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Memory of Social Defeat Is Facilitated by cAMP Response Element-Binding Protein Overexpression in the Amygdala

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The cAMP-responsive element binding protein (CREB) is a transcription factor that regulates synaptic plasticity and memory formation. Studies that have used conditioned fear models have established that CREB is important for the acquisition and consolidation of fear learning. The authors demonstrate that overexpression of CREB within the basolateral amygdala (BLA) of animals that are exposed to social defeat enhances subsequent defeat-induced changes in social behavior. This effect is specific to the acquisition of defeat-induced behaviors; overexpression of CREB has no effect on the expression of these behaviors if the overexpression occurs after the initial defeat. These data demonstrate that CREB is important for regulating learning not only to explicit cues but also for mediating behavioral plasticity in ethologically relevant social contexts.

Keywords: conditioned defeat, fear, anxiety, CREB, viral vector

The neural circuitry underlying aversive learning in mammals has been well characterized, and the data indicate that the basolateral complex of the amygdala (BLA) is an essential part of this neural circuit. Specifically, the BLA is important for the acquisition and expression of conditioned fear responses (Campeau, Miserendino, & Davis, 1992; Miserendino, Sananes, Melia, & Davis, 1990 Rodrigues, Schafe, & LeDoux, 2001). Moreover, several studies demonstrate that long-term changes in synaptic plasticity within the BLA accompany fear learning. Such synaptic plasticity may be critical for the formation of long-term memories (LTM) and is known to require protein synthesis (Bailey, Kim, Sun, Thompson, & Helmstetter, 1999; McKernan & Shinnick-Gallagher, 1997; Schafe et al., 2000).

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The cAMP response element-binding protein (CREB) is a major transcription factor involved in regulating the synthesis of proteins necessary for the formation of LTM in a variety of species. Induction of a blocking isoform of CREB (dCREB2-b) in *Drosophila* abolishes the formation of LTM (Perazzona, Isabel, Preat, & Davis, 2004; Yin et al., 1994;). Mice with a disruption of the alpha and delta isoforms of CREB (CREB$\alpha^{\delta -/-}$) are specifically deficient in the formation of LTM, whereas short-term memory (STM) is left intact (Bourtchuladze et al., 1994). Also, inducible and reversible disruption of CREB function blocks memory for contextual fear, cued fear, spatial and object recognition, as well as conditioned taste aversion (Bozon et al., 2003; Josselyn, Kida, & Silva, 2004; Kida et al., 2002). These studies provide evidence that CREB is essential in long-term memory formation. In order to determine the role of CREB in memory formation with both neuroanatomical and temporal specificity, Josselyn et al., (2001) overexpressed CREB specifically within the BLA of rats by using herpes simplex virus (HSV) vector-mediated gene transfer. This led to a facilitation of LTM, but not STM, as measured by fear-potentiated startle, providing the first evidence that increasing CREB levels within a specified mammalian brain region enhances memory.

Although a large number of studies have investigated the mechanisms underlying aversive learning tasks under highly controlled, but largely artificial circumstances, relatively little is known about how animals learn about their social environment. Therefore, we tested the hypothesis that increasing CREB levels within the BLA

in hamsters would enhance the behavioral effects of social defeat. We have examined learning by using a behavioral model called *conditioned defeat*, which may be a naturalistic measure of fear. Male Syrian hamsters are normally aggressive animals that readily defend home territories by attacking intruding conspecifics (Nowack & Paradiso, 1983). However, if these animals are placed in a situation in which they experience a mild social defeat by a larger, more aggressive animal, they subsequently become highly submissive and are virtually unable to reverse their subordinate social status (Huhman et al., 2003; Jasnow, Banks, Owens, & Huhman, 1999; Jasnow & Huhman, 2001; Potegal et al., 1993). Following this experience, defeated hamsters no longer defend their home territory, even against nonthreatening opponents that they would have previously attacked and defeated. Instead, defeated hamsters vigorously avoid social interaction and submit to intruders without provocation.

In the present study, we tested the hypothesis that increasing CREB levels specifically within the BLA would enhance the acquisition of conditioned defeat as measured by an increase in the display of submissive–defensive behaviors. We accomplished this by using a defeat procedure that normally does not produce robust levels of conditioned defeat. We also examined whether increasing CREB levels within the BLA after the initial defeat would have an effect on the production, or expression, of conditioned defeat.

Method

Animals and Housing Conditions

Adult male Syrian hamsters (*Mesocricetus auratus*) were obtained from Charles River Laboratories and weighed 120–130 g at the beginning of each experiment. Hamsters were housed individually for two weeks prior to testing in a temperature-controlled (20 °C ± 2°) colony room on a 14:10 hr light/dark cycle with lights off at 1100. Additional hamsters weighing 150–180 g were used as resident aggressors for conditioned defeat training, and hamsters weighing 100–110 g at the beginning of each experiment were used as nonaggressive intruder stimulus animals during behavioral testing. All animals were housed in polycarbonate cages (20 cm × 40 cm × 20 cm) with wire mesh tops, and food and water were available ad libitum. Resident aggressors were housed individually to increase aggressiveness, whereas nonaggressive intruders were group housed (5 animals per cage). All procedures and protocols were approved by the Georgia State University Institutional Animal Care and Use Committee.

Virus Preparation

CREB and LacZ cDNAs were inserted into the HSV amplicon HSV-PrpUC and packaged with the helper 5 dl1.2 (Carlezon et al., 1997, 1998; Neve & Lim, 1999). The virus was purified on a sucrose gradient, pelleted and resuspended in 10% sucrose. The average titer of the recombinant virus stocks was 4.0×10^7 infectious U/ml and was similar for HSV-CREB. Transgene expression was regulated by the constitutive promoter for the HSV immediate-early gene *IE 4/5*.

Surgical Procedures and Virus Infusion

At the beginning of each experiment, adult male Syrian hamsters were anesthetized deeply with ketamine/xylazine/acepromazine cocktail and were placed in a stereotaxic instrument. Stereotaxic coordinates for the basolateral complex of the amygdala were 0.4 mm posterior and ± 3.9 mm lateral to bregma and 6.3 mm below dura. Stereotaxic coordinates for the caudate nucleus were 1.7 mm anterior and ± 3.0 mm lateral to bregma and 3.6 mm below dura. The head was positioned in the stereotaxic frame so that the skull was level between lambda and bregma. The infusion procedure used in the present study was similar to the procedure used by Josselyn et al. (2001), and produced localized transgene expression within the basolateral complex of the amygdala and the control region (caudate nucleus). Briefly, bilateral infusions of HSV-CREB or HSV-LacZ (2 μL per side) were delivered over a 10-min period through a 30-gauge injection cannula attached by polyethylene tubing to a Hamilton microsyringe attached to an infusion pump. The infusion cannula was left in place for an additional 5 min after infusion to ensure diffusion of the injectate.

Histology and Immunocytochemistry

After completion of conditioned defeat training and testing, hamsters were given an overdose of sodium pentobarbital and perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1M PBS. Brains were removed and stored in 20% sucrose solution for at least 2 days. Sections were cut at 40 μm on a freezing microtome. Immunocytochemistry was performed on free-floating coronal sections. Briefly, brains that were infused with LacZ were reacted for β -galactosidase and counterstained with neutral red. Sections were placed overnight in a solution comprised of potassium ferrocyanide, potassium ferricyonide, $MgCl_2$, PBS and 5-bromo-4chloro-3-indolyl-β-D-galactopyranosidase (0.2 mg/ml; Boehringer Mannheim, Indianapolis, IN). Brain sections from HSV-CREB-infused animals were incubated with H_2O_2 and Triton X-100, blocked with bovine serum albumin, normal goat serum, and incubated with rabbit anti-CREB antibody overnight (1:1000; Upstate Biotechnology, Lake Placid, NY). Sections were then incubated with biotinylated goat anti-rabbit IgG secondary antiserum (1:200 Vector Laboratories, Burlingame, CA) followed by avidin–biotin–peroxidase complex (ABC) reagent (Vector Laboratories). Immunoreactivity was visualized by using a diaminobenzidine reaction.

Social Defeat Testing

All hamsters were matched by weight and randomly assigned to experimental or control groups. In the conditioned defeat model, male Syrian hamsters display greatly increased submissive–defensive behaviors when tested with a nonthreatening animal in their territory (their own home cage) 1 day following the experience of social defeat in the cage of a larger, dominant hamster. We find that when hamsters are defeated by a dominant resident for 15 min, they subsequently appear virtually unable to reverse their subordinate social status (Jasnow, Cooper, & Huhman, 2004; Jasnow et al., 1999; Jasnow & Huhman, 2001; Jasnow, Huhman, & Davis, 2004). This behavioral change is so profound that defeated hamsters subsequently fail to defend their territory even when a smaller, nonaggressive animal is introduced. The behavior displayed by defeated hamsters is characterized by a total absence of territorial aggression accompanied by frequent displays of submissive and defensive behavior. To examine the effect of increasing CREB levels on the acquisition of conditioned defeat, we used a defeat procedure that normally does not result in robust levels of conditioned defeat. This procedure consisted of a single resident/intruder pairing in which animals were placed into the cage of a resident aggressor for only 5 min. All aspects of the defeat procedure were identical to the 15-min defeat procedure used previously (Jasnow, Cooper, & Huhman, 2004; Jasnow & Huhman, 2001; Jasnow, Huhman, & Davis, 2004) except for the duration of the session. Because maximal expression of this transgene occurs within 2–4 days (Carlezon et al., 1997 Carlezon et al., 1998; Neve & Lim, 1999), social defeat began 2 days after infusion of the virus. On the day of defeat training, animals were transported from the colony room to the behavioral room for the procedure. All behavioral procedures occurred during the first 2 hr of the dark phase of the daily light/dark cycle to control for circadian rhythmicity of behavior. During the 5-min defeat session, experimental animals were routinely attacked by resident aggressors, and

displayed submissive and defensive behaviors toward the resident aggressors. Behavioral testing began one day after the acquisition procedure and was again completed during the first 2 hr of the dark phase of the daily light/dark cycle. A similar resident–intruder model was used for testing but in this case a nonaggressive intruder was placed into the home cage of the defeated animal for 5 min. We have previously shown that 5-min testing sessions are sufficient to observe an adequate sample of behaviors.

To examine the effect of increasing CREB levels on the expression of conditioned defeat, we used the same defeat and testing procedure as described above except that the time between defeat and testing was 3 days instead of 1 day. This procedure was used in order to carry out the testing session at the time of the maximal expression of the transgene, which occurs 2–4 days after infusion. All training and testing sessions were recorded on VHS tape, transferred to CD-ROM, and scored by an observer blind to experimental condition with Noldus Observer (Version 4; Noldus Information Technology, Wageningen, Netherlands). During each testing trial, we measured the duration of all behaviors exhibited by the defeated hamsters in the presence of a nonaggressive intruder. We also scored aggression displayed by the resident aggressors during the initial defeat sessions to ensure that there were no systematic differences in the nature of the defeat among groups. The following behaviors were recorded as total duration in seconds: submissive–defensive, that is upright and side defense, tail lift, flee, tooth chatter, full submissive posture, risk assessment, attempted escape from cage. In the conditioned defeat model, a statistically significant reduction in the duration of submissive–defensive behavior and/or a return of territorial aggression signifies a reduction in, or blockade of, conditioned defeat, respectively. To determine the effect of increasing CREB levels on activity, animals were placed in a novel cage and allowed to explore for 5 min. The cage was divided into 9 equal squares and hamsters were placed in the center of the middle square, to begin the test session. Total grid crossings were counted during the 5-min period. A grid crossing was counted when a hamster's entire body crossed completely from one square into another square.

Statistical Analyses

For all experiments, the total duration (in seconds) of submissive–defensive behavior was determined. In Experiment 1, the mean total duration of each behavioral category observed during the testing session was analyzed with a one-way between-subjects analysis of variance (ANOVA) with treatment as the between-subjects factor. The mean total duration of aggression displayed by resident aggressors during defeat training was analyzed with a one-way ANOVA with treatment as the between-subjects factor. Activity measurements (mean number of grid crossings) were analyzed by using a Student's t test between treatment groups. Behaviors displayed by the nondefeated control hamsters were analyzed with a Student's t test between treatment groups. In Experiment 2, the mean total duration of behavior during the testing session was analyzed with a Student's t test between treatment groups. Significant differences for all analyses were ascribed at $p < .05$. Statistically significant differences determined by the ANOVAs were analyzed further with a Fisher LSD post hoc test.

Results

Experiment 1: Overexpression of CREB in the Amygdala Facilitates the Acquisition of Memory for Social Defeat

In order to test the hypothesis that increasing CREB levels within the BLA in hamsters would enhance the behavioral effects of social defeat, animals were infused with HSV vectors encoding the CREB protein (HSV-CREB; $n = 21$) or HSV encoding a control protein (HSV-LacZ encoding β-galactosidase; $n = 15$) into the BLA or HSV-CREB into a control region (caudate nucleus, HSV-CREB/caudate; $n = 7$) and then placed back in their home cage. Because maximal expression of this transgene occurs within 2–4 days (Carlezon et al., 1997, 1998), social defeat began 2 days after infusion of the virus. Infusion of the HSV-LacZ vector produced localized transgene expression throughout the BLA. Similarly, infusion of the HSV-CREB vector produced strong CREB immunostaining throughout the BLA (Figure 1a) and in the control region (caudate nucleus). Although there may have been some infection of neurons within the central amygdala, the histological examination did not reveal robust CREB immunostaining or β-gal staining in this region suggesting that the infusions were specific to the BLA. The pattern of transgene expression in terms of number and localization of cells overexpressing CREB in the basolateral complex of the amygdala was similar to the expression observed in a previous study (Josselyn et al., 2001). The number of neurons overexpressing CREB is likely underestimated in this preparation because the immunocytochemical conditions were adjusted so as to be minimally sensitive to the endogenous levels of CREB.

Increasing CREB levels specifically within the BLA enhanced the acquisition of conditioned defeat as measured by an increase in the display of submissive–defensive behaviors, $F(2, 43) = 3.86$; $p < .05$. This effect was observed in animals that were exposed to a suboptimal defeat procedure that normally does not result in the expression of conditioned defeat. The effect of increasing CREB levels on submissive–defensive behavior was neuroanatomically specific. Post hoc analysis revealed an increase in submissive–defensive behavior displayed by animals infused with HSV-CREB within the BLA compared with animals infused with HSV-LacZ in the BLA or animals infused with HSV-CREB within the caudate nucleus ($p < .05$; Figure 1b).

To test whether CREB overexpression altered agonistic behavior in the absence of prior defeat we also tested animals that did not experience social defeat. Animals that received infusions of HSV-CREB ($n = 6$ per group) but were not defeated displayed virtually no submissive behavior when tested, indicating that CREB overexpression alone is not sufficient to alter agonistic behavior; a prior defeat is necessary (Figure 1b). It is important to note that no differences were observed in the degree of defeat by the resident aggressors among all treatment groups, $F(1, 42) = 1.47$, $p > .05$, (Figure 1c), and no significant difference was observed in activity levels between HSV-CREB and HSV-LacZ-infused animals, $t(11) = 0.927$, $p > .05$, (Figure 1d), indicating that the observed increase in submissive–defensive behavior in HSV-CREB-infused animals was not due simply to a more vigorous defeat or to any changes in general activity. Taken together, these data indicate that increasing CREB levels specifically within the BLA facilitated the acquisition of conditioned defeat.

Experiment 2: Overexpression of CREB in the Amygdala Does Not Facilitate the Expression of Submissive–Defensive Behaviors

If CREB is a transcription factor important for the synthesis of proteins necessary for memory formation, then overexpression of CREB following the social defeat training should not affect the expression of submissive/defensive behavior. To examine whether increasing CREB levels within the amygdala affects expression of conditioned defeat, we exposed animals to the same social defeat

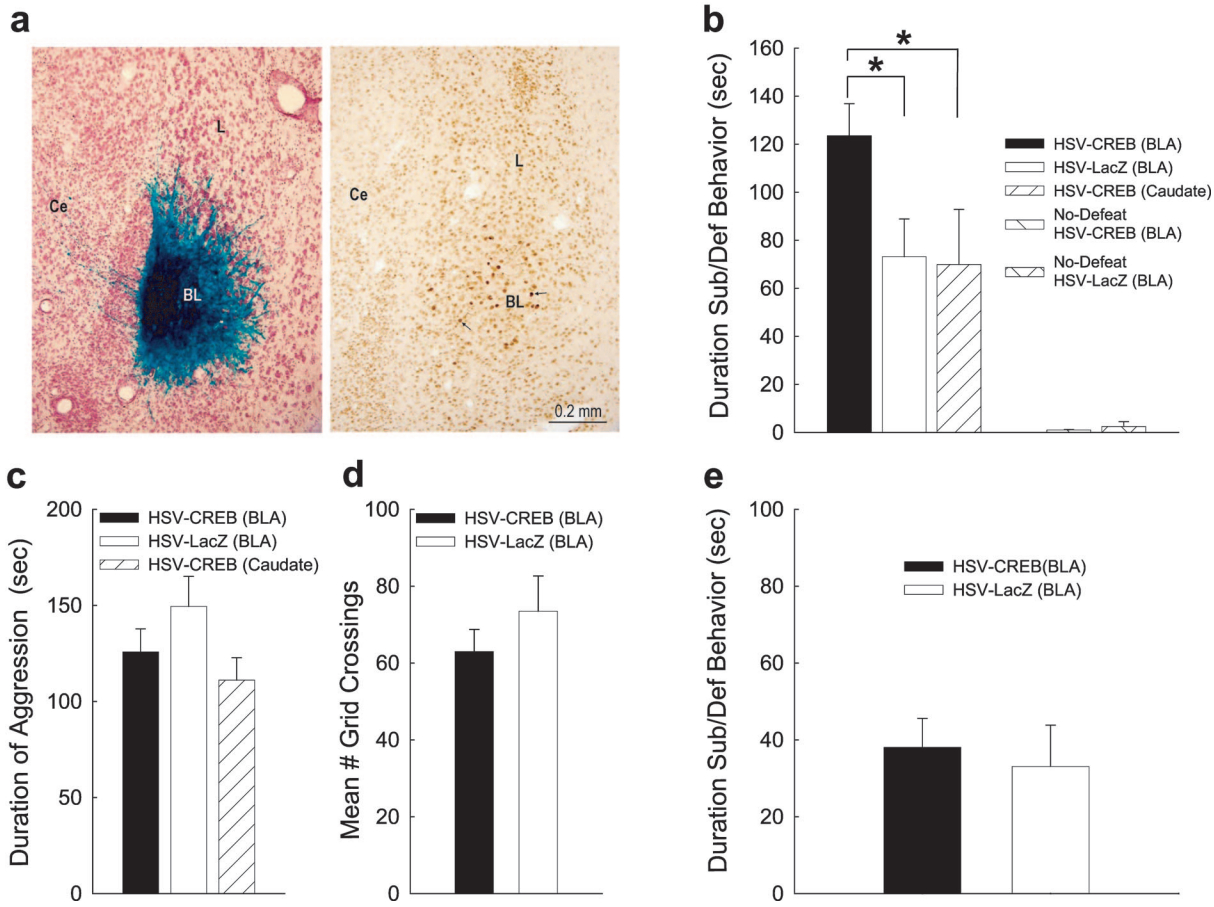

Figure 1. (a) Immunohistochemical examination of the BLA after infusion of HSV vectors. Expression of β-galactosidase 3 days after infusion of HSV-LacZ into the BLA in a section counterstained with neutral red (left panel). Overexpression of CREB 3 days after infusion of HSV-CREB into the BLA (right panel). *Ce*, Central nucleus of the amygdala; *BLA*, Basolateral nucleus of the amygdala; *L*, Lateral nucleus of the amygdala; (b) overexpression of CREB in the BLA facilitates the acquisition of conditioned defeat. Effect of infusion of HSV vectors into the BLA and caudate nucleus on total duration ($M \pm SEM$) of submissive–defensive behavior displayed by previously defeated and nondefeated hamsters during a 5-min test with a nonaggressive intruder. Animals received infusion of either HSV-CREB or HSV-LacZ into the BLA of the amygdala or HSV-CREB into the caudate nucleus 2 days before being defeated or placed in a novel arena. An asterisk (*) denotes significant differences ($p < .05$); (c) total duration ($M \pm SEM$) of aggression displayed by resident aggressor during a 5-min defeat training period with the experimental animals. There were no differences among groups in the degree of defeat experienced by the experimental animals; (d) effect of infusion of HSV vectors into the BLA on activity over 5 min. There were no significant differences observed in the number of grid crossings between HSV-CREB and HSV-LacZ infused animals; (e) overexpression of CREB in the BLA had no effect on expression of conditioned defeat. Effect of infusion of HSV vectors into the BLA on total duration ($M \pm SEM$) of submissive–defensive behavior displayed by defeated hamsters during a 5-min test with a nonaggressive intruder. Animals received infusions of either HSV-CREB or HSV-LacZ into the BLA 1 day following defeat and 2 days before being tested with a nonaggressive intruder.

session as described above. One day following the defeat, HSV-CREB ($n = 8$) or HSV-LacZ ($n = 7$) was infused into the BLA. The overall baseline levels of submissive/defensive behaviors displayed by hamsters infused with HSV-LacZ in this experiment were lower than those displayed by hamsters in the previous experiment. This was expected because there was a longer time period between the defeat training and testing in Experiment 2. No differences were observed in submissive–defensive behavior, $t(13) = 0.386$, $p > .05$, between animals receiving HSV-CREB and animals receiving HSV-LacZ into the BLA (Figure 1e), indicating that increasing CREB levels specifically within the BLA after social defeat had no effect on the subsequent expression of conditioned defeat.

Discussion

The present data provide the first evidence that increasing CREB levels within a specific brain region enhances an organ-

ism's memory about its social environment. We have demonstrated that overexpression of CREB within the basolateral complex of the amygdala enhances behavioral responses to social defeat by using a procedure that normally does not produce robust behavioral changes. Moreover, the present data demonstrate that overexpression of CREB has specific effects on memory formation of social defeat and not on the actual production of submissive–defensive behaviors. Increasing CREB levels during the defeat procedure enhanced the subsequent display of submissive–defensive behaviors, whereas increasing CREB levels only during behavioral testing had no effect on submissive–defensive behavior. There are some differences between the two experiments that temper somewhat the strength of our conclusions. In particular, there is a difference in the number of days between social defeat training and behavioral testing in the acquisition and expression experiments (1 day as opposed to 3 days, respectively). In the expression experiment, animals had to be trained before virus infusion. It was important, however, to test the animals during the window of optimal transgene expression so we waited 3 days before testing to ensure that CREB overexpression was comparable to that observed in the acquisition experiment. Thus, it is still possible, although unlikely, that overexpression of CREB may have some effect on the expression of conditioned defeat if animals are tested 1 but not 3 days after defeat. It is important to note that the observed increase in submissive–defensive behavior in HSV-CREB-infused animals was not simply due to a more vigorous defeat or due to a nonspecific increase in general activity (Figure 1c and 1d), nor was it likely due to an increase in the pain associated with the defeat, as shock reactivity has been demonstrated to be no different between HSV-CREB-infused animals and control animals (Josselyn et al., 2001). In addition, animals that received infusion of HSV-CREB but were not defeated did not display any submissive behavior when tested, indicating that CREB overexpression did not alter agonistic behavior in the absence of prior defeat. Finally, we also demonstrated that the effect of increasing CREB levels on submissive–defensive behavior was neuroanatomically specific. Overexpression of CREB specifically within the BLA, but not a control region, enhanced the display of submissive–defensive behavior. It is important to note that the level of submissive–defensive behavior displayed by animals receiving HSV-CREB infusions in the BLA prior to a 5-min defeat was similar to the levels seen in our previous studies using a longer, 15-min defeat procedure (Jasnow, Cooper, & Huhman, 2004; Jasnow & Huhman, 2001; Jasnow, Huhman, & Davis, 2004).

The present data extend, and are consistent with, a previous report that used the same HSV-CREB virus. Josselyn et al. (2001) used this vector to increase CREB levels within the basolateral complex of the amygdala and demonstrated enhanced LTM as measured by fear-potentiated startle. Furthermore, they demonstrated enhanced LTM, but not STM, following a training procedure that normally does not produce robust fear conditioning. Likewise, in the present study, we observed enhanced defeat-responsive behavior following a modified training procedure that normally does not produce conditioned defeat. The present data are also consistent with previous reports that have used transgenic animals showing an important role for CREB in LTM formation. Inducible and reversible disruption of CREB function blocks memory for contextual fear, cued fear, spatial and object recognition, as well as conditioned taste aversion (Bozon et al., 2003; Josselyn et al., 2004; Kida et al., 2002). Collectively, these data along with the findings of the present study demonstrate that altering CREB levels globally, as well as in temporally specific and neuroanatomically specific regions, has profound effects on memory in a wide variety of models.

Similar to conditioned fear, NMDA receptor-dependent plasticity within the BLA is essential for the acquisition of conditioned defeat (Jasnow, Cooper, & Huhman, 2004). We have also shown, however, that conditioned defeat involves mechanisms related to anxiety-like behavior. This is evidenced by the dependence of conditioned defeat on corticotropin-releasing factor (CRF) receptor activation in the BNST and not in the amygdala (Jasnow, Huhman, & Davis, 2004). The present data are in agreement with previous data from our laboratory and further strengthens the hypothesis that conditioned defeat is partially reliant upon amygdala-dependent learning that may generalize to different contexts and opponents. Taken together, these data suggest that conditioned defeat is a naturalistic measure of fear learning with which researchers can explore how animals learn about important events in their social environment.

One issue that remains unresolved is which downstream targets of CREB are activated following binding to CRE (cyclic AMP response element) elements in the promoter region of these target genes. A number of genes have been identified to have CRE elements within their promoter region, and they appear to be regulated by CREB activation. These include brain-derived neurotrophic factor and CRF, although not in the BLA (Cheng et al., 2000; Conti, Cryan, Dalvi, Lucki, & Blendy, 2002; Spengler, Rupprecht, Van, & Holsboer, 1992), both of which have been implicated in fear conditioning and social defeat (Jasnow et al., 1999; Jasnow, Huhman, & Davis, 2004; Lee & Davis, 1997; Radulovic, Fischer, Katerkamp, & Spiess, 2000; Rattiner, Davis, French, & Ressler, 2004). Thus, known target genes that are dependent on CREB activation and binding to CRE elements on their promoters are also implicated in fear conditioning and conditioned defeat. It is important to note, however, that although overexpression of CREB facilitated the acquisition of conditioned defeat we do not know whether this manipulation resulted in increased activated CREB (pCREB) levels within the BLA. In addition, overexpression of CREB may also result in CREB acting nonspecifically on multiple genes and not specifically on CRE-regulated genes. In summary, the data presented in this article along with previous data provide evidence that CREB plays an essential role in the formation of LTM not only for specific tasks or responses but also for mediating behavioral plasticity in ethologically relevant social contexts.

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