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Threat bias in mice with inactivating mutations of *Prkar1a*

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Abstract

Anxiety disorders are associated with abnormalities in the neural processing of threat-related stimuli. However, the neurobiological mechanisms underlying threat bias in anxiety are not well understood. We recently reported that a *Prkar1a* heterozygote (*Prkar1a*^{+/-}) mouse with haploinsufficiency for the main regulatory subunit (R1α) of protein kinase A (PKA) exhibits an anxiety-like phenotype associated with increased cAMP signaling in the amygdala. *Prkar1a*^{+/-} mice provide a novel model to test the *direct* effect of altered PKA expression and subsequent anxiety-like behavioral phenotype on the response to threat. We hypothesized that *Prkar1a*^{+/-} mice would exhibit a bias in threat detection since increased amygdala activity during emotional stimuli is associated with a maladaptive response. We measured behavior and PKA activity in brain areas after exposure to predator or control odor exposure in male *Prkar1a*^{+/-} and wild-type (WT) littermates. Indeed, there were significant differences in the behavioral response to threat detection; WT mice showed the expected response of decrease in exploratory behavior during predator vs. control odor exposure, while *Prkar1a*^{+/-} mice did not alter their behavior between conditions. Basal and total PKA activity was independently associated with genotype, with an interaction between genotype and threat condition. *Prkar1a*^{+/-} mice had higher PKA activity in amygdala and ventromedial hypothalamus in response to predator odor. In contrast, WT mice had higher PKA activity in amygdala and orbitofrontal cortex after exposure to control odor.

Dysregulated PKA activity in the amygdala- prefrontal cortex circuitry in *Prkar1a*^{+/-} mice is associated with behavioral phenotype of anxiety and a bias for threat. This is likely related to a failure to inhibit the amygdala response, which is an effect of the genotype. These results suggest that the alteration in PKA signaling in *Prkar1a*^{+/-} mice is not ubiquitous in the brain; tissue-specific effects of the cAMP/PKA pathway are related to threat detection and fear sensitization.

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The authors state they have no conflict of interest.

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Keywords

Protein kinase A; amygdala; orbitofrontal cortex; anxiety; *Prkar1a*; threat detection

INTRODUCTION

Anxiety and fear are defensive responses that are essential to survival. Also important is the ability to attenuate the defensive response when the threat is no longer present. An extensive body of literature has established that pathological anxiety is associated with abnormalities in threat- detection and fear learning (Charney, 2004; Adamec et al., 2005; Kalin et al., 2004; Blanchard et al., 2011). The cAMP/Protein kinase A (PKA) molecular pathway is crucial in the response to fear and fear memory (long- term potentiation (LTP)) (Abel et al., 1997; Bernabeu et al., 1997; Bourtchouladze et al., 1998; Kelly et al., 2007). Activity in neural circuits prior to or immediately after stimuli may influence PKA activity and LTP and therefore affect fear learning and memory (Huang et al., 1992; Cohen and Abraham, 1996; Abel et al., 1997). Studies of *in vivo* manipulation of PKA and protein synthesis have advanced our understanding of the neural processes involved in fear learning and threat detection, however gaps remain in our understanding of the neurobiological mechanisms in psychopathologies, such as anxiety, and response to threat (Abel et al., 1997; Cohen and Abraham, 1996; Huang et al., 1992; Parsons and Davis, 2012).

Transgenic mice with alterations in PKA signaling provide a model to investigate the rodent defensive response. Recently, we reported a mouse model bearing one inactivated allele of the *Prkar1a* gene exhibited an anxiety-like behavioral phenotype associated with increased PKA activity in the amygdala compared to wild type (WT) littermates (Keil et al., 2012). Although *Prkar1a*^{+/-} mice showed a difference in spatiotemporal measures in a test of anxiety (elevated plus maze), no genotype difference was found in risk assessment or exploratory behavior, which suggests that arousal behavior was similar between the *Prkar1a*^{+/-} and WT mice. Risk assessment is an adaptive behavioral pattern (i.e. freeze, flight, defensive attack) associated with the detection and analysis of threat stimuli and related environmental contexts (Blanchard et al., 2011). Prior studies report that the prefrontal cortex - amygdala neural pathway has a significant role in risk assessment and response to threat (Blanchard et al., 2011; Grillon and Charney, 2011; LeDoux, 2000; Pine, 2004; Robinson et al., 2012). Results of our study of *Prkar1a*^{+/-} mice showed no difference in baseline PKA activity in the prefrontal cortex, an area known to have extensive reciprocal connections with the amygdala. This finding suggests that the alteration of PKA activity noted in the amygdala of these mice may be associated with threat bias and an anxiety-like phenotype (trait anxiety) (Blanchard et al., 2011; Keil et al., 2012).

Recent studies on the neural mechanisms of anxiety and threat-bias propose two mechanisms. Robinson et al. (2012) proposes that anxiety significantly increases prefrontal cortex-amygdala connectivity during processing of a threat, which if recruited too readily or often may lead to pathology such as general anxiety disorder or post-traumatic stress disorder (PTSD). An alternate view proposes that the increased threat-bias associated with pathological anxiety may be the result of a failed top-down inhibition of the prefrontal cortex, which results in reduced inhibition of the amygdala aversive response (Rauch et al., 2006; Bishop, 2008; Etkin, 2012; Mogg et al., 1993). In the current study, we used *Prkar1a*^{+/-} mice to test the direct effect of altered PKA expression and resultant anxiety-like behavioral phenotype on the subsequent threat-detection response. (Kirschner et al., 2005). Functionally, loss of *PRKAR1A* is associated with excess PKA signaling in specific tissues so this transgenic mouse provides a model to study the direct effect of increased PKA signaling in neuronal pathways crucial to threat processing. It is likely that disease-related

symptoms are related to long term changes in neuronal function; here, we tested the following hypothesis: chronic elevation of PKA activity in the amygdala and related neural pathways involved in mediating anxiety and fear, which we have shown is associated with an anxiety-like phenotype, will result in a maladaptive behavioral response and atypical activation of these pathways in response to threat exposure.

Predator stress, which is an ethologically relevant form of psychosocial stress, has been used as a model of hyperarousal and threat-related paradigm, with a principal target of the amygdala (Adamec et al., 2006; Dielenberg et al., 2001a; Dielenberg et al., 2001b; Figueiredo et al., 2003; Roseboom et al., 2007). The use of transgenic mice in threat-related paradigms may be useful in elucidating neurobiological mechanisms underlying anxiety and related threat detection/precautionary responses, in particular PTSD. We focused on the extent to which predator odor or control odor (novelty) exposure elicits detection of and response to potential threats in *Prkar1a*^{+/-} mice compared to WT littermates.

EXPERIMENTAL PROCEDURES

Animals

Adult male *Prkar1a*^{+/-} and WT mice (background:129/Sv, C57BL/6,FVB) were housed three to four per cage with *ad libitum* access to food and water and maintained on a 12:12 light schedule (lights on at 0600 h). Throughout the experimental period, the mice were handled (two to three times per week) and weighed (weekly) to acclimate to the investigator. All animal work in this study was carried out in accordance with standards approved by the NIH Guide for the Care and Use of Laboratory Animals. All animal protocols received prior approval at the NIH.

Prkar1a heterozygous mice (*Prkar1a*^{+/-}, which contain one null allele of *Prkar1a*^{A2}) were generated in our laboratory as previously described (Kirschner et al., 2005). R1α haploinsufficiency leads to increased total PKA activity in response to cAMP in addition to increased PKA-II to PKA-I ratio (Griffin et al., 2004a; Griffin et al., 2004b; Amieux et al., 1997; Robinson-White et al., 2006). *Prkar1a*^{+/-} and WT mice were interbred, maintained on a mixed C57BL/6, 129Sv/B6 hybrid background, and post-weaning littermates were reared together in same-sex groups. Three primers (5'-AGCTAGCTTGGCTGGACGTA-3', 5'-AAGCAGGCGAGCTATTAGTTTAT-3' and 5'-CATCCATCTCCTATCCCCCTTT-3') were used for *Prkar1a* genotyping: the WT allele generated a 250 base pair (bp) fragment and the null allele generated an 180 bp product. Primers and conditions for the PCR reactions are available upon request (Kirschner et al., 2005; Nesterova et al., 1996).

Adrenal tumor or corticosterone overproduction was not identified in *Prkar1a*^{+/-} mice. Genotype-specific cardiac and adrenal lesions were not seen in *Prkar1a*^{+/-} mice; however benign and malignant thyroid neoplasias were observed in some older mice. In our hands, there were no differences in basal corticosterone levels between WT and *Prkar1a*^{+/-} mice measured during the light phase and during the transition from light to dark phase (Keil, unpublished data). It was not uncommon for *Prkar1a*^{+/-} mice to develop nonpigmented schwannomas and fibro-osseous bone lesions beginning at approximately six months of age; however, no other physical abnormalities were noted. A few transgenic mice that were used in this study had early tail tumors; however none of the mice used in this study had evidence of thyroid or other tumors. Only male *Prkar1a*^{+/-} and WT mice were tested in these experiments. Standard assessment of neurological function revealed no deficits in *Prkar1a*^{+/-} mice. Specifically, testing revealed no differences in neuromuscular, righting reflex, taste, nociception, olfaction between WT and *Prkar1a*^{+/-} mice (data not shown).

Treatment groups

Mice were randomized into three groups: (1) home cage controls (no odor exposure), (2) control odor (cotton swab with physiological saline), and (3) predator odor (cotton swab with fox urine, Buck Stop Lure, Stanton, MI). All behavioral testing was performed between the hours of 1300-1700 h. Mice were moved to testing room in the home cage 1.5 h prior to experiment to acclimate to the experiment room. In a clean cage with 3 cm of sawdust bedding, two cotton-tipped swabs containing either 300 μ l of fox urine or physiological saline (control odor) were placed in a corner of the cage with the opening of the beaker horizontal to the floor of the cage. The mouse was placed in the cage, the cover was replaced, and behavior was recorded for 5 min with standard room lighting (overhead fluorescent). After 5 min of odor presentation the mouse was placed in a clean cage, which was covered and kept in a quiet area of the test room for 90 min, prior to euthanization by CO₂. The time point of 90 min was chosen to coincide with the expected second peak of PKA activity post-stress (Schafe et al., 2000). Brains were removed and immediately frozen (-80 C) until cryosectioning. Mice in the home cage control group were moved to the testing room in the home cage and kept in a quiet area for 1.5 h to acclimate to the room change prior to euthanization.

Behavioral assays

Four behaviors were scored and summated for the 5-min time in the testing cage: 1) vigilance, defined as the number of times mouse reared on hind legs (without upper extremity weight bearing on the side wall of the cage, vertical position), (2) exploratory, defined as the number of times mouse reared on hind legs (with upper extremity weight bearing on the side wall of the cage, vertical position) (3) number of digging episodes, (4) number of grooming episodes, (5) number of times the mouse touched the beaker containing the swabs. The behavioral parameters selected were based on previous studies as components of behavior that typically change in response to exposure to a predator or predator odor (Blanchard and Blanchard, 1989; Blanchard et al., 2011). Predator odor is associated with a reduction of exploratory activity as well as suppression of non-defensive behaviors (Blanchard and Blanchard, 1989).

PKA assay

Whole brains were sectioned at 250 μ m and punch biopsies (0.5mm diameter stainless steel punch) of tissue from the following brain regions were obtained using the Allen brain atlas of the mouse (Dong, 2008) to identify the following nuclei: basolateral, central, and medial amygdala (BLA, CA, MA, respectively) (corresponding to Figs. 64-70, Bregma -0.955 to -1.555mm); ventromedial and paraventricular hypothalamus, and thalamus (VMH, PVH and T, respectively; corresponding to Figs. 64 and 70, Bregma -0.955 to -1.555mm); and orbitofrontal cortex (OFC); corresponding to Figs. 64 and 70; Bregma -0.955 to -1.555mm). Olfactory bulb and cerebellum (olf and cb, respectively) were dissected in entirety. All dissected tissues were stored in cryotubes at -80 C until assayed.

PKA enzymatic activity was measured following the protocol described earlier (Nesterova et al., 1975). PKA assay was performed in the presence of Kemptide (a highly specific substrate of PKA). Basal levels of PKA activity represent the non-stimulated PKA activity. Total PKA activity reflects the PKA activity after the addition of cAMP, which stimulates the PKA holoenzyme and the catalytic subunit dissociates from the complex, becoming fully active (Nesterova et al., 1981; Clegg et al., 1987), whereas PKA-specific activity is what remains after the application of the PKA-specific inhibitor (PKI). PKA values were normalized by protein content of each sample.

Statistical analyses

The results were analyzed using SPSS software (SPSS version 18, IBM Corp., NY). Data were assessed for normality, outliers, and homogeneity of variances. Data were analyzed for effect of genotype and treatment group by multifactorial ANOVA with Bonferroni *post-hoc* comparisons where appropriate. All values are reported as means $\pm$ SEM. Behavioral measures during the odor exposures were analyzed by a multifactorial ANOVA, with between subject factors of genotype and treatment. To confirm any differences between groups of conditions for PKA data, a two (group: WT, *Prkar1a*^{+/-}) $\times$ three (treatment: predator odor (fox urine)), control odor (novelty (saline)) and home cage (control undisturbed in home cage)) ANOVA was performed, and *post-hoc* comparisons with Bonferroni correction where appropriate. Significance was determined at $p < 0.05$.

RESULTS

Behavioral analysis

There were significant differences in the behavioral response of male *Prkar1a*^{+/-} and WT mice to predator vs. control odor ($F_{2,47} = 9.939$, $p < 0.04$). WT mice showed the expected response of decrease in exploratory behavior (stretch attend posture against wall of cage) during predator vs. control odor ($p < 0.009$). *Prkar1a*^{+/-} mice did not show the expected alterations in behavior (exploratory or defensive) with exposure to different odors compared to WT littermates. ANOVA analysis showed genotype ($F_{1,48} = 15.16$, $p < 0.001$); and threat ($F_{1,48} = 5.066$, $p < 0.03$) effects for digging, with *Prkar1a*^{+/-} engaging in more digging episodes overall compared to WT mice ($p < 0.001$). In addition, *Prkar1a*^{+/-} mice did not alter defensive behavior (digging) with exposure to predator vs. control odor, while WT mice showed the expected decrease in defensive behavior during exposure to control odor (novelty) ($p < 0.01$) (Figure 1). Neither *Prkar1a*^{+/-} nor WT mice showed a change in grooming behavior or vigilance (rearing and sniffing without touching cage wall) during predator vs. control odors. Little, if any stationary/freezing behavior was observed in either WT or *Prkar1a*^{+/-} mice, which is consistent with prior studies of threat exposure in confined environment (Blanchard and Blanchard, 1989; Blanchard et al., 2000) (Data not shown).

PKA activity in controls

To investigate possible differences in anatomical sites associated with loss of one *Prkar1a* allele we measured PKA activity in the brain of WT and *Prkar1a*^{+/-} mice in a home cage (non-stressed) situation ($n = 10 - 18$ /genotype). Data for all parameters are shown in Table 1. The loss of one *Prkar1a* allele led to an increase in basal and cAMP-stimulated kinase activity in the CA ($p < 0.003$ and $p < 0.002$, respectively) and BLA ($p < 0.04$ and $p < 0.012$, respectively), and thalamus ($p < 0.001$) compared to the WT mice ($n = 5 - 12$ per genotype/treatment group). No genotype effect was seen for the other brain or sensory areas measured ($p > 0.10$) (Tables 1, 2).

Amygdala PKA activity: predator vs. control odor exposure

Multivariate AVOVA analysis of basal and total PKA activity in amygdala areas (basolateral, central, and medial) revealed a main effect for genotype ($F_{6,13} = 14.37$, $p < 0.001$), treatment ($F_{12,26} = 3.213$, $p < 0.005$), and a significant interaction for genotype and treatment ($F_{12,26} = 2.73$, $p < 0.014$) with significantly higher PKA activity in *Prkar1a*^{+/-} compared to WT mice. *Post-hoc* comparisons of treatment groups showed significantly higher PKA levels after predator odor exposure in both WT and *Prkar1a*^{+/-} mice in both the CA ($p < 0.05$) and BLA ($p < 0.05$) amygdala versus respective control groups. In the MA, a significant main effect was shown for genotype in basal PKA activity ($p < 0.01$); however there was no treatment effect ($p > 0.10$) (data not shown).

In *Prkar1a*^{+/-} male mice, post-hoc comparison showed a significant difference in basal and total PKA activity in the CA (predator odor vs. home cage, $p<0.03$; $p<0.02$, respectively). Post-hoc comparisons of the BLA showed a significant difference in basal PKA activity (predator odor vs. home cage, $p<0.01$) with a trend for predator vs. control odor, $p<0.056$, and total PKA activity (predator vs. control odor $p<0.012$). Post-hoc comparisons in WT male mice showed a significant difference in CA basal PKA activity (predator and control odor vs. home cage, $p<0.013$, $p<0.041$, respectively); and total PKA activity (control odor vs. home cage, $p<0.013$). In the BLA post-hoc comparisons showed significant differences in basal PKA activity (control odor vs. home cage, $p<0.016$) with a trend for predator odor vs. home cage, $p<0.057$; and total PKA activity (control odor vs. home cage, $p<0.014$) (Figures 2).

Hypothalamic and thalamic nuclei PKA activity: predator vs, control odor exposure

Results of multivariate ANOVA analysis of PKA activity in thalamus (T) and ventromedial hypothalamus (VMH) areas revealed a statistically significant main effect for treatment group ($F_{12, 8} = 6.562, p<0.01$); a trend for genotype ($F_{6, 3} = 7.295, p<0.06$); and a significant interaction for genotype and treatment ($F_{12, 8} = 4.906, p<0.02$). Genotype and treatment effect was shown for total PKA activity in the T and VMH ($F_{1, 14} = 6.083, p<0.01$; $F_{1, 14} = 25.63, p<0.001$; $F_{2, 14} = 8.838, p<0.01$; $F_{2, 14} = 12.259, p<0.004$; respectively), with higher PKA activity in *Prkar1a*^{+/-} mice and significantly higher PKA activity in treatment-exposed groups compared to home cage controls.

In *Prkar1a*^{+/-} male mice, post-hoc comparison showed a significant difference in VMH basal PKA activity (predator odor vs. home cage, $p<0.05$) and total PKA activity (predator and control odors vs. home cage, $p<0.001$). Post-hoc comparison in WT mice showed a significant difference in VMH basal (predator and control odors vs. home cage, $p<0.001$) and total PKA (predator odor vs. control odor and home cage ($p<0.001$) (Tables 1, 2).

PKA activity in other brain areas: predator vs. control odor exposure

ANOVA analysis showed a significant main effect for treatment ($p<0.001$; $F_{2, 20} = 8.057$) and genotype ($F_{1, 20} = 6.86, p<0.013$;) in basal and total PKA activity in the OFC. In WT mice, significant differences were shown in basal PKA activity (predator odor vs. home cage, $p<0.03$) and total PKA activity (control odor vs. home cage, $p<0.004$) in the OFC, whereas in *Prkar1a*^{+/-} mice no treatment effect was found. ANOVA analysis showed a significant main effect for treatment in basal and total PKA activity in olf ($F_{2, 16} = 7.811, p<0.004$; $F_{2, 16} = 6.636, p<0.008$; respectively); importantly, no genotype effect was found. Post-hoc comparisons showed a significant difference for basal and total PKA activity in olf between predator odor vs. home cage controls in both WT and *Prkar1a*^{+/-} mice ($p<0.05$) (Tables 1, 2).

DISCUSSION

Behavioral responses to threat-detection

Previously we reported that mice with the loss of one *Prkar1a* allele had elevated PKA activity in the amygdala that was associated with an anxiety-like phenotype (Keil et al., 2012). Therefore, we hypothesized that *Prkar1a*^{+/-} mice would exhibit an atypical response to threat detection since increased amygdala activity during emotional stimuli is associated with a maladaptive response (Goosens and Maren, 2004; Fecteau et al., 2007; Phelps and LeDoux, 2005). As predicted, *Prkar1a*^{+/-} mice failed to exhibit behavioral changes (exploratory or defensive) to distinguish between predator versus control odor exposure, which was an effect of the genotype. The behavioral response of the WT mice confirmed prior findings of laboratory and field studies that exposure to predator odor would reduce

exploratory and non-defensive behaviors (Blanchard and Blanchard, 1989; Farmer-Dougan et al., 2005; Dielenberg et al., 2001a), thus providing an important control for this study. Therefore, the behavioral response of *Prkar1a*^{+/-} mice is consistent with the “prediction error” hypothesis of anxiety disorders, specifically a deficit in the ability to discriminate between an ambiguous versus a predator threat.

PKA activity in treatment groups

While PKA activity is ubiquitous in the brain, results from this study confirm our previous findings that significant differences were found specifically in the central and basolateral amygdala and thalamus in *Prkar1a*^{+/-} compared to WT (Keil et al., 2012). Since these brain regions are known to have a crucial role in regulating the anxiety response, we speculate that PKA is important for mediating the anxiety phenotype described in *Prkar1a*^{+/-} mice due to the imbalance of excitation/inhibition in the neuronal network underlying pathological anxiety (Wu et al., 2008).

In the present study, PKA activity increased in response to threat detection (predator odor) for both WT and *Prkar1a*^{+/-} mice in the BLA and CA, T, VMH, OFC, and olf. However, an interesting difference in the pattern of PKA activity response was found only in specific brain areas when compared between WT and *Prkar1a*^{+/-} mice. For WT mice, basal and total PKA activity was higher in the amygdala and OFC after exposure to control odor (novelty), whereas total PKA activity was higher in the VMH and T after predator odor. These results parallel the behavioral response of WT mice that showed a decrease in defensive and increase in exploratory behavior during control odor compared to predator odor exposure. In contrast, *Prkar1a*^{+/-} mice showed higher PKA activity in the amygdala after predator odor exposure compared to home cage controls, and no difference in PKA activity in OFC between predator odor compared to control odor (although levels in treatment groups were higher than home cage controls). These results also parallel the behavioral response of *Prkar1a*^{+/-} mice where no change in exploratory or defensive behavior was detected between control odor (novelty) or predator odor exposure. Also, in *Prkar1a*^{+/-} mice total PKA activity in the VMH was higher in control odor (novelty) compared to the predator odor group, which is contrast to the findings with the WT mice. These findings suggest that the loss of one *Prkar1a* allele results in specific neurobiological modifications in brain areas with a crucial role in the integration and expression of innate fear responses to predatory threat.

The significant difference found in the pattern of response in PKA activity between WT and *Prkar1a*^{+/-} mice in the OFC, and VMH is congruent with the crucial roles of the VMH in defensive response and the prefrontal cortex control of the amygdala. PKA signaling in *Prkar1a*^{+/-} mice occurs in a tissue-specific manner and results of this study suggest that unchecked PKA activity in the amygdala is associated with anxiety, which is a threat-bias disorder, and subsequent maladaptive response to threat detection. Recent studies report that anxiety can promote the detection of and defensive response towards fearful stimuli (Blanchard et al., 2011; Keil et al., 2012; Bishop, 2008; Rauch et al., 2006). Proposed mechanisms include anxiety potentiated coupling of the prefrontal cortex-amygdala or failure of prefrontal cortex inhibition of the amygdala (Robinson et al., 2012; Grillon and Charney, 2011). Our findings suggest that a maladaptive threat response is associated with failure of “top down” inhibition of the amygdala by the prefrontal cortex. This is demonstrated by the finding in WT mice of an increase in PKA in the PFC activity during control odor (novelty) exposure with a parallel decrease in defensive behavioral response compared to predator odor exposure. However, in *Prkar1a*^{+/-} mice, no difference was shown in PKA activity in the PFC between control odor (novelty) or predator odor exposure, which parallels the behavioral findings and is consistent with prior studies that report increased amygdala activity during emotional stimulation is associated with maladaptive response to threat (McEwen, 2007).

Electrophysiological studies suggest that changes in the amygdala circuitry and dendritic morphology affect fearful responses and highly correlates with basolateral amygdala transmission, and that the degree of anxiogenic effect of predator stress is positively correlated with the degree of potentiation of amygdala circuitry (Vyas et al., 2004; Adamec et al., 2004; Adamec et al., 2005; Adamec et al., 2006). Recent studies provide evidence to support the major role of the prefrontal – amygdala circuitry in the behavioral response to threat, such that the circuit is more positively coupled in anxious vs. non-anxious conditions (Robinson et al., 2012; Blanchard et al., 2011; Eilam et al., 2011). In our study, the behavioral response to novelty in the WT mice is consistent with a “top-down” inhibition of the amygdala, whereas the response of *Prkar1a*^{+/-} mice is not.

Alterations in PKA activity in the OFC may also contribute to the atypical response to threat detection in *Prkar1a* heterozygote mice since a key function of the OFC is to inhibit prepotent (habitual) behaviors and promote task relevant operations (Roberts and Wallis, 2000). Results of our study provide evidence that the loss of one allele of the regulatory subunit of PKA is associated with increased PKA activity in the amygdala and importantly no change in PKA activity in the orbitofrontal cortex between threat conditions, as seen in WT littermates. Our results are congruent with other studies, which note that dysfunction in OFC – amygdala neural pathways are associated with atypical behavioral response to threat detection and subsequent increased vulnerability to development of psychopathology (Arnsten and Rubia, 2012; Yuen et al., 2012; Graybeal et al., 2012; Arnsten, 2009). Our findings suggest that increased PKA signaling in *Prkar1a*^{+/-} mice is associated with anxiety and hyperarousal symptoms and failure to extinguish fear response (Blanchard et al., 1979; Kalin et al., 2004). These results are consistent with prior studies of transgenic mice with increased cAMP signaling that report an association with anxiety-like behavior (Favilla et al., 2008; Kelly et al., 2007; Zhang et al., 2008).

It is interesting to note that PKA is a naturally occurring holoenzyme containing two regulatory (R) and two catalytic (C) subunits. The R subunits have 4 main isoforms (RI α , RI β , RII α and RII β) that exhibit major differences in the tissue distribution, biochemical and physical properties encoded by four genes. Studies of PKA gene expression in the adult mouse brain report that RI α and RI β mRNA co-localize in most brain areas, while RII α and RII β mRNA are expressed only in very discrete brain areas. Ca mRNA expression, similar to RI α and RI β , shows preferential hybridization in the neocortex, caudate-putamen, amygdala, hypothalamus, thalamus, and hippocampus, while C β has a distinct pattern of reduced level of hybridization in the thalamus and pyramidal cell layers CA1-3 of the hippocampus (Cadd and McKnight, 1989; Ventra et al., 1996; Mucignat-Caretta and Caretta, 2002). Since PKA activity is affected by and affects various neurotransmitter systems (i.e. GABAergic, glutamergic, serotonergic, noradrenergic, dopamine, gamma-aminobutyric acid, and corticotropin releasing hormone systems), it is possible that the interaction with specific neurotransmitter systems may result in the anxiety disorder observed (Brandon et al., 1997; Wu et al., 2008). Therefore we speculate that disruptions in the PKA pathway in *Prkar1a*^{+/-} mice lead to abnormal excitability of the neuronal pathway, leading to aberrant anxiety responses. The *Prkar1a*^{+/-} mouse, along with knockouts of specific R and C subunits will allow us to dissect the dysregulation leading to these observations.

CONCLUSION

Non-pathological anxiety is adaptive and functions to facilitate sensory processing in order to rapidly activate defense mechanisms to promote survival. Recent studies in rodents support the crucial role of the medial prefrontal cortical (prelimbic)-amygdala circuit in behavioral response to threat (Sierra-Mercado et al., 2011; Robinson et al., 2011; Grillon and Charney, 2011; Rauch et al., 2006) via top-down regulation of the amygdala by the

PFC. Results of our study provide evidence to support that dysregulated PKA activity in the amygdala- prefrontal cortex circuitry is associated with behavioral phenotype of anxiety and sustained bias for threat likely related to failure to “top-down” inhibit the response in non-threatening encounters. In support of the crucial role of PKA activity in anxiety, we recently reported that transgenic mice with a null allele for the C-alpha subunit of PKA had no differences in PKA activity in the amygdala or measures of anxiety-like behavior compared to WT littermates (Briassoulis, 2011). This suggests that the finding of increased anxiety-like behavior noted in *Prkar1a*^{+/-} mice may be related to the increased activity of the catalytic subunit of PKA.

Our results parallel findings from human neuroimaging studies, which have shown enhanced responsiveness of the amygdala and attenuated responsiveness of the prefrontal cortex in patients with PTSD, major depressive illness, and Cushing's syndrome (Maheu et al., 2008; Roberson-Nay et al., 2006; Pine et al., 2004; Merke et al., 2005; Hariri et al., 2002; Bourchouladze et al., 1998). Elucidation of the neurobiological mechanisms in the response of the amygdala to unconditioned fear is relevant to understanding the pathophysiology underlying endogenous stress-related disease in order to develop novel targets for treatment of anxiety and prevention of chronic PTSD. Neuropsychological assessment of patients with germline *PRKARIA* testing is in process, however, results are confounded by co-morbidities in this patient population (i.e. chronic endocrine, cardiac, other). Preliminary data from patients show an increased incidence of early history of learning or attention problems, and increased prevalence of anxiety, depression, bipolar disorder, or obsessive-compulsive disorder (Stratakis, unpublished data).

In summary, results from this study suggest that the effect of down-regulation of the regulatory subunit of PKA is localized to brain areas involved in the processing of emotionally salient stimuli, including the BLA, CA, VMH, and OFC. This suggests that the chronic dysregulation of PKA activity in these areas in *Prkar1a*^{+/-} compared to WT mice result in specific neurobiological modification of behaviors involved in anxiety and fear response. These results support our prior findings that increased PKA activity noted in *Prkar1a*^{+/-} mice is related to a behavioral phenotype of anxiety leading to dysregulated threat-detection and precautionary behavior (Keil et al., 2012). An amygdala-OFC localized effect on response to threat-detection associated with the loss of one *Prkar1a* allele is consistent with extensive data on the importance of the function of the amygdala- OFC neural pathway as a mediator of emotional arousal and threat-detection.

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Highlights

- *Prkar1a* mice exhibit an anxiety-like phenotype associated with increased Protein kinase A (PKA) activity in the amygdala.
- *Prkar1a* mice are a model to investigate direct effect of increased PKA activity in the amygdala and response to threat.
- *Prkar1a* mice did not alter behavior between predator odor vs. control, associated with parallel responses in PKA activity.
- Dysregulated PKA activity in the amygdala-prefrontal cortex is associated with an anxiety phenotype and a bias for threat.

Figure 1.

Behavioral responses of WT and *Prkar1a*^{+/-} mice: comparison of the number of exploratory (vertical postures) vs. defensive (digging) episodes in WT and *Prkar1a*^{+/-} mice during predator odor versus control odor exposure; a genotype- ($p < 0.001$) and stressor-specific ($p < 0.02$) effect for digging, with *Prkar1a*^{+/-} engaging in more digging episodes compared to WT mice; WT mice showed the expected response of decrease in exploratory behavior (vertical postures) during predator (fox urine) vs. control odor (novelty) exposure ($p < 0.02$); however, *Prkar1a*^{+/-} mice did not show alterations in behavior (exploratory or defensive) with exposure to different stressors (predator vs. control odor). * = genotype difference and ^ = treatment difference ($p < 0.05$).

Figures 2.

PKA activity in amygdala areas of WT and *Prkar1a*^{+/-} mice: comparison of basal (-cAMP) (Figure 2a) and total (+cAMP stimulated) (Figure 2b) activity in central amygdala and basolateral amygdala of WT and *Prkar1a*^{+/-} mice: home cage control (non-stressed) vs. control odor (saline) vs. predator odor exposed; overall, significantly higher PKA activity in *Prkar1a*^{+/-} vs. WT and significantly higher PKA levels after predator odor vs. control odor in both genotypes. *, p<0.05 WT vs *prkar1a*^{+/-}. *, p<0.05.

ANOVA post-hoc comparisons of basal (-cAMP) and total (+cAMP)-stimulated PKA activity in **WT mice** in various brain/sensory areas: home cage (no odor) vs control odor (saline) vs. predator odor (fox urine) exposed

Table 1

	Basal PKA activity (- cAMP Normalized PKA/mcg protein) Mean $\pm$ SEM				Total PKA activity (+ cAMP Normalized PKA/mcg protein) Mean $\pm$ SEM			
	Home cage	Control odor	Predator odor	p-value	Home cage	Control odor	Predator odor	p-value
Central amygdala	100 $\pm$ 54	2689 $\pm$ 697 [*]	3091 $\pm$ 752 [*]	0.04 [*]	2039 $\pm$ 536	14302 $\pm$ 4075	9971 $\pm$ 1522	0.01 [*]
Basolateral amygdala	171 $\pm$ 82	3241 $\pm$ 601 [*]	2397 $\pm$ 765 [*]	0.01 [*]	2639 $\pm$ 819	19344 $\pm$ 4827 [*]	12814 $\pm$ 3313	0.01 [*]
Medial amygdala	532 $\pm$ 216	1047 $\pm$ 198	942 $\pm$ 157	NS	7522 $\pm$ 1789	21100 $\pm$ 1958	13762 $\pm$ 2156	NS
Ventromedial hypothalamus	383 $\pm$ 81	2421 $\pm$ 275 [*]	2211 $\pm$ 417 [*]	0.0001 [*]	2205 $\pm$ 403	3438 $\pm$ 1598	16922 $\pm$ 1903 [*]	0.0001 ^{^*}
Paraventricular hypothalamus	211 $\pm$ 92	2585 $\pm$ 501 [*]	2398 $\pm$ 630 [*]	0.05 [*]	681 $\pm$ 349	15795 $\pm$ 4042	2398 $\pm$ 3034 [*]	0.04 [*]
Thalamus	52 $\pm$ 11	1039 $\pm$ 77	2498 $\pm$ 1038 [*]	0.02 [*]	1235 $\pm$ 591	10806 $\pm$ 2469	31820 $\pm$ 8226 [*]	0.001 ^{^*}
Orbitofrontal cortex	91 $\pm$ 30	921 $\pm$ 336	1075 $\pm$ 421 [*]	0.03 [*]	522 $\pm$ 110	14451 $\pm$ 4921 [*]	8907 $\pm$ 3526	0.004 [*]
Cerebellum	375 $\pm$ 131	173 $\pm$ 47	391 $\pm$ 77	NS	743 $\pm$ 244	469 $\pm$ 127	1199 $\pm$ 249	NS
Olfactory	194 $\pm$ 53	424 $\pm$ 98	1356 $\pm$ 552 [*]	0.003 [*]	2211 $\pm$ 598	4562 $\pm$ 1555	7893 $\pm$ 2009 [*]	0.007 [*]

* treatment group(s) vs. home cage controls

[^] predator odor vs. control odor

Table 2

ANOVA post-hoc comparisons of basal (-cAMP) and total (+cAMP)-stimulated PKA activity in *Prkar1a^{+/Δ}* mice in various brain/sensory areas: home cage controls (no odor) vs. control odor vs. predator odor exposed.

	Basal PKA activity (- cAMP Normalized PKA/mcg protein)			Total PKA activity (+ cAMP Normalized PKA/mcg protein)		
	Home cage	Control odor	Predator odor	Home cage	Control odor	Predator odor
Central amygdala	702±144	7771±1812	17285±3830 *	6712±679	62773±19142	106565±18383 *
Basolateral amygdala	1017±417	7184±1042	17550±3198 *	7757±1289	44739±7985	73862±12554 *
Medial amygdala	2285±867	1495±336	2412±821	11991±1502	25231±7308	16443±3972
Ventromedial hypothalamus	720±195	4585±1612	5703±1442 *	3386±1058	49189±4622 *	36546±3783 *
Paraventricular hypothalamus	293±72	3879±1907	6173±2069	2434±987	28089±11441	25221±4208
Thalamus	507±87	2700±1324	8760±3459	5491±725	55101±6252 *	69656±13023 *
Orbitofrontal cortex	117±25	4987±2620	4680±1255	897±174	29659±14148	33944±9654
Cerebellum	158±44	254±22	295±41	644±128	744±18	733±260
Olfactory	192±39	653±151 *	590±42 *	1098±123	3748±549	7876±2707 *

^ predator odor vs. control odor

*
treatment group(s) vs. home cage controls