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Inhibition of Protein Kinase G Facilitates the Consolidation of Fear Extinction

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An abstract of
A thesis submitted to the Faculty of Emory College of Arts and Sciences
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Abstract

Inhibition of Protein Kinase G Facilitates the Consolidation of Fear Extinction

By Kelsey S. Zimmermann

Previous studies have reported that the activation of PKG by cGMP is necessary for the successful acquisition and consolidation of fear memories. However, little definitive information is available on whether or not the acquisition of fear extinction is similarly affected by exogenously affecting PKG activation. To this end, we chose to explore the effect of administering the PKG inhibitor Rp-8-Br-PET-cGMPS or the PKG activator 8-Br-cGMP into the basolateral amygdala (BLA) of fear conditioned mice immediately after they completed extinction training. When tested 24 hours later, the animals that received the activator showed extinction levels similar to vehicle controls, but those that were given the inhibitor exhibited significantly less fear to the conditioned stimulus (CS) when compared to the vehicle group, indicating that there exists a facilitatory effect of PKG inhibition on extinction. These data suggest that PKG activation may be critical for the maintenance fear memories; that inhibition of PKG may be important during the consolidation of extinction of fear; and that inhibiting PKG might be a novel therapeutic approach to enhancing extinction in fear-related disorders.

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INTRODUCTION

Disorders of Fear and Anxiety

Anxiety disorders appear at an alarming rate in the general population of the United States and many other countries. A 2008 assessment published by the National Institute of Mental Health (NIMH) found that in a given year, approximately 40 million American adults (approximately 18.1% of the population) would be afflicted with an anxiety disorder. These diagnoses include panic disorder, obsessive-compulsive disorder (OCD), post-traumatic stress disorder (PTSD), generalized anxiety disorder (GAD), and a spectrum of phobias (social phobias, agoraphobia, and specific phobias). Anxiety disorders often present comorbidly with one another and/or with a diagnosis of depression or substance abuse (NIMH). The debilitating effects of anxiety disorders can severely impact the personal and professional lives of the millions who suffer, and a variety of treatments have consequently become available to help those that are afflicted cope with their symptoms. Cognitive-behavioral therapy (CBT) can prove beneficial for many, while pharmacological treatments with benzodiazepines like diazepam (Valium) and alprazolam (Xanax) or selective serotonin reuptake inhibitors (SSRIs) have significant anxiolytic properties that are helpful for others (often in conjunction with CBT). Though these approaches are successful in many cases, some forms of anxiety, like PTSD and phobias, can prove more resistant to traditional treatments than others, and methods utilized in CBT must be adjusted accordingly to accommodate the specific needs of the individual patient.

Posttraumatic stress disorder (PTSD) represents a specific constellation of

symptoms experienced by many individuals after suffering or being exposed to a traumatic event. Prominent among the qualifying criteria listed in the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV) is an acute fear response generated by a reminder of the initial trauma. Subjects with PTSD may experience exaggerated startle responses when in the presence of salient sensory stimuli, and may even be compelled to change their daily routines in order to avoid such triggers entirely. As with phobias, the most severe anxiety responses in PTSD are typically generated by exposure to a relevant trigger.

Many who suffer from PTSD or phobias may benefit significantly from a particular form of CBT known as exposure therapy. With this approach, the patient is presented with the fear-eliciting stimulus (in the case of PTSD, typically the memory of the trauma) repeatedly, until the trigger itself no longer holds the salience necessary to provoke a visceral or behavioral fear response. This process, wherein the subject learns to dissociate the presence of the trigger from the trauma itself, is known as "extinction". Evidence has shown that extinction is in fact a form of learning, rather than simply a loss of the initial fear memory. A study published by Cammarota et al. (2007) states that "extinction requires neural activity, signaling pathways, gene expression and protein synthesis" (Cammarota et al. 2007), and another publication by Liu et al. (2009) found that an NMDA receptor (a very important component in the learning of new memories) was necessary for the consolidation of extinction learning (Liu et al. 2009). If all the elements necessary for extinction are functioning normally, it can be a very effective approach. However, other studies have found a decreased ability of individuals with PTSD to learn and recall the memories of extinction training (Milad et al. 2006). It is

therefore important to understand the molecular processes involved in the learning of extinction more comprehensively. By establishing more definitively the pathways involved in the extinction of fear, it is possible to identify potential pharmacological targets that could act as adjuncts to exposure therapy. In this way it may be possible to compensate for the deficits in extinction learning seen in subjects with PTSD. In order to identify the relevant molecular activity, it is necessary to create models that can accurately reflect the pathology seen in presentations of human fear and anxiety. Mice and rats have therefore been used pervasively in laboratory settings to replicate the common behavioral phenotypes that appear in individuals with anxiety disorders so that the contributing physiological factors may be analyzed.

Animal Models of Fear and Anxiety

Animal models of fear have proved invaluable in understanding many fundamental aspects of fear as well as identifying its essential neurocircuitry. The paradigm of extinction training to reduce the expression of fear in response to a cue previously paired with an aversive stimulus is frequently used as a model that accurately simulates the process of exposure therapy. Through Pavlovian conditioning, animals (most commonly mice and rats) can arguably be made to acquire, consolidate, and extinguish fear in the same manner as humans. In a controlled experimental environment, these respective learning processes can be isolated and considered in detail from many useful perspectives, including both the respective behavioral expressions associated with each stage and the molecular activity that influences long- or short-term alterations in neuronal structure and connectivity.

By employing adaptations of these basic conditioning paradigms in studies using animal models, it is possible to observe not only the spectrum of natural emotional and biological processes involved in fear learning, but also the modifications of behavioral phenotypes that may be induced by exogenous manipulation of the established neurophysiology. There is currently a widespread effort to better understand how the activity of molecular pathways precipitates altered responses in neuronal function and morphology known to influence the acquisition, consolidation, and extinction of fear. Based on the burgeoning developments in this area of research, there is now a search for pharmacological adjuncts to CBT that may augment and/or hasten its curative effects. For example, the recent discovery that the response of fear-conditioned rats to extinction training can be enhanced by administration of the NMDA receptor agonist D-cycloserine (DCS) (Walker et al. 2002) has been and continues to be translated with great success in human trials that persistently yield significant and promising results for the treatment of a wide variety of psychological disorders, particularly when administered in the context of CBT. Thus far, these include phobias (Ressler et al. 2004; Aupperle et al. 2009), Social Anxiety Disorder (Hofmann et al. 2006; Guastella et al. 2008) Obsessive Compulsive Disorder (Kushner et al. 2007; Wilhelm et al. 2008), anxiety and depression (Britton et al. 2007), the negative symptoms of schizophrenia and effects on memory consolidation (Goff et al. 2008), addiction (Santa Ana et al. 2009), and Panic Disorder (Otto et al. 2010). The efficacy of DCS in animal work and the evidently replicable results found thus far in clinical studies strongly encourage the continuation of research in this area; further understanding of the physiological mechanisms of action involved in fear learning will very likely provide many additional benefits to the millions who struggle with

disorders of fear and anxiety.

The Amygdala

Considerable evidence suggests that the amygdala may play an important role in the pathologies associated with fear and anxiety. In the case of PTSD, abnormal amygdalar activity (Shin et al. 1997; Rauch et al. 2000; Bryant et al. 2005; Shin et al. 2005; Liberzon and Martis 2006; Bryant et al. 2008), connectivity (Rauch et al. 2000; Gilboa et al. 2004; Shin et al. 2004; Williams et al. 2006) and volume (Pavlisa et al. 2006) have been notably and consistently established as prominent features in clinical presentations of the disorder. This structure, largely responsible for the learning and expression of fear, has also been strongly implicated in the process of learning fear extinction. Many of the alterations in signaling and protein synthesis found by Cammarota et al. were identified in the basolateral amygdala (BLA) (Cammarota et al. 2007), and Amano et al. found that synaptic inhibition was altered in the central amygdala (CeA) in response to extinction training (Amano et al. 2010). Consequently, many animal studies target the amygdala directly in order to manipulate aspects of fear learning and memory; some use lesions while others apply various exogenous substances through microinjection.

The amygdala is composed of several nuclei, and the functions of each are implicated in specific aspects of emotional learning and the associated behavioral responses. The connectivity between the nuclei of the amygdala and the respective brain regions to and from which they send and receive projections have been extensively investigated and well-mapped (Figure 1).

The lateral and basolateral nuclei are largely responsible for the integration of sensory cues, and much of the associative learning process occurs in these two regions. The central nucleus receives input from the other two nuclei, and projects to a wide array of brain regions responsible for initiating appropriate behavioral responses to various fear-inducing stimuli (Figure 1). The consolidation and extinction of conditioned fear are therefore largely dependent upon neuronal communication and synaptic plasticity in the lateral and basolateral complexes (Muller et al. 1997; Cousens and Otto 1998; Laurent and Westbrook 2010) and their efferent projections to the central nucleus are necessary for the resultant behavior (Jimenez and Maren 2009). Many genes of interest in the amygdala are responsible for long-term potentiation (LTP), which is necessary for the consolidation of fear memories. Administering certain drugs to the basolateral nucleus of the amygdala can have either inhibitory or facilitatory effects on the expression of these proteins. This can be done in several manners; in some cases the protein of interest itself (or one of its critical subunits) is directly affected, while other approaches preferentially target the protein's known transcription factors or other upstream molecular influences. Both can be viable methods of blocking or enhancing the consolidation or extinction of fear, ultimately exerting their full effects by altering activity at the synapses and manipulating the natural processes needed for learning-induced dendritic arborization. These particular pathways active in the amygdala have generated much interest, particularly as they may potentially suggest sites on which therapeutic drugs may act.

PDE-cGMP-PKG Pathway (Figure 2)

Within the last year, our lab collected data from a gene array study that involved excising the amygdalas from mice two hours after they were given extinction training and identifying any genes that showed significantly different expression levels when compared with control animals that had not received extinction (Ressler et al., unpublished data). The most robust changes included several families of protein phosphatases (PPPs) and phosphodiesterases (PDEs), the majority of which were upregulated. Among the PDEs affected by extinction training, the isoforms 1c, 1b, 8b, 7a, 10a, and 4d showed the most significantly increased expression. Certain families of PDEs exert inhibitory effects on cAMP and/or cGMP, and the noted upregulation of the PDEs after extinction therefore most likely indicates training-induced decreased activation of cAMP and cGMP, as well as PKA and PKG, their respective downstream targets. Those data coincide appropriately with previous work that has shown that the activity of amygdalar PKA and PKG is necessary for the consolidation of fear (Nijholt et al. 2008; Ota et al. 2008), and also provides supportive evidence for two studies that found that the inhibition of PKA has a facilitatory effect on the extinction of fear (Isiegas et al. 2006; Nijholt et al. 2008).

Amygdalar cGMP and PKG, upon which this study focuses, have been recently implicated in fear consolidation (Ota et al. 2008), and three of the PDEs found to be upregulated in the amygdala after fear extinction also show affinity for cGMP (1c, 1b, and 10a) (Deng et al. 2007). Though the literature concerning the impact of cGMP and PKG on learning and memory is not nearly as prevalent as publications concerning cAMP and PKA, there is accumulating evidence that they do in fact have considerable

influence on these processes. Most of the effects of cGMP and PKG on learning and memory explored thus far have been attributed to the nitric oxide pathway (reviewed briefly in the following section), but investigating the possibility of additional modulation by way of the inhibitory action of cGMP-associated PDEs could provide novel approaches to potential treatments of fear and anxiety disorders as well as contribute to a more holistic model of fear conditioning and extinction.

The data from the gene array strongly suggested a role for the PDEs in the extinction of fear, and based on these results and the recognized necessity of PKG activity for the process of consolidation, we asked if blocking or enhancing the endogenous effects of the PDEs on cGMP/PKG could respectively blunt or facilitate the effects of extinction training on fear-conditioned mice.

NOS-NO-cGMP-PKG Pathway (Figure 3)

The excitatory molecular pathway involving nitric oxide (NO), cGMP, and PKG has been implicated in several studies of fear learning (Chapman et al. 1992; Ota et al. 2008; Kelley et al. 2009; Overeem et al. 2010), and the action of NO as a retrograde signal involved in LTP is well-established. A recent publication also found that blocking or enhancing the activity of PKG respectively inhibited or facilitated the consolidation of conditioned fear (Ota et al. 2008). Inhibiting nitric oxide synthase (NOS) blocks NO-induced activation of cGMP/PKG, and this approach has been shown to impair both spatial learning in rats and conditioned eyeblink response in rabbits (Chapman et al. 1992).

Aims of Study: PKG and Extinction of Fear

In the current study, we explored the effects of cGMP/PKG manipulation on the consolidation of extinction learning. We used the PKG inhibitor Rp-8-Br-PET-cGMPS and the PKG activator 8-Br-cGMP, two of the drugs used by Ota *et al* (2008) to affect the consolidation of fear in their study of the NO-cGMP-PKG signaling pathway. The inhibitor would putatively augment the enhanced inhibition of cGMP caused by the extinction-induced increase in PDE levels, while the activator would block or reduce the natural molecular response.

Given the data collected from our lab and others concerning the activity of the signaling pathways mentioned above, we hypothesized that **1) the administration of a PKG inhibitor to mice via microinjection within the BLA immediately after extinction training would result in diminished expression of fear to the conditioned stimulus (CS) in subsequent tests** and **2) the similar administration of a PKG activator would conversely decrease the efficacy of the extinction training, resulting in comparatively high retention of the original aversive memory trace and a maintained expression of fear to the CS in subsequent tests.**

The results of the experiments significantly supported our first hypothesis, but the PKG activator interestingly has thus far shown no effect.

METHODS AND MATERIALS

Subjects:

Adult (6-8 weeks of age) male C57BL/6J mice weighing 20-30 g (Jackson Laboratories, Bar Harbor, ME, USA) were used for all experiments. Animals were housed in standard group cages (four per cage) and were given *ad libitum* access to food and water. All experiments were performed during the light portion of a 12-h light/dark cycle. All experiments were approved by Emory University Institutional Review Board following Institutional Animal Care and Use Committee (IACUC) standard with accordance to the Yerkes Primate Research Center regulations.

Drugs:

The PKG inhibitor Rp-8-Br-PET-cGMPS (1.0μg/μl) (Sigma Chemical, St Louis, MO, USA) or the PKG activator 8-Br-cGMP (1.0μg/μl) (EMD Chemicals, Gibbstown, NJ, USA) were administered through microinjection to the basolateral amygdala (BLA) immediately after extinction training (0.25-0.3μl/side). Drugs were dissolved in 1XPBS, which was also administered alone for the vehicle injections.

Cohorts:

Experiments were carried out in three cohorts. Cohort 1 (n=17) received extinction training and either vehicle or Rp-8-Br-PET-cGMPS. Cohort 2 (n=30) contained a vehicle group and an Rp-8-Br-PET-cGMPS group that both received extinction training, as well as a drug group that was given reconsolidation training. Cohort 3 (n=36) all received

extinction training, and were divided into a vehicle group, an Rp-8-Br-PET-cGMPS group, and an 8-Br-cGMP group. In analyzing data for the PKG inhibitor, vehicle groups and drug groups from all three cohorts were included.

Surgeries:

For our purposes, we aimed all microinjections at the basolateral complex, primarily due to its well-documented role in consolidation and extinction as well as its comparatively large size relative to the lateral nucleus, which made it a more feasible target. Prior to surgery, mice were anesthetized by intraperitoneal (i.p.) injections of ketamine-dormitor (ketamine: 80mg/kg; dormitor: 1.0 mg/kg). They were then mounted in a stereotaxic apparatus (Stoeling Instruments, Wood Dale, IL, USA), and implanted bilaterally with 4-mm stainless-steel guide cannulas (26 gauge; Plastics One, Roanoke, VA, USA), which were fixed to the skull using dental acrylic and jeweler's screws. The following stereotaxic coordinates from bregma were used: anteroposterior (AP) = -1.5, mediolateral (ML) = $\pm$3.4, dorsoventral (DV) = -5.1. Dorsoventral coordinates were measured from the skull surface with the internal cannula extending 2mm beyond the end of the guide cannula. Coordinates were based on the mouse brain atlas of Paxinos and Franklin (2001). After surgery, mice were given i.p. injections of antisedan (4.0mg/kg, dormitor-reversing agent) and meloxicam (1.0mg/kg). All animals were monitored closely following surgeries; if any discomfort was noted, an additional injection of meloxicam was administered 24 hours later.

Conditioning Apparatus:

Med associate mouse conditioning chambers (SRLAB, San Diego Instruments) were used for all training and testing. The unconditioned stimulus (US) consisted of a 1-second, 0.8mA footshock delivered to the stainless-steel shock grid floor via Med Associate shockers. The conditioned stimulus (CS) consisted of a 75dB, 30-second 6kH tone (cohorts 1 and 2) or 12kH tone (cohort 3), generated by Tektronix function generator and delivered to high frequency speakers mounted on the side of the conditioning chambers.

Procedures (Figure 4):

Training- A mouse was placed in a chamber and exposed to five 30-second tones that coterminated with a 1-second footshock. The first tone was presented after three minutes in the chamber and the intertrial interval was 2.5 minutes.

Matching- Twenty-four hours later, mice were placed back in the same chambers and exposed to three 30-second tones (no shocks) presented over the course of a 7.5 minute trial (tones at 180, 300, and 420 seconds). Freezing was averaged for each animal over the three tones, and they were placed into groups for different treatments so that each group began with a comparable level of freezing (fear).

Extinction Training- Extinction training was given twenty-four hours after matching. Animals were placed in the chambers and exposed to fifteen 30-second tone-alone cues over the course of approximately 32 minutes (first tone at minute 3, intertrial interval 2.5 minutes). After training, animals were removed from the chamber and received their designated treatments.

Reconsolidation Training- (Cohort 2 only) Twenty-four hours after matching, these animals were placed in the chambers and exposed to only one 30-second tone (given at minute 3 of a 5 minute trial). After the trial ended, the mice were removed and given an injection of the PKG inhibitor.

Testing- Either two or three post-training tests were given, the first starting at 24 hours after extinction or reconsolidation training and the subsequent tests continuing on consecutive days. Mice were placed in the chambers and exposed to 15 tone-alone cues (same as extinction training).

Reinstatement Training and Testing- Reinstatement training and testing was given two days after the last test day. Mice were placed in the chambers for 10 minutes, and at minute 5 received a 1-second shock (0.8mA) 5 minutes after the start of training. Twenty-four hours later, freezing to the tone was once again assessed over three trials.

Retraining and Retesting- Two or three days following reinstatement, animals were placed in the chambers again and given the initial training protocol again (five tone-shock pairings). The following day, they were given the 15-tone trial once again to measure conditioned freezing.

Histology:

Animals were sacrificed by anesthetic overdose with an i.p. injection (0.4ml) of 8% Chloral Hydrate, and intracardially perfused. Brains were removed, frozen on dry ice, and stored at -80º until sectioning. For Cohort 1, microinjections of green dye (0.2-0.3µl/side) were given immediately prior to sacrifice, and cannula placements were mapped according to the injection sites of the dye. Cohorts 2 and 3 were stained with

Cresyl violet, and injection sites were mapped based on the location of the guide cannula.

Statistical Analyses:

Data were analyzed with T-tests and 2-way Analysis of Variance (ANOVAs). Any animal freezing less than 3% upon testing the first day was excluded based on inferred amygdala damage or injection abnormalities (these exclusions did not affect group averages). Significance was set at alpha=0.05

RESULTS

Inhibiting activity of PKG in the BLA with Rp-8-Br-PET-cGMPS after extinction training enhances consolidation of extinction learning. (Figure 5)

Microinjections of the PKG antagonist Rp-8-Br-PET-cGMPS into the basolateral amygdala immediately after mice received extinction training significantly reduced their freezing response to the tone CS in the first subsequent test ($p<0.05$) 24 hours later (Figure 5a), as well as the first three trials of the second post-extinction training test ($p<0.05$) 48 hours later (Figure 5b). They also showed a decreased response to the CS in a test given 24 hours after they received a one-shock reinstatement training ($p<0.05$), indicating a true learned dissociation of the CS and the US (Figure 5c). After being retrained according to the original paradigm, the difference between the Rp-cGMPS and vehicle groups was non-significant ($p=0.23$) in an assessment of tone-induced freezing given the following day (Figure 5d). This suggests that the drug did not reduce fear expression by causing damage to the amygdala.

Rp-8-Br-PET-cGMP enhances learning of extinction training rather than disrupting the previously consolidated fear memory. (Figure 6)

Exposing animals to a paradigm of reconsolidation (1 tone-alone trial) instead of extinction did not disrupt the original fear memory trace. If the PKG antagonist exerted its function by weakening the synaptic communications that resulted from the fear acquisition and consolidation phases, we would expect that administering the drug after the memory had been recalled by a reminder cue (the single tone) would disrupt the initial trace and result in comparable deficits in tone-induced freezing as those seen in animals that received full extinction training (for an explanation of reconsolidation, see Discussion). However, a reminder cue alone was not enough to facilitate the effects of the drug; animals in the reconsolidation group still showed robust fear to the tone in the test given on the subsequent day (Figure 6). Only mice that were given the drug after the full 15-trial extinction training showed stable and significant decreases in freezing to the CS on subsequent test days. This suggests that PKG inhibition, when acting in the temporal context of extinction training, strengthens the learning of the training rather than directly weakening the extant trace of previously acquired fear.

Post-extinction training administration of 8-Br-cGMP showed no effect on subsequent assessments of tone-induced freezing. (Figure 7)

Giving mice a post-extinction training microinjection of the PKG agonist had no significant effect on freezing to the CS in subsequent tests (Figure 7a-b). Animals actually showed a trend (non-significant) toward comparatively decreased freezing, but further tests are needed to determine the presence or absence of a definitive effect in

either direction. Ability to learn fear was still intact (Figure 7d), suggesting that the lower levels of fear were not likely due to amygdala damage.

DISCUSSION

Rp-8-Br-PET-cGMPS

The significant decrease in CS-elicited freezing exhibited by animals that received post-extinction injections of the PKG inhibitor supports our initial hypothesis, and suggests that blocking the activation of PKG's downstream effectors in the period of time immediately after fear initial fear extinction serves to enhance the effect of the training. Past gene array data that found increased levels of PKG- and PKA-inhibiting PDEs after extinction reinforces the idea that this inactivation may be a necessary part of the process that facilitates the inhibition of behavioral responses to fear memories (Ressler et al., unpublished data). More information concerning the molecular activity upstream of PKG and its downstream targets will be needed to further understand the molecular mechanisms that precipitate the neuronal changes involved in extinction, but continual progress is being made in this area. Our results and those of Ota et al. show that affecting the cGMP/PKG pathways using the same drug can have opposing effects on memory formation depending on the context; when given during the acquisition phase, Rp-8-Br-PET-cGMPS disrupts learning and inhibits the consolidation of the fear memory (Ota et al. 2008), while our data indicates that administering the same drug at the time of extinction enhances consolidation of the training and the ability of the animal to inhibit fear to the tone. This may suggest the existence of a pathway for acquisition and a

pathway for extinction that function in direct opposition to each other. It is possible, for instance, that downstream targets of PKG in the lateral/basolateral complex are responsible for forming the associations necessary for auditory fear conditioning, and that the inactivation or suppression of such targets is required for molecular components of an extinction pathway to exert their function. Further exploration of the functions of the PPPs found to be upregulated in gene array studies may provide more information about a pathway necessary for extinction. The data from our reconsolidation group in Cohort 2 provides evidence that the effect of inhibiting PKG is indeed a process of learning, rather than simply a disruption of the original memory trace, while the maintenance of the diminished response to the CS in the drug groups after reinstatement training implies that the memory of extinction is both stable and stronger than vehicle controls.

8-Br-cGMP

There are several possible reasons for the lack of an observable effect in the group receiving the PKG agonist. Although Ota et al. did find behavioral effects in rats at 1μg/side, a dose comparable to what we used, the most effective dosage was determined to be 10μg/side, which was significantly higher. A follow-up study involving a dosage response curve will be necessary before drawing any definitive conclusions about the lack of effect with the PKG activator. If, however, our results are consistent at a higher dosage, it could indicate that normal post-extinction inhibition by PDE is strong enough to override the agonistic action of the drug. 8-bromo-cGMP functions as an active analog of cGMP that is more resistant to degradation by PDEs than the endogenous form of cGMP, but in a situation where excessive PDEs are present and the dosage of the drug is

low enough, it is conceivable that inhibition could be robust enough to neutralize the agonistic effects. Furthermore, though the drug is cited as being more resistant to PDEs than cGMP, perhaps it is inhibited differently by different PDE isoforms. The primary families of PDEs in the brain responsible for hydrolysis of cGMP are 2, 5, and 9 (Domek-Lopacinska and Strosznajder 2010), none of which were found significantly upregulated by extinction. It may be possible that the other subtypes are more effective at degrading the analog.

Limitations

One limitation in this study was the comparatively small size of the PKG agonist group (n=12); this drug was tested only in the third cohort after initially finding a response with the antagonist, which was administered to animals in all three cohorts. After considering the literature in more detail, a higher dosage would likely have been more appropriate, and more work is still needed to confirm the lack of an effect.

In these initial studies we did not control for the potential effects of training and testing contexts; namely, animals underwent all training and testing in the same conditioning chambers with no contextual alterations. This is of course an issue that should be addressed in the future to verify that our results are indeed specifically dependent upon the extinction of the tone CS.

Finally, the gene array data was taken two hours following extinction training, while we administered the drugs immediately after the training had ended. There could conceivably be changes in the expression of influential molecular components within this time span that are responsible for short-term memory and the early stages of

consolidation.

Future Directions

While this study offered strong evidence in support of PKG inhibition as an active contributor to the consolidation of extinction learning, more studies are needed to establish the influence of various upstream activators and inhibitors. For instance, it is not yet known with complete certainty whether the effects we found were a result of enhancing the endogenous inhibition of PKG activity by the PDEs (Figure 2) or by blocking PKG activation through the nitric oxide pathway (Figure 3). Due to the gene array data that specifically implicated PDEs in the extinction process, we consider it likely that the activity in this inhibitory process is significant, and merits further investigation.

Of the three PDE isoforms that had a regulatory effect on PKG (1b, 1c, and 10a), 1b and 10a are the more compelling potential targets for the purposes of fear learning and extinction. PDE1b has been implicated in the modulation of dopamine in the brain (Reed et al. 1998), which is an activator upstream of the PDE-cGMP-PKG pathway (Figure 2), and PDE10a holds particular interest due to the use of its inhibitors in treating the positive symptoms of schizophrenia (Grauer et al. 2009). Further studies may examine the inhibition of PDE10a through i.p. administration of papaverine or MP-10 (both specific to the 10a subtype), which would theoretically result in a blunted effect of extinction training. If this proved successful, and the extinction pathway is mediated according to our initial hypothesis of PDE-induced inactivation of PKG, concomitant microinjections of Rp-8-Br-PET-cGMPS may even reverse the effect of the 10a inhibitor.

It will also be important to give further consideration to the potential impact of the nitric oxide pathway in the process of extinction. Future studies would likely include post-extinction administration of NOS inhibitors and/or NO scavengers. In this way, we would be able to identify the respective influences of the pathways depicted in Figures 2 and 3 in the consolidation of extinction learning.

Significance

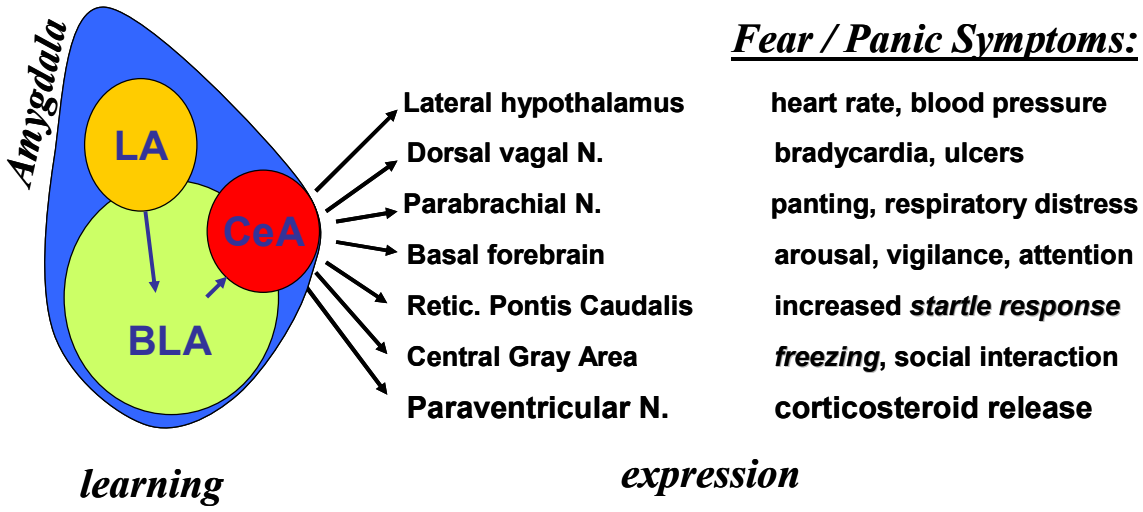
In addition to previous work that implicates PKG and its upstream regulators in the process of fear consolidation, the results of this study provide novel evidence that suggests an active role for these factors in the extinction of fear as well. While past investigations have specifically examined the impact of NO on PKG activity within the context of fear conditioning, the influence of this pathway on extinction has not yet been specifically addressed. Furthermore, the regulatory effects of PDE-induced inhibition of PKG in regard to the extinguishing of learned fear have not, to our knowledge, been explored. When considered in conjunction with the data collected from the post-extinction gene array, the behavioral findings presented above offer strong incentive for continual research in this avenue.

A Brief Note on Reconsolidation

The single tone-alone paradigm we used in the second cohort as a model of reconsolidation is likely an adequate approach, but its validity is somewhat limited due to the previous exposure of animals to the tone during the matching session carried out on the previous day. Reconsolidation is a process in which subjects are exposed to the CS

briefly, but not for a long enough period of time to extinguish their fear. This "reminder" of the initial training appears to result in a temporarily labile state of the fear memory. Research has shown that by introducing a protein synthesis inhibitor during reconsolidation, the initial fear memory may be disrupted (Nader et al. 2000). Our implementation of this behavioral paradigm was therefore necessary to **a)** confirm that learning of extinction was necessary for the drug to exert its action and **b)** establish that it had no direct effect on reconsolidation, thereby supporting its fear-reducing effects as specifically attributable to increased extinction learning as opposed to inhibited reconsolidation.

FIGURES



Adapted from (Davis 1992)

Figure 1: *Function and Connectivity of Amygdala Nuclei*

The lateral and basolateral nuclei send inputs to the central nucleus, which projects to brain regions involved in the expression of fear.

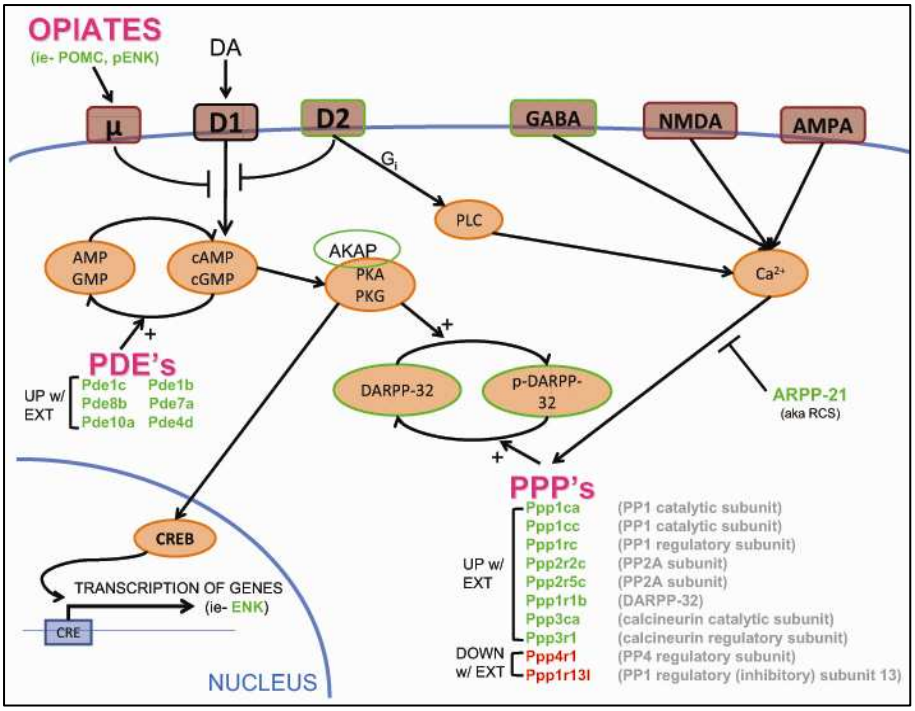


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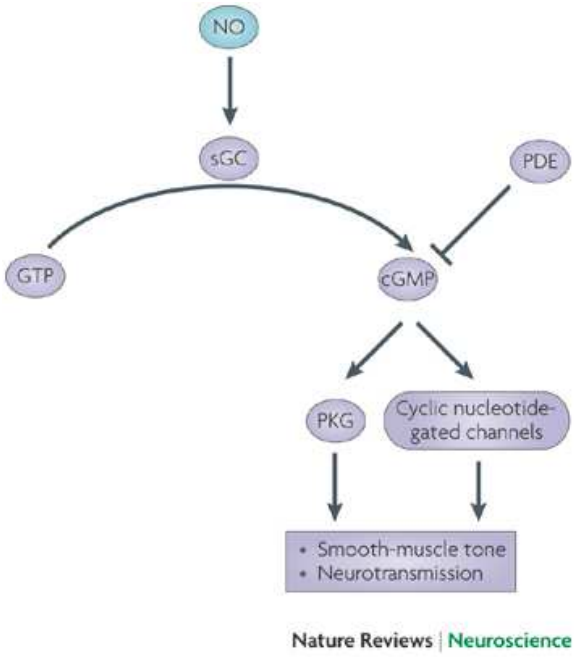
Figure 2: *Phosphatase, phosphodiesterase, and kinase pathways potentially involved in extinction of fear.*

Data from microarray: Relevant to the current study, several PDEs, which exert inhibitory effects on cAMP/cGMP and therefore PKA/PKG, were upregulated two hours after extinction.



(Calabrese et al. 2007)

Figure 3: *Nitric Oxide Activation of cGMP*

Nitric oxide is an additional factor upstream of cGMP that affects its activation.

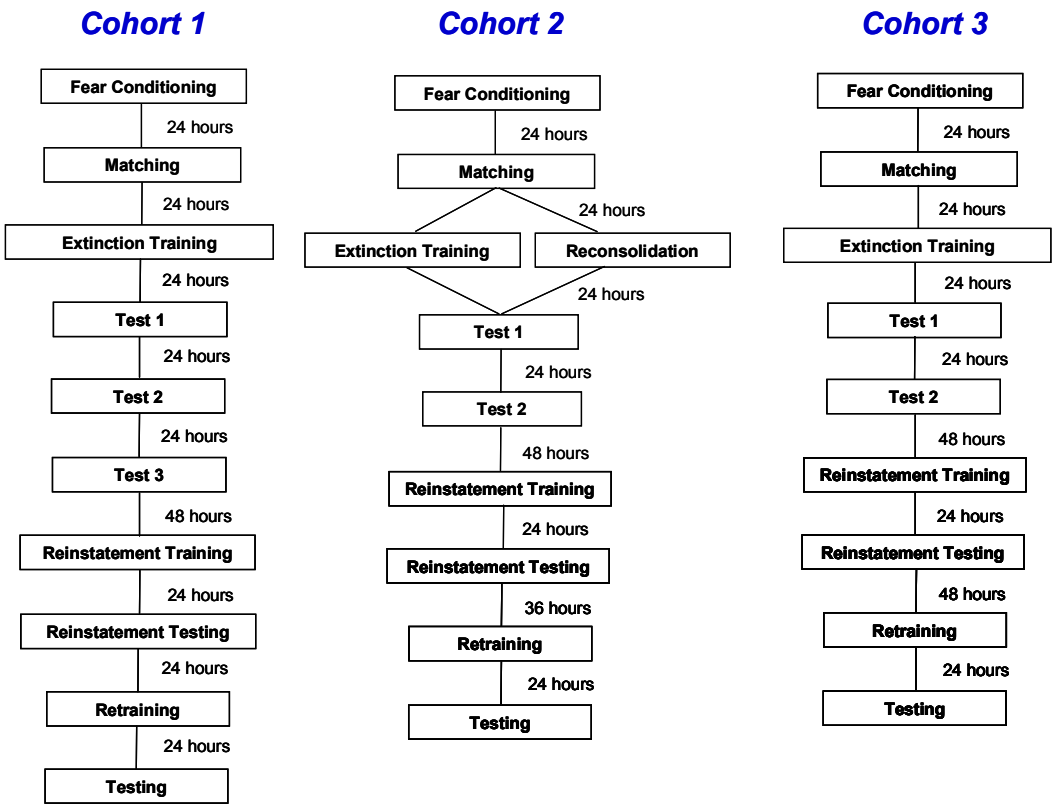


Figure 4: *Behavioral Procedures According to Cohort*

Rp-8-Br-PET-cGMPS, 8-Br-cGMP, or vehicle were administered by microinjection directly after extinction training or reconsolidation.

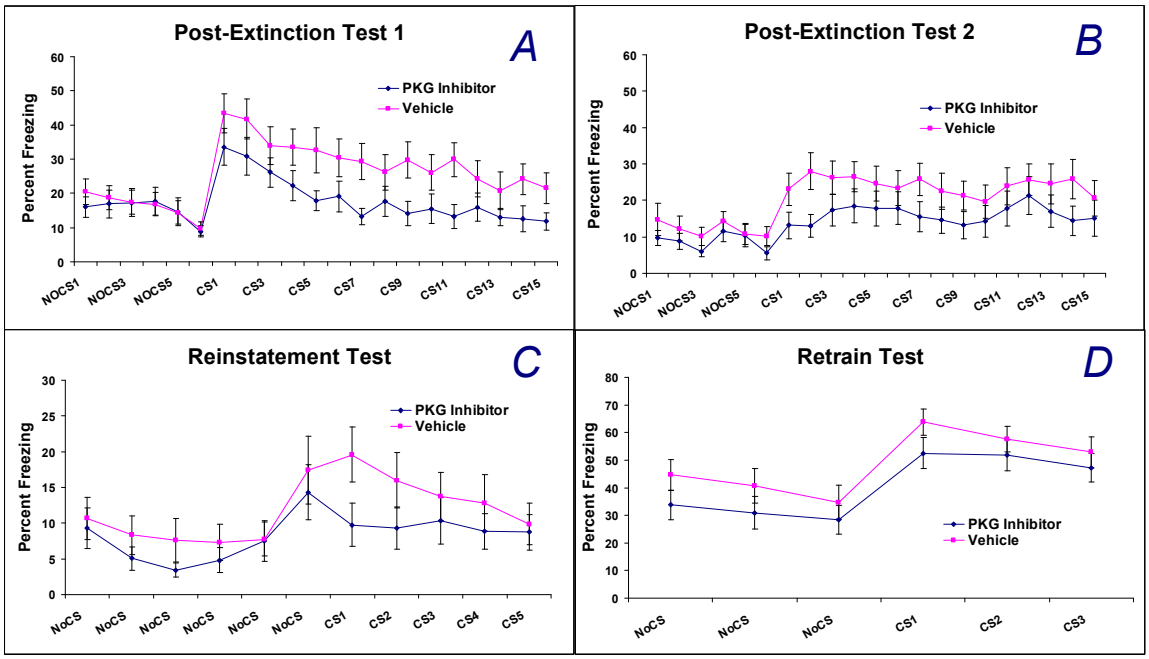


Figure 5: *Rp-8-Br-PET-cGMPS enhances the consolidation of extinction learning.* Data was taken from vehicle and Rp-8-Br-PET-cGMPS groups in all three cohorts: n=26 for drug group, n=28 for vehicle group. Dosage was set at 0.5µg/side.

A) *Assessment of tone-induced freezing 24 hours after extinction training and drug administration*: the vehicle group shows significantly higher freezing in response to the CS than the group that received drug (p=0.038). **B)** *Tone-induced freezing in a second test given 24 hours after the first*: freezing was still significantly lower in the drug group for the first five trials (p=0.039), and a non-significant trend remains for the overall length of the trial (p=0.086). **C)** *Test of tone-induced freezing 24 hours after one-shock reinstatement training*: fear shown to the tone was still significantly lower in the drug group as measured by freezing to the CS (p=0.031). **D)** *Test for freezing to the CS given 24 hours after animals were retrained according to original fear-conditioning paradigm (5 tone-shock pairings):* the difference between the groups was non-significant, indicating that the capacity for fear consolidation was not impaired by the drug.

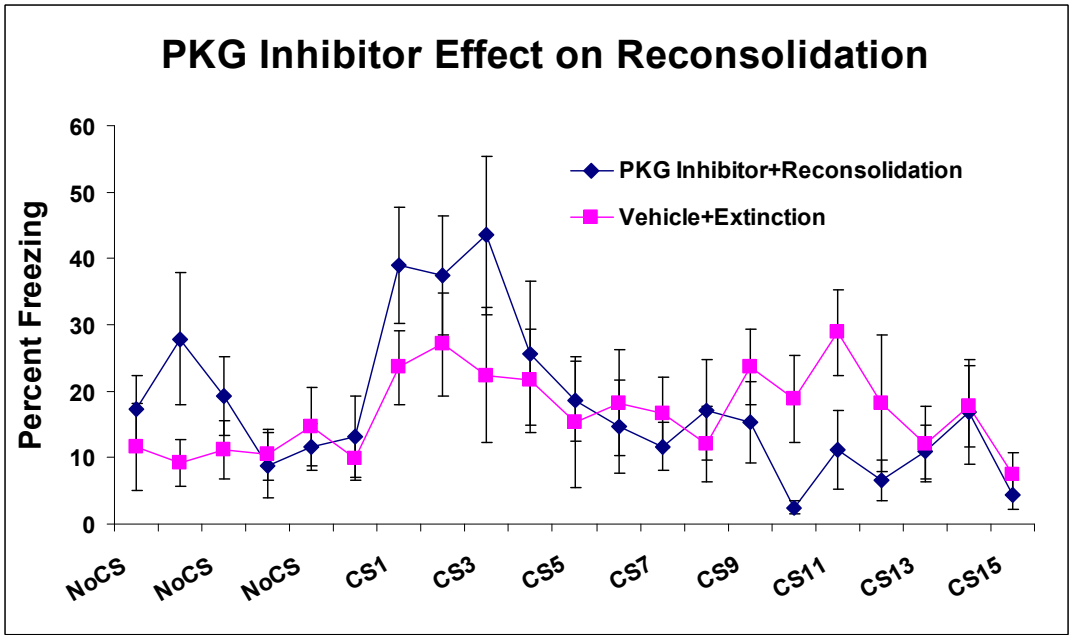


Figure 6: *Retained fear to CS in animals that received Rp-8-Br-PET-cGMPS (0.5μg/side) after one tone-alone reconsolidation trial.*

Mice that were exposed to a trial consisting of only a single tone on the previous day still show estimable freezing to the first few tones compared to a vehicle group that received the full 15-trial extinction training. Drug and vehicle data shown are from Cohort 2 only: n=9 for drug group, n=9 for vehicle group.

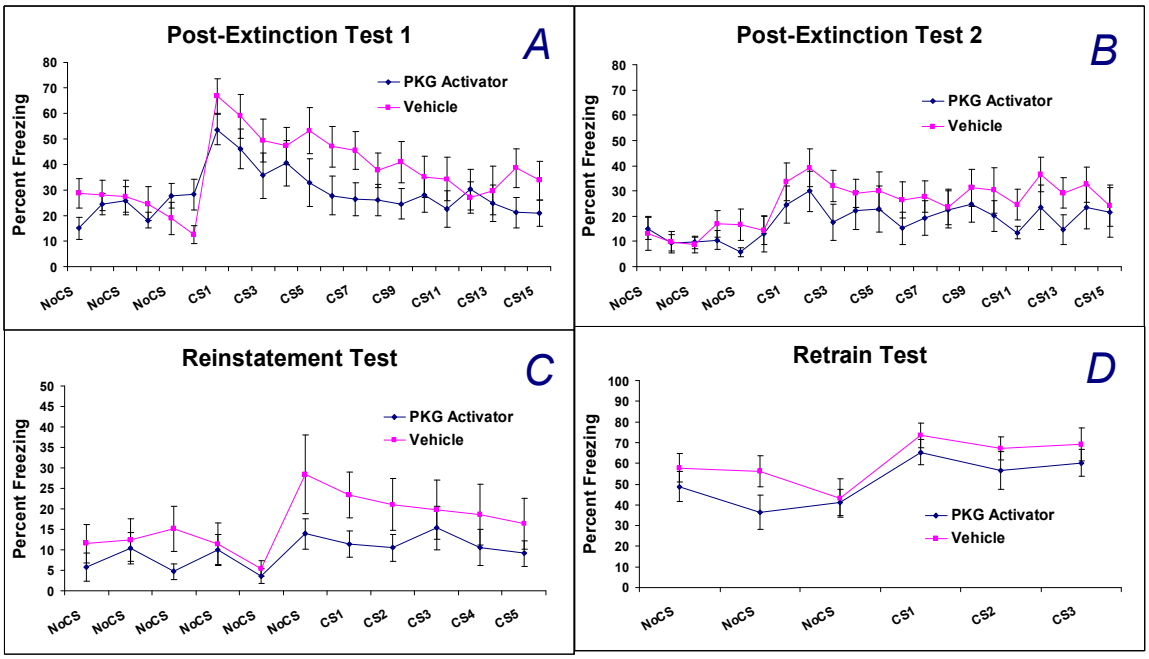


Figure 7: *Non-significant effects on freezing of post-extinction training administration of 8-Br-cGMP*

Data was taken from Cohort 3 only: n=12 for drug group, n=12 for vehicle group. Dosage was set at 0.5µg/side.

A) *First test after extinction training:* the PKG agonist did not produce significant effects (p=0.16), but the drug group did show an unexpected trend towards decreased freezing. **B)** *Second test after extinction training:* non-significant trend (p=0.29). **C)** *Test for freezing to the CS after one-shock reinstatement:* the non-significant trend was preserved (p=0.19), with the drug group appearing to have a stronger retention of learned CS-US dissociation. **D)** *Test for tone-induced freezing 24 hours after retraining:* both groups were still able to consolidate fear acquisition.

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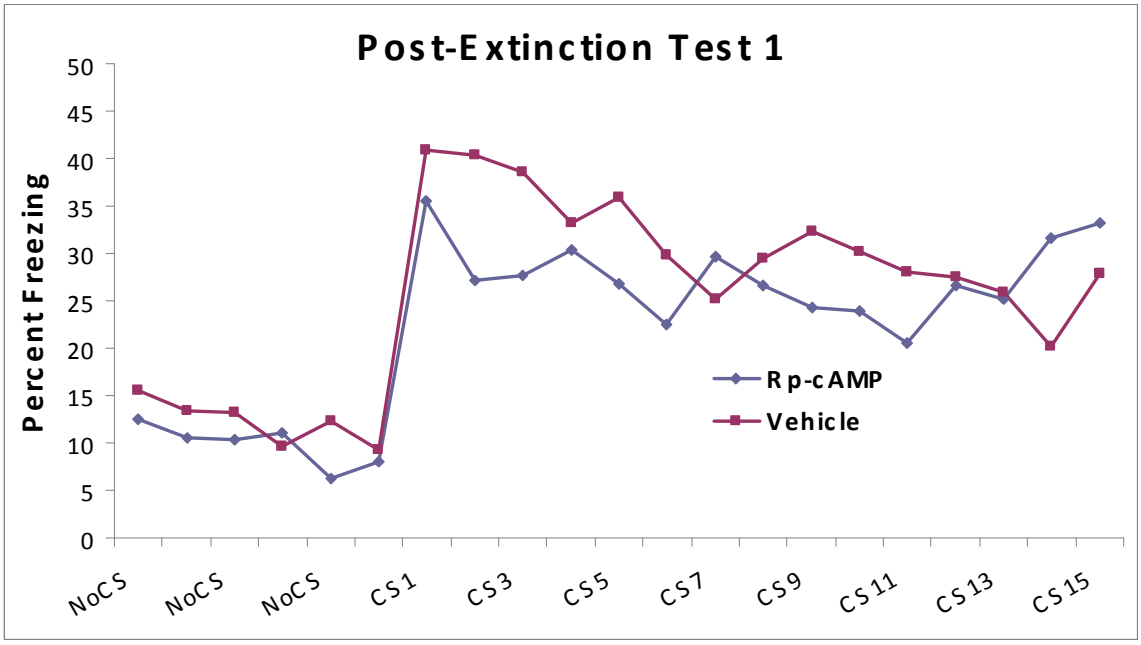
APPENDIX

PKG versus PKA (Figure 8)

Preliminary data collected in a similar paradigm using BLA microinjections of the PKA inhibitor Rp-8-Br-cAMP (an inactive analogue of cAMP) immediately after extinction training did not yield significant results, nor did the i.p. administration of rolipram, a PDE4-specific inhibitor (therefore a cAMP/PKA agonist), when given at several time points before and after extinction training (Zimmermann et al., unpublished data). In this set of experiments, we expected that the PKA inhibitor, which has been shown to interfere with LTP in fear consolidation (Schafe and LeDoux 2000), would enhance extinction, while rolipram, found to enhance the consolidation of fear (Monti et al. 2006), would inhibit extinction. Though adjustment of dosages as well as the time and method of administration is necessary to make an accurate comparison, the negative results of these behavioral experiments offer potential evidence in favor of a largely PKG-mediated effect in extinction training. Despite the lack of significant behavioral changes induced by Rp-8-Br-cAMP or rolipram, there was a stronger trend using the PKA inhibitor, which moderately increased extinction (Figure 8). The PKA agonist, which was expected to decrease extinction, showed only a minimal trend toward increasing freezing in post-extinction training tests. This could again be due to robust levels of PDEs in response to extinction training that neutralize efforts to suppress their action. However, rather than directly agonizing PKA, rolipram functions directly on a cAMP-inhibiting PDE. Considering the aforementioned data that successfully used rolipram to enhance LTP in fear learning (Monti et al. 2006), the minimal effects that we

saw may suggest a role for cAMP-associated PDEs in fear consolidation that may not be as essential in fear extinction. In contrast, the significance we found in the current study by inhibiting PKG may also suggest that the increased PDEs that degrade cGMP are more influential in extinction than those that affect PKA, though experimentation with a cGMP-associated PDE inhibitor (to function with the same basic mechanism of action as rolipram) is necessary to accurately test this theory.



Zimmermann et al., unpublished data

Figure 8: *Preliminary findings with Rp-8-Br-cAMP (10μg/side)*

(Data shown from two waves: n=19 for drug group, n=18 for vehicle group)

Early tests showed a trend towards decreased freezing in the first post-extinction test when animals had received post-extinction training microinjections of a PKA inhibitor, but the findings were not significant.