et al., 2004, Shen et al., 2004a, Kwon et al., 2006). However, other studies examining more general markers of neuronal activation seem to suggest that restraint and swim stress engage the brain in different ways. For example, local cerebral glucose utilization is decreased following restraint stress in the frontal cortex and hippocampus, whereas swim stress leads to increases in these brain regions (Duncan et al., 1993, Warnock and Steckler, 2011). Along with these differences in general activation, restraint and swim stress can lead to different engagement of immune, hormone and neurotransmitter responses. For example, restraint stress has been shown to increase cytokine activity, while swim stress does not (Shintani et al., 1995, Nguyen et al., 1998, Nguyen et al., 2000, Deak et al., 2003, Deak et al., 2005). Additionally, repeated restraint stress leads to increased endocannabinoid signaling in the amygdala, whereas repeated swim stress does not (Patel et al., 2005, Roberts et al., 2012). Lastly, while both restraint and swim stress can affect memory retention, only swim stress has been shown to affect learning on the Morris Water Maze task (Kim et al., 2005, Warner and Drugan, 2012). More directly related to the current findings is the fact that footshock will only induce reinstatement of drug seeking when performed within the drug taking context, whereas swim and restraint will elicit reinstatement in a context-independent manner (Erb et al., 2006, Briand et al., 2010, Conrad et al., 2010).

Although there are some commonalities, the current findings on swim-elicited zif268 expression differed from our previously published results examining Fos expression. Specifically, we saw equivalent zif268 expression within the lateral septum of both wildtype and CREB mutant mice exposed to forced swim stress, where differences were seen in Fos expression. Additionally, we saw differences between wildtype and CREB mutant animals in their swim-elicited zif268 expression in the prelimbic cortex, where none were seen in Fos expression. This is surprising given that expression of both these IEGs has been correlated with neuronal activity (Worley et al., 1991, Worley et al., 1993). However, both these genes have multiple signaling cascades that lead to their expression, some of which require CREB and some of which do not (Sheng and Greenberg, 1990, Hai and Curran, 1991, Silverman and Collins, 1999). In fact, there is some evidence that zif268 and Fos have

differential sensitivity to synaptic activation (Worley et al., 1993). Furthermore, differences in stress-evoked expression of these two IEGs have been noted in the amygdala (Rosen et al., 2005). The current study suggests that these differences seen in the amygdala, may extend to other brain regions, specifically the prelimbic cortex and lateral septum, two areas involved in reinstatement.

It should be noted that while CREB does not appear to be necessary for restraint-induced zif268 expression, developmental compensatory mechanisms could play a role in this effect. Further work utilizing inducible mouse models, in which CREB is deleted in adulthood only, may provide more insight. Additionally, the current studies utilized cocaine-naïve mice to examine stress-induced zif268 expression. Although it is true that parasympathetic tone is altered in cocaine-experienced animals, previously published work demonstrates that swim-induced Fos expression is not altered by the cocaine conditioning procedure (Briand et al., 2010). Further work would have to be done to confirm that the same would be true for restraint stress as well as zif268 expression but these findings suggest that stress-induced IEG expression in drug naïve animals can be related to the reinstatement paradigm.

Taken together, our findings suggest that not all stressors engage the same circuits to elicit drug craving. Therefore, it is important to consider that the conclusions drawn from one stressor may not be universal for all stressors. Further work will need to be done to elucidate how CREB plays a differential role in the ability of these stressors to engage cocaine seeking.

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Highlights

Fifteen-minute restraint stress can elicit reinstatement of cocaine conditioned reward

Unlike swim stress, CREB is not necessary for restraint-induced reinstatement of cocaine CPP

CREB is necessary for swim-stress-elicited zif268 expression

CREB is not necessary for restraint-stress-elicited zif268 expression

Figure 1. Reinstatement of cocaine conditioned reward following restraint stress

Both wildtype and mutant mice paired with cocaine showed a significant place preference to the cocaine-paired side on test day. CREB $\alpha\Delta$ mutant mice exhibited a higher preference than wildtype animals. Following extinction training this preference was no longer present in either group. On reinstatement test day, a 15-minute restraint stress elicited a significant preference for the drug-paired side in cocaine-treated mice regardless of genotype.

*Indicates significant differences from saline controls; #Indicates differences between wildtype and CREB $\alpha\Delta$ mice.

Figure 2. Zif268 immunoreactivity following restraint stress

Example photomicrographs demonstrating that wildtype mice exhibit an increase in zif268 expression within the NAc shell (a, b, d), prelimbic cortex (f, g, i), and infralimbic cortex (k, l, n) following both restraint and forced swim stress. While, CREB $\alpha\Delta$ mutant mice show equal levels of expression following restraint (c, h, m), they exhibit lower levels of expression within the NAc shell (e) and prelimbic cortex (j) following swim stress.

Figure 3. CREB mutant mice exhibit decreased zif268 protein expression in response to swim stress but not to restraint stress

Wildtype and CREB Δ mutant mice exhibit an increase in zif268 protein following restraint stress in the prelimbic (a; PrL), infralimbic (b; IL), Nucleus accumbens (NAc) shell (c) and core (d), lateral septum (e; LS) and the central amygdala (f; CeA). In contrast, while wildtype mice exhibit a swim stress-induced (FST) increase in zif268 in all the regions mentioned above, CREB Δ mutant mice do not exhibit increases within the PrL and the NAc shell. *Indicates significant differences from non-stressed (NS) controls; #Indicates differences between wildtype and CREB Δ mice.