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Anxiety phenotype in mice that overexpress protein kinase A

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Summary The role of cyclic adenosine monophosphate/protein kinase A (cAMP/PKA) signaling in the molecular pathways involved in fear and memory is well established. Prior studies in our lab reported that transgenic mice with an inactivating mutation in *Prkar1a* gene (codes for the 1-alpha regulatory subunit (R1α) of PKA) exhibited behavioral abnormalities including anxiety and depression. In the present study, we examined the role of altered PKA signaling on anxiety-like behaviors in *Prkar1a*$^{+/-}$ mice compared to wild-type (WT) littermates. The elevated plus maze (EPM) and marble bury (MB) tests were used to assess anxiety-like behavior. The hotplate test was performed to evaluate analgesia. We further examined the impact of the *Prkar1a* inactivating mutation on PKA activity in specific nuclei of the brain associated with anxiety-like behavior. Results for the MB test showed a genotype effect, with increased anxiety-like behavior in *Prkar1a*$^{+/-}$ mice, compared to WT littermates ($p < 0.05$). MANOVA analysis showed a significant genotype difference in anxiety-like behavior in the EPM between WT and *Prkar1a*$^{+/-}$ mice on combined dependent variables (open arm time and open to total time ratio; $p < 0.05$). Results of hotplate testing showed no genotype effect however; the expected sex difference was noted. Analysis of PKA activity showed the loss of one *Prkar1a* allele led to an increase in basal and cAMP-stimulated kinase activity in both the basolateral and central amygdala. These results suggest that the alteration in PKA signaling in *Prkar1a*$^{+/-}$ mice is not a ubiquitous effect; and supports the importance of cAMP/PKA pathway in neurobiological processes involved in anxiety and fear sensitization.
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1. Introduction

The hypothalamic pituitary adrenal (HPA) axis and the autonomic nervous system have been the primary foci of research to elucidate the neurobiological mechanisms involved in the stress response. Typically the stress response has been identified as a "fight or flight" reaction, but may also include an increased state of vigilance, which is often accompanied by increased anxiety. Prolonged or repeated exposure to stress often results in physiological, psychological, and behavioral morbidities (i.e. anxiety, depression, post-traumatic stress disorder) in animals and humans. Anxiety is a normal response to a potentially threatening situation, which serves a physiological protective function. However, behavioral flexibility is advantageous in many situations, and this ability may be impaired in anxiety disorders (Schiller et al., 2008). Anxiety is considered pathological when there is a bias to interpret ambiguous situations as threatening, with behavioral responses of avoidance or exaggerated reactions to potential threats (Wood and Toth, 2001).

There is ample evidence to support the role of various neurotransmitters systems in the pathophysiology of anxiety and stress-activated behaviors (y-aminobutyric acid, serotonin, epinephrine, dopamine, corticotrophin-releasing factor, cholecystokinin, and neuropeptide Y), and pharmacological agents are targeted to these systems to treat anxiety disorders. Recently, transgenic mice with an anxiety-like phenotype were developed by targeted inactivation of genes associated with either chemical or neuronal signaling between neurons. This work has identified two groups of anxiety-relevant molecules, one that is involved with neuronal development and cell-to-cell communication (neurotrophic-type molecule) and the other with the regulation of intracellular signaling and gene expression (Wood and Toth, 2001). Research using mice with targeted inactivation of genes related to these molecules are valuable tools to provide insight regarding how abnormal development and/or function of neuronal networks affect the manifestation of anxiety disorders.

The role of cAMP/PKA signaling in molecular pathways involved in fear and fear memory is well established. PKA is a naturally occurred holoenzyme known to have a critical role in anxiety and formation of fear memories. PKA is a four-membered structure with two regulatory (R) and two catalytic (C) subunits. The R subunit isoforms exhibit major differences in the tissue distribution, biochemical and physical properties encoded by four genes (RI-Alpha, RI-Beta, RII-Alpha and RII-Beta) (Tasken et al., 1993). Several research groups have reported CNS effects in knock-out models for RI-Beta (Brandon et al., 1995, Huang et al., 1995), RII-Alpha (Rao et al., 2004) and RII-Beta (Brandon et al., 1998), as well as catalytic unit knockouts (Huang et al., 1995; Qi et al., 1996; Howe et al., 2002). RI-Alpha ($R1a^{-/-}$), RII-Alpha($RIIa^{-/-}$), & R1-Beta ($R1B^{-/-}$), – subunit deficient mice were shown to have reduced cAMP – stimulated (and PKI-inhibited) PKA activity, while only the RI-Alpha ($R1a^{-/-}$) mice showed significantly increased baseline (non cAMP-stimulated, but PKI inhibited) PKA activity. The absence of a severe phenotype in RII-Alpha, RII-Beta and R1-Beta, but not in RI-Alpha ($R1a^{-/-}$) deficient mice supports the essential role of the R1-Alpha regulatory subunit on maintaining the catalytic subunit under cAMP control. (Adams et al., 1997; Planas et al., 1999; Cummings et al., 1996; Schreyer et al., 2001; Brandon et al., 1998; Amieux et al., 1997; Burton et al., 1997, 1999).

Studies with Gsalpha transgenic mice have shown that increased cAMP signaling is associated with an anxiety-like phenotype (Favilla et al., 2008). Zhang et al. (2008) recently reported that mice with reduced phosphodiesterase 4B activity, the enzyme that degrades cAMP and interrupts the negative feedback of PKA pathway resulting in increased PKA activity, displayed anxiogenic behavior. In addition, transgenic mice with overexpression of the striatially enriched cAMP-producing adenyl cyclase 5, showed increased anxiety-related behavior (Kim et al., 2008). Results of the studies reviewed above indicate that increased cAMP signaling is associated with an anxiety-like phenotype, and provide indirect evidence that an increase in PKA activity may be associated with an increased risk for anxiety.

Prior studies in our lab showed that transgenic mice with a down-regulated *Prkar1a* gene (tTA/X2AS, antisense transgene, codes for the 1-alpha regulatory subunit (R1α) of PKA) (Griffin et al., 2004a) exhibited behavioral abnormalities, including anxiety (Batista et al., 2005) and depression. A knockout mouse heterozygous for a null allele of *Prkar1a* was recently developed in our lab as a model to investigate Carney complex (Kirschner et al., 2005). Carney complex is an autosomal dominant multiple neoplasia syndrome that is associated with inactivating mutations of the PRKAR1A gene, resulting in a net increase in PKA activity.

The null allele functionally results in increased PKA signaling. It is likely that disease-related symptoms are related to long term changes in neuronal function, therefore a transgenic mouse model with down-regulation of *Prkar1a* provides a research tool to test for the first time the effect of altered PKA expression on anxiety-like behavior. The objective of this study was to examine the role of altered PKA signaling on anxiety-like behaviors using behavioral assays known to be sensitive to anxiolytics in *Prkar1a* mice (HZ) compared to wild-type (WT) littermates. In addition, we measured the expression in PKA activity (basal and cAMP stimulated) in various brain areas in $Prkar1a^{+/-}$ mice and WT control littermates to evaluate possible associations of altered PKA activity with the behavioral phenotype. The inclusion of a nocioceptive assessment is important to in the behavioral phenotype of the *Prkar1a* knock-out mouse since the amygdala is known to have a key role in the emotional–affective dimension of pain as well as anxiety behavior.

2. Methods

2.1. Animals

All mice were housed three to four per cage with same-sex littermates with *ad libitum* access to food and water and maintained on a 12:12 light schedule (lights on at 0600 h). All animals were adults at the time of testing (2–10 months old). Throughout the entire experimental period, the mice were handled and weighed to acclimate to the investigator. All animal procedures were conducted in accordance with the standards approved by the NIH Guide for the Care and Use of Laboratory Animals. All animal protocols received prior approved at the NIH.

$Prkar1a^{+/-}$ mice (which contain one null allele of $Prkar1a^{\Delta 2}$) were previously generated in our laboratory

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(Kirschner et al., 2005). R1α haploinsufficiency leads to increased total PKA activity in response to cAMP in addition to increased PKA-II to PKA-1 ratio (Griffin et al., 2004a,b; Amieux et al., 1997; Robinson-White et al., 2006). All mice were bred into a mixed C57BL/6 129Sv/B6 hybrid background to generate *Prkar1a*$^{+/-}$ and control (wild type, WT) mice were used from the same litters. Adrenal tumor or cortisol overproduction was not identified in Prkar1a knock-out 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 (Kirschner et al., 2005). Standard assessment of neurological function revealed no deficits in *Prkar1a*$^{+/-}$ mice. It was not uncommon for *Prkar1a*$^{+/-}$ mice to develop nonpigmented schwannomas and fibro-osseous bone lesions beginning at approximately 6 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.

Heterozygous (*Prkar1a*$^{+/-}$) and wild-type (WT) males and females were tested with various behavioral assays in order to characterize the phenotype of *Prkar1a*$^{+/-}$ mice. All behavioral testing was performed between the hours of 1300–1700 h. One behavioral test per day was performed, with a span of at least 2 days between tests. The order of behavioral tests performed was randomly distributed, since confounding effects have not been reported for EPM, marble bury, and hotplate tests (Espejo, 1997a). Two scorers performed behavioral testing and scoring of results in a blinded fashion.

2.2. Genotyping analysis

Initial genotyping of founders was by polymerase chain reaction (PCR of tail DNA) that was then used to genotype our mice containing one null allele (*Prkar1a*$^{\Delta 2}$) and those with the NEO cassette within *prkar1a* gene: three primers (5′-AGCTAGCTTGGCTGGACGTA-3′, 5′-AAGCAGGCGAGCTATTAGTTTAT-3′ and 5′-CATCCATCTCCTATCCCCTTT-3′) were used for *prkar1a* genotyping: the WT allele generated a 250 bp fragment and the null allele generated an 180 bp product (Kirschner et al., 2005).

2.3. Measurement of anxiogenic-like behavior

Marble bury test was performed as previously described (Treit et al., 1981; Njung'e and Handley, 1991; Broekkamp et al., 1986). Mice were transported in their home cages to the testing room 2 h prior to acclimate to the room prior to the experiment. Standard rodent sawdust bedding was placed in standard mouse cages (38 cm × 22 cm × 16 cm) and eight dark colored marbles were placed on top of the bedding in two evenly spaced rows and the cage was closed with standard lid. No food or water was present during the 30-min test period. Lights were turned off in the room and after 30-min the number of marbles buried (>2/3 marble covered by bedding material) was recorded.

Elevated plus maze (EPM) testing was performed as described previously (Pellow et al., 1985; Lister, 1987). Mice were transported in their home cages to the testing room 2 h prior to acclimate prior to testing. The EPM consists of two open arms (30 cm × 5 cm) and two enclosed arms (30 cm × 5 cm), with end and side-walls (15 cm height), and a center platform (5 cm × 5 cm). The maze was raised to a height of 38 cm above the counter and illuminated (100 lux) from above. The mouse was placed in the center area of the EPM, facing an open arm, and allowed to explore the maze for 5 min. Tests were video recorded and analyzed by ANY-maze software© (Stoelting Co., Wood Dale, IL, USA). Arm entry was defined as all four paws in an arm or center area. After 5 min, the mouse was removed from the EPM, the number of boli recorded, the maze cleaned with 70% ethanol and allowed to dry prior to testing the next mouse. In addition, hand scoring was performed to validate time and entries into arms, as well as record risk assessment behavior (calculated by dividing number of protected stretch attend postures by total closed arm time) and exploratory behavior (head dips). Measures scored included: open and closed arm time, open and closed arm entries, open to total time ratio (open arm time/open arm time + closed arm time), number of head dips, and number of protected stretch attend postures (defined as two hind feet remaining in closed arm while the mouse elongated its head and shoulders, followed by retraction), and risk assessment ratio (number of protected stretch attend postures/amount of closed arm time). Time spent in center area of the maze was not counted (Weiser et al., 2009).

The number of closed arm entries is used as a measure of locomotor activity. A video recording device and automated scoring software (ANY-MAZE©) allow standardization and objectivity for behavior in the EPM.

2.4. Measurement of nocioception

This test was performed as described previously (Ballou et al., 2000). Mice were transported in their home cages to the testing room 1–2 h prior to acclimate to the room change prior to testing. The mouse was placed on a hotplate inside a clear plastic cylinder, with the temperature of the hotplate set to 50 °C. Latency to lick the hind-paw was recorded. If no response was observed after 45 s, the mouse was removed from the hotplate, to avoid any tissue injury.

2.5. PKA assay

Prkar1a$^{+/-}$ and WT littermates were moved to testing room 2 h prior to euthanization by CO_2. The brains were removed and immediately frozen (−80 °C) until cyrosectioning. Cyrosections of 250 μm and punch biopsies (0.5 mm diameter stainless steel punch) of tissue from the following brain regions were obtained using the punch method of Palkovits (1983): central and basolateral amygdala, ventromedial hypothalamus, paraventricular hypothalamus, thalamus, and orbitofrontal cortex. Olfactory bulb, eyes, and cerebellum were dissected in entirety and stored in cyrotubes at −80 prior to homogenization and measurement of PKA activity. The mouse brain atlas of Paxinos and Franklin (2001) was used to guide the dissections.

PKA enzymatic activity was measured following the protocol described earlier by Nesterova et al. (1975, 2008). The assays were carried out in a total volume of 50 μL for 15 min at 37 °C in the reaction mixture containing 1 mol/L Tris–HCl (pH 7.5), 1 mol/L DTT, 1 mol/L MgCl2, 60 μmol/L Kemptide (a phosphate acceptor peptide; Leu-Arg-Arg-Ala-Ser-Leu-Gly),

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20 μmol/L [γ-32P] ATP (25 Ci/mmol), with or without 5 μmol/L cAMP and 10 μL of the cell extracts. After incubation, the reaction mixtures were spotted onto 0.23-mm phosphocellulose discs and washed thrice in 0.5% phosphoric acid. Filters were air dried and counted by liquid scintillation counter. Basal levels of PKA activity represent the non-stimulated PKA activity. Total PKA activity reflects the PKA activity after the addition of cAMP. PKA values were normalized by protein content of each sample.

2.6. Statistical analysis

Data were analyzed for effect of genotype and sex by ANOVA and Bonferroni post hoc comparisons where appropriate using SPSS statistical software. Significance was determined at $p < 0.05$. All values are reported as means ± SEM. Behavioral measures in the EPM were analyzed by a multifactorial ANOVA, with between subjects' factors of sex and genotype.

3. Results

3.1. Measurement of anxiogenic-like behavior

To differentiate sex and/or genotype effect on anxiety-like behavior on novelty induced locomotor activity, the marble bury test was performed. *Prkar1a*$^{+/-}$(males and females) mice were compared to WT (males and females) littermates. ANOVA analysis showed a significant difference, with increased marble burying for *Prkar1a*$^{+/-}$ compared to WT mice In addition, an interaction between sex and genotype was noted, with *Prkar1a*$^{+/-}$females burying more marbles than all other groups ($F = 4.345$; $p < 0.05$) (WT vs. *Prkar1a*$^{+/-}$ males: 4.3 ± 0.5 vs. 5.9 ± 0.4; WT vs. *Prkar1a*$^{+/-}$ females: 5.2 ± 0.5 vs. 6.5 ± 0.4; $n = 19$—26 per group) (Fig. 1).

To examine sex differences and the impact of the null mutation on anxiety behaviors the EPM, a test of unconditioned response to novelty, was performed. A one-way between groups multivariate analysis was performed to investigate genotype differences in anxiety-like behavior in the EPM. Four dependent variables, open arm time, open to total arm time ratio, risk assessment, and total head dips were used. A significant difference was noted between WT and *Prkar1a*$^{+/-}$ mice on the combined dependent variables (F 2.892), $p = 0.03$, Wilks' lambda = 0.874; partial eta squared = 0.126). There was a significant difference between WT and *Prkar1a*$^{+/-}$ mice for open arm time (WT 24.1 ± 3.9 s, vs. *Prkar1a*$^{+/-}$ 10.1 ± 4.2; $p < 0.05$) (Fig. 2a) and open to total arm ratio (WT 0.1 ± 0.02 vs. *Prkar1a*$^{+/-}$ 0.04 ± 0.02; $p < 0.05$) (Fig. 2b). However, no genotype or gender effect was found for risk assessment behavior (WT 0.05 ± 0.004 vs. *Prkar1a*$^{+/-}$ 0.05 ± 0.005) or total head dips (WT 6.9 ± 0.7 vs. *Prkar1a*$^{+/-}$ 6.2 ± 0.7) ($n = 37$—50 per group). The number of closed arm entries was used as an indicator of locomotor activity and no genotype or gender differences were found (Males: WT 8 ± 0.7, *Prkar1a*$^{+/-}$ 7.6 ± 1; females: WT 7 ± 0.8, *Prkar1a*$^{+/-}$ 8.4 ± 0.9). These results indicate that locomotion did not influence the measures of anxiety-like behavior.

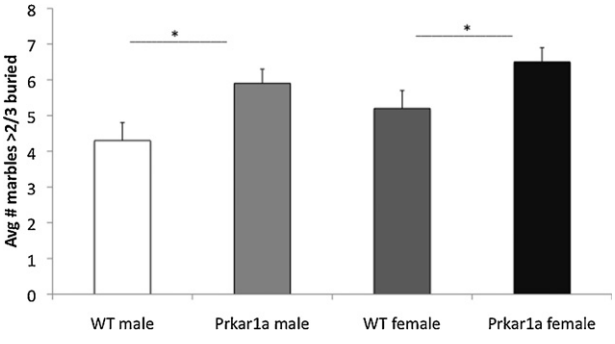

Figure 1 Marble Bury Test. Mean (±SEM) number of marbles buried 2/3 or greater after 30 min. *Prkar1a*$^{+/-}$ mice buried significantly more marbles than WT littermates (*$p < 0$).

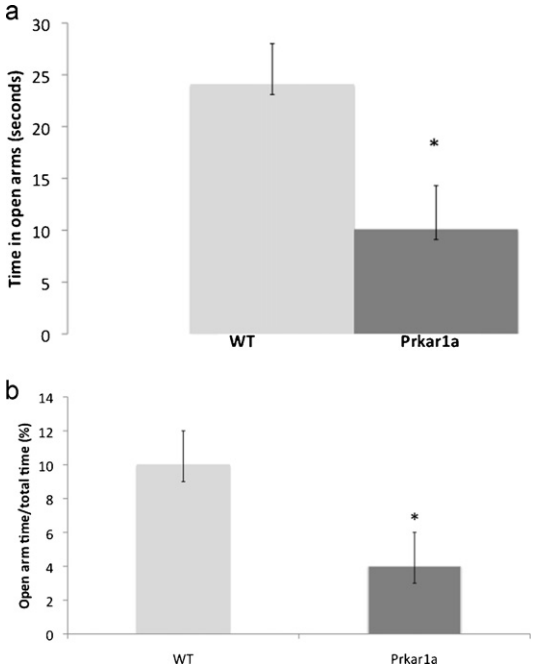

Figure 2 (a) Elevated plus maze: open arm time. Mean (±SEM) time in seconds of time mouse spent in open arm of elevated plus maze test (total test time 5 min.) *Prkar1a*$^{+/-}$ mice spent significantly less time in open arm than WT littermates (*$p < 0.05$). (b) Elevated plus maze: ratio of open arm time to total time. Mean (±SEM) percentage of time that mouse spent in open arm of elevated plus maze test (i.e. open arm time/(open arm + closed arm time). *Prkar1a*$^{+/-}$ mice had significantly lower percentage of open to total time than WT littermates (*$p < 0.05$).

3.2. Measurement of nocioception

To investigate the potential role of *Prkar1a* in nocioception we examined the response in the hot-plate test. ANOVA analysis of latency to lick response in the hotplate test showed the expected sex differences, with a longer latency in females compared to males (males: WT 28.8 ± 1.6, *Prkar1a*$^{+/-}$ 23.6 ± 1.8 s vs. females: WT 32.6 ± 1.9, *Prkar1a*$^{+/-}$ 32.6 ± 1.7 s; $p < 0.05$; $n = 15$—22 per group). No genotype effect was found. This suggests that the alteration in PKA signaling is not a ubiquitous effect, similar to what has been described in the *Prkar1a* mouse model, and provides an important positive control for this study.

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3.3. PKA activity

To investigate possible anatomical sites associated with changes in anxiety-like behavior we studied PKA activity in the brain of WT and *Prkar1a*$^{+/-}$ mice. ANOVA analysis showed no differences in PKA activity between male and female mice and no relation to age so all data were pooled together for analysis. In a control (non-stressed) situation the loss of one *Prkar1a* allele led to an increase in basal kinase activity in both the basolateral (WT 2867 ± 518 vs. *Prkar1a*$^{+/-}$ 12124 ± 3841 − cAMP/1 mcg, $p < 0.03$) and central amygdala (WT 3196 ± 693 vs. *Prkar1a*$^{+/-}$ 10923 ± 3097 − cAMP/1 mcg, $p < 0.05$) (Fig. 3a) and cAMP-stimulated kinase activity (WT 16717 ± 2938 vs. *Prkar1a*$^{+/-}$ 510230 ± 14301 + cAMP/1 mcg, $p < 0.03$; WT 21448.1 ± 6380 vs. *Prkar1a*$^{+/-}$ 68329 ± 16916 + cAMP/1 mcg; basolateral and central amygdala respectively, $p < 0.02$) (Fig. 3b) compared to the WT mice ($n = 13$–14 per group). The thalamus exhibited the highest basal and total PKA activity among WT mice, whereas the paraventricular hypothalamus and orbitofrontal cortex PKA activity did not parallel the increased activity of basolateral and central amygdala in the *Prkar1a*$^{+/-}$ group (Fig. 3a and b). No differences in basal or stimulated (cAMP-stimulated kinase) activity were found between WT and *Prkar1a*$^{+/-}$ mice in: ventromedial hypothalamus, cerebellum, olfactory bulb, or eyes (data not shown). In both genotypes, however, basal and cAMP-stimulated kinase differed significantly ($p < 0.05$) in all studied brain areas.

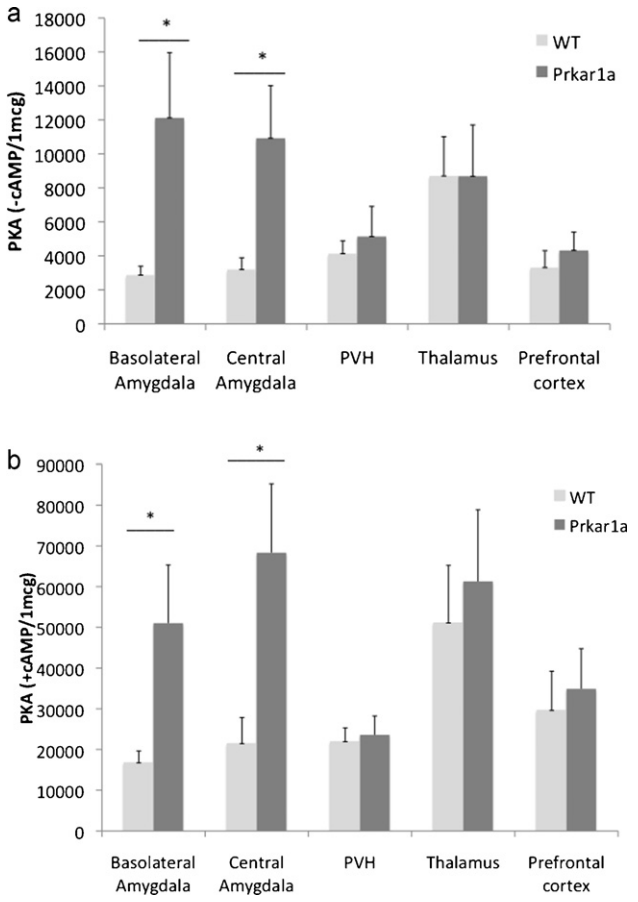

Figure 3 (a and b) Means (±SEM) of: (a) basal and (b) cAMP-stimulated PKA activity between wild type mice and mice with loss of one *Prkar1a* allele in a control (non-stressed) situation in basolateral and central amygdala, paraventricular hypothalamus, thalamus, and orbitofrontal cortex. Significantly increased basal and total PKA activity was found in basolateral and central amygdala in *Prkar1a*$^{+/-}$ mice compared to WT littermates (*$p < 0.05$).

4. Discussion

4.1. Behavioral responses to novel stressors

The present findings demonstrate that mice with a down-regulation of the regulatory subunit of PKA, exhibit behavioral changes in tests that measure anxiety (EPM and marble) suggesting a key role of PKA in modulating anxiety-related behaviors. Compared to WT mice, *Prkar1a*$^{+/-}$ mice had higher basal and stimulated (cAMP) PKA activity levels in the central and basolateral amygdala, brain areas known to have a critical role in the processing of sensory information related to anxiety and emotion as well as regulation of arousal level. The EPM relies on the innate motivational conflict between the drive to explore a novel environment (approach behavior) in opposition to the fear of open space (avoidance behavior). Anxiolytic compounds increase the proportion of open arm exploration relative to total arm exploration, whereas anxiogenic compounds reduce open arm exploration (spatiotemporal measures) (Lapiz-Bluhm et al., 2008). In this study *Prkar1a*$^{+/-}$ mice displayed a decrease in the number of entries into the open arms of the maze as well as an increase in the amount of time spent in the closed arms (spatiotemporal measures) when compared to WT littermates. Consistent with the findings of the EPM, results of the marble bury test, another test of novelty that involves approach-avoidance behavior, also showed a genotype effect. These findings suggest that the observed behavior of *Prkar1a*$^{+/-}$ mice relates to "trait" rather than "state" anxiety, since these tests discriminate between approach and avoidance responses to novel environments.

In contrast with the results of the spatiotemporal measures of the EPM, it is interesting that no genotype difference was found in risk assessment or exploratory behaviors in the EPM, which implies that arousal behavior was similar between the two groups. Recent studies report that risk assessment behavior is a highly sensitive index of anxiety, based on ethological and pharmacological manipulations (Carobrez and Bertoglio, 2005; Espejo, 1997b; Cole et al., 1995), while head dipping is as an index of exploratory behavior (head dip over side arm of the maze) (Rogers and Johnson, 1995). Factor analysis studies support the segregation of behavioral and neural mechanisms controlling risk assessment and open arm exploration in the EPM (Adamec et al., 1999, 2001). In our study, although exposure to the open arm area functioned as a stressor that was sufficient to elicit increased anxiety-like (avoidance) behavior *Prkar1a*$^{+/-}$ mice, the similarities in risk assessment and exploratory behaviors of WT and *Prkar1a*$^{+/-}$ suggest that alteration in PKA activity discriminated between anxiogenesis and sedation. Importantly, these behaviors were observed in the absence of changes to total arm entries, suggesting that the observed changes in spatiotemporal-related measures

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in the EPM were behaviorally selective. The finding of low open arm time, but no difference in risk assessment behavior of *Prkar1a*$^{+/-}$ mice support findings of prior studies that note differences in the anxiety pathways related to spatiotemporal and risk assessment measures of EPM.

Using c-Fos immunocytochemistry to map neural circuits underlying behavioral responses, various studies have reported that the prefrontal cortex and amygdala are the main brain areas activated after exposure to the EPM (Rubino et al., 2007; Hinks et al., 1996). Results of our study showed no difference in baseline PKA activity in the prefrontal cortex, an area known to have extensive reciprocal connections with the amygdala, which suggests that the alteration of PKA activity noted in the amygdala of HZ mice may play a role in the inhibition of approach in situations of fear and promotes risk assessment behavior at the expense of flight. Mcnaughton and Corr (2004) proposed a two-dimensional hierarchal view of defensive behavior that provides a clear distinction between fear and anxiety. Fear functions to move the animal away from danger (defense avoidance system), the neural control is more elaborated at lower levels of the neural system, and is insensitive to anxiolytics. Anxiety functions to move the animal toward danger (defensive approach system), the neural control is more elaborated at higher levels of the neural system, and is sensitive to anxiolytics. In addition, anxiety involves inhibitory behavior and increased risk assessment (vigilance). The discrepancy noted between the increased anxiety-like behavior noted in spatiotemporal measures in the EPM and marble bury test, with no difference in risk assessment behavior, suggests that alterations in PKA activity independently affect neural pathways associated with various aspects of anxiety behavior.

Data presented here suggest that the effect of down regulation of the regulatory subunit of PKA is localized to the amygdala, since *Prkar1a*$^{+/-}$ mice showed increased anxiety-like behaviors and increased PKA activity in the amygdala, but not in the cortex. The role of the amygdala in the control of fear and anxiety is well established. Studies of anxiolytic compounds show that these agents act to reduce the arousal associated with anxiety, and that the intensity of amygdala activation is equated with arousal level, which is not mediated by the septo-hippocampal system (Mcnish et al., 1997; Davis, 2000, 1992). This does not rule out the possibility that increased PKA activity is not acting elsewhere in the brain (i.e. paraventricular hypothalamus, ventromedial hypothalamus, or other brain areas involved with neural pathways of anxiety) through compensatory mechanisms; but an amygdala localized effect is consistent with extensive data on the role of the amygdala in anxiety and fear-related behaviors.

4.2. Behavioral response to nocioceptive stimuli

An assessment of response to nocieceptive stimuli is relevant in the behavioral phenotype of the *Prkar1a*$^{+/-}$ mouse since the amygdala has a key role in both the emotional—affective processing of pain and anxiety behavior. Specifically the central nucleus of the amygdala receives nocioceptive information directly from the spinal cord and brainstem, and indirectly through the basolateral amygdala, thalamus, and cortex. The amygdala is important in nociofensive behavior as well as for pain inhibition. There is a paucity of data regarding the conditions that result in a pro- or anti-nocioceptive amygdala response and the molecular mechanisms involved. Little is known about the pain-related functions and interactions of various protein kinases, including PKA, PKC, and ERK in the amygdala (Fu et al., 2008). The hotplate pawlick test is a well-validated measure of analgesia in rodents that measures the spontaneous reaction to thermal nocioception. Unknown differences may pre-exist in a transgenic model that may affect pain sensitivity, so a baseline assessment is important in order to determine whether alterations in PKA activity affect pain response. In the present study, it is interesting that although basal and stimulated PKA activity in the basolateral and central amygdala were increased in HZ compared to WT, no genotype effect was found for nocioception; however, the expected sex difference in latency response remained intact.

Data from this study show no difference in basal or total PKA activity between WT and *Prkar1a*$^{+/-}$ mice in the ventromedial hypothalamus, cerebellum, olfactory bulb, or eyes, which suggests that alteration in PKA signaling is not a ubiquitous effect, similar to what has been described in the *Prkar1a*$^{+/-}$ mouse model (Tsang et al., 2010; Kirschner et al., 2005, 2009; Griffin et al., 2004c; Yin et al., 2009). Results from this study are consistent with prior studies that showed R1b mice had reduced injury-induced inflammation and pain, without apparent differences in PKA brain activity, likely due to compensatory increases in levels of *Prkar1a* protein (Brandon et al., 1995; Amieux et al., 1997; Huang et al., 1995; Malmberg et al., 1997). This provides an important positive control for the noted behavioral differences noted between WT and HZ, which are also mediated by the amygdala. This data suggests that downstream targets of PKA may be important mediators of the response to thermal nocioception and related to the pain-related plasticity in the amygdala.

4.3. Implications for neurobiology of anxiety research

We conclude that alterations in PKA signaling, as those that have been described in the *Prkar1a* mouse model, result in specific neurobiological modifications of behaviors involved in anxiety and fear sensitization in mice. The cAMP response element (CRE) is present in many genes (Impey et al., 2004; Zhang et al., 2005) and functions as a promoter/enhancer element in many brain areas that responds to environmental stimuli such as psychological stress (Konradi et al., 1994). PKA activity is also affected by various neurotransmitter systems that are involved with alertness, anxiety, emotion or mood through the actions of G-protein coupled receptors that regulate adenyl cyclase. Our results suggest that a chronic increase in PKA activity in the amygdala regulates the anxiety response, and a better understanding of the downstream targets of increased PKA activity may identify novel therapeutic targets to treat anxiety.

Conflict of interest

The authors state they have no conflict of interest.

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Margaret Keil designed the study, wrote the protocol, performed data analysis, and wrote the first draft of the manuscript. Nirmal Gokarn, George Briassoulis, and Maria Nesterova contributed to completion of experiments and data collection. And analysis. Constantine Stratakis and T. John Wu provided advice regarding the experimental design and contributed to the editing of the manuscript. All authors contributed to and have approved the final manuscript.

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