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Studies of mice with cyclic AMP-dependent protein kinase (PKA) defects reveal the critical role of PKA's catalytic subunits in anxiety

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Abstract

Cyclic adenosine mono-phosphate-dependent protein kinase (PKA) is critically involved in the regulation of behavioral responses. Previous studies showed that PKA's main regulatory subunit, R1 α , is involved in anxiety-like behaviors. The purpose of this study was to determine how the catalytic subunit, C α , might affect R1 α 's function and determine its effects on anxiety-related behaviors.

The marble bury (MB) and elevated plus maze (EPM) tests were used to assess anxiety-like behavior and the hotplate test to assess nociception in wild type (WT) mouse, a *Prkar1a* heterozygote (*Prkar1a*^{+/-}) mouse with haploinsufficiency for the regulatory subunit (R1 α), a *Prkaca* heterozygote (*Prkaca*^{+/-}) mouse with haploinsufficiency for the catalytic subunit (C α), and a double heterozygote mouse (*Prkar1a*^{+/-}/*Prkaca*^{+/-}) with haploinsufficiency for both R1 α and C α . We then examined specific brain nuclei involved in anxiety.

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Results of MB test showed a genotype effect, with increased anxiety-like behavior in *Prkar1a*^{+/-} and *Prkar1a*^{+/-}/*Prkaca*^{+/-} compared to *WT* mice. In the EPM, *Prkar1a*^{+/-} spent significantly less time in the open arms, while *Prkaca*^{+/-} and *Prkar1a*^{+/-}/*Prkaca*^{+/-} mice displayed less exploratory behavior compared to *WT* mice. The loss of one *Prkar1a* allele was associated with a significant increase in PKA activity in the basolateral (BLA) and central (CeA) amygdala and ventromedial hypothalamus (VMH) in both *Prkar1a*^{+/-} and *Prkar1a*^{+/-}/*Prkaca*^{+/-} mice.

Alterations of PKA activity induced by haploinsufficiency of its main regulatory or most important catalytic subunits result in anxiety-like behaviors. The BLA, CeA, and VMH are implicated in mediating these PKA effects in brain.

Keywords

anxiety; amygdala; protein kinase A; knockout mice

Introduction

In brain, genetic manipulations of cAMP-dependent protein kinase A (PKA) suggest its critical role in a wide spectrum of neurobiological and psychiatric disorders. The PKA is a multi-substrate serine/threonine protein kinase responsible for modulating a variety of cellular processes [1-4]. It is a heterotetramer composed of two N-terminally dimerized regulatory (R) subunits bound to two catalytic (C) subunits [5, 6]. PKA activation involves simple subunit dissociation upon binding of cAMP to the R subunits; thereby freeing catalytically active C subunits, which in turn phosphorylate protein substrates that contain appropriate consensus phosphorylation motifs [7]. One target of PKA is the cAMP responsive element (CRE) binding protein (CREB). Phospho-CREB in turn binds to CREs to mediate gene expression that has been linked to numerous behavioral reactions including those to stressors [8-10]. The response generated by cAMP can be terminated by the hydrolysis of cAMP into 5'AMP by phosphodiesterases (PDEs). Various studies indicate that increased cAMP signaling is associated with an anxiety-like phenotype and suggest that elevated cAMP activity is associated with abnormal reactivity to novel environments [11, 12], stress-coping responses [13, 14], and an overall increased anxiety [15-18]. Thus, therapeutic manipulation of PKA activation, such as decreasing phosphorylation of cAMP-CREB might lead to new treatment strategies for anxiety, addiction, or psychiatric disorders [3, 19-21].

Our laboratory has studied the consequences of R and C subunit dysregulation [22]. The *Prkar1a* and *Prkaca* genes encode the type 1A regulatory (R1α) and type A catalytic (Cα) subunits of PKA [23]. Regulatory subunits form a homodimer that binds two catalytic subunits (Cα, Cβ, or Cγ coded by the *PRKACA*, *PRKACB*, and *PRKACG* genes, respectively) in the PKA tetramer (R2C2) [8]. When the R and C subunits form a complex, the cAMP-catalytic activity is suppressed. As shown in knockout (KO) mouse studies, these four genes function in a tissue and cell-type specific manner to regulate the activity of the catalytic subunits [5]

Recently, we reported that loss of one *Prkar1a* allele in mice (*Prkar1a*^{+/-}) led to an augmentation of anxiety-like behaviors in association with an increase in PKA activity in both the basolateral (BLA) and central amygdala (CeA) [24]. These regions of the amygdala coordinate fear- and anxiety-like behavioral responses [24-27] as well as external cues, responding to threats via direct and indirect projections to the paraventricular nucleus of the hypothalamus (PVH) and brainstem regions [27-29]. PKA activity is increased in *Prkar1a*^{+/-} mice because of a deficiency in overall Ca inhibition, although the net effect varies widely in different tissues [25-30].

We reasoned that the introduction of half-null alleles of Ca into the *Prkar1a*^{+/-} mice might abrogate the excess Ca activity caused by R1α haploinsufficiency. Thus, we crossed *Prkar1a*^{+/-} and *Prkaca*^{+/-} mice to generate double heterozygous *Prkar1a*^{+/-}/*Prkaca*^{+/-} mice and expected a reversal of the anxiety phenotype that we observed previously [24, 27] in *Prkar1a*^{+/-} mice, consistent with what has been reported in PKA activity regulation [25, 26]. The data perhaps not unexpectedly support attenuation but not elimination of the anxiety phenotype noted in *Prkar1a*^{+/-} knockout mice (given the variable expression of other PKA subunits in brain) and highlight the importance of even modest changes in total PKA activity in the regulation of anxiety behaviors and the fact that Ca is not the sole determinant of PKA's cAMP signaling effects.

Materials and Methods

Animals

All mice were housed three to four per standard barrier cages on a ventilated rack in a room with a constant temperature (~22±1 °C) with same-sex littermates with *ad libitum* access to food and water and maintained on a 12:12 light schedule (lights on at 0600h). All animals were adults at the time of testing (2-7 months old at time of behavioral testing; 6-10 months at time of biochemical testing). Throughout the entire experimental period, the mice were handled daily and weighed weekly 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 approval at the NIH. All behavioral testing was performed, as previously reported [31-33], between the hours of 1300-1700 h. One behavioral test per day was performed, with a span of at least two days between tests. The order of behavioral tests was randomly assigned [27]. Two scorers performed behavioral testing and obtained scoring of all results in a blinded fashion (without knowledge of the genotypes of the mice under observation).

Generation of *Prkar1a*^{+/-}/*Prkaca*^{+/-} double heterozygous mice

Prkar1a heterozygous mice (*Prkar1a*^{+/-}) with one null allele of *Prkar1a* 2 were previously generated in our laboratory [28]. *Prkaca* heterozygous mice (*Prkaca*^{+/-}), which have a neomycin-resistance cassette to replace exons 6-8 of the *Prkaca* gene [29], were purchased from Mutant Mouse Regional Resource Centers (MMRRC) (strain name: B6; 129×1-*Prkacatm1Gsm/Mmnc*). We used heterozygotes *Prkaca* mice because the majority of Ca knockouts are either embryonic lethal or not viable and die in the immediate postnatal period [29, 34]. In addition, using heterozygotes for both *Prkar1a*^{+/-} and *Prkaca*^{+/-}, and also

double heterozygotes, we ensured equivalent genotyped groups, more appropriate for multiple comparisons. *Prkar1a*^{+/-} and *Prkaca*^{+/-} mice were crossed to generate the *Prkar1a*^{+/-}/*Prkaca*^{+/-} double heterozygous mice, which were maintained on a mixed C57BL/6 129Sv/B6 hybrid background (REF). All mice were genotyped using tail DNA. Control (wild-type, *WT*) mice were used from the same litter and were matched for age. Genotyping was conducted by polymerase chain reaction (PCR) using primers previously validated [30]. Three primers (5'-AGCTAGCTTGGCTGGACGTA-3', 5'-AAGCAGGCGAGCTATTAGTTTAT-3' and 5'-CATCCATCTCCTATCCCCTTT-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 [28, 35]. Genotyping of *prkaca* was done with two pairs of primers: the first pair (5'-CTGACCTTTGAGTATCTGCAC-3' and 5'-GTCCCACACAAGGTCCAAGTA-3') was used to detect the *WT* allele by amplification of the intron between exons 6 and 7 with a product of 250 bp; the second pair was used to detect the knockout allele (5'-AGACTACTGCTCTATCACTGA-3' and 5'-GTGGTTTGTCCAACTCATCAATGT-3') by amplification of a 270-nucleotide fragment of the region between the 3'-end of the neomycin resistance gene and a portion of the intron just 3' to exon 8. The wild-type allele generated a 6.0-kb fragment, and the knockout allele generated a 2.7-kb fragment.

Mouse phenotyping, numbers, behavioral tests, necropsies

Adrenal tumors or corticosterone overproduction were not identified in *Prkar1a*^{+/-} or *Prkar1a*^{+/-}/*Prkaca*^{+/-} mice by the age they were used in this study; likewise, thyroid neoplasms, schwannomas and other tumors were observed only in older *Prkar1a*^{+/-} mice, as reported previously [28]. No mice with tumors were used in this study. In addition, standard assessment of neurological function [24] revealed no deficits in mice enrolled in the study. A total of 55 control (wild-type, *WT*), 40 *Prkar1a*^{+/-}, 29 *Prkaca*^{+/-}, and 33 *Prkar1a*^{+/-}/*Prkaca*^{+/-} mice (all littermates) were handled for the purposes of this project.

Measurement of anxiety-like behavior and nociception

Heterozygous (*Prkaca*^{+/-}, *Prkar1a*^{+/-}), double heterozygous (*Prkar1a*^{+/-}/*Prkaca*^{+/-}), and *WT* mice (both males and females) were tested with various behavioral assays in order to characterize the phenotype. The marble bury test [31] and elevated plus maze (EPM) were performed as described previously [31-33]. The hotplate test was performed as described previously to assess nociception [36].

Marble bury test

Marble bury test was performed as previously described [31, 37, 38]. Mice were transported in their home cages to the testing room two hours prior to the experiments, to acclimate to the new environment. Rodent sawdust bedding was placed in standard mouse cages (38×22×16cm) and eight dark colored marbles were placed on top of the bedding in two evenly spaced rows and the cage was closed with a lid. No food or water was present during the 30-min test period. Lights were turned off in the room for 30-min and then the number of marbles buried 2/3 or greater was recorded.

Elevated plus maze

Elevated plus maze (EPM) testing was performed as described previously [24, 27, 32, 33]. Mice were transported in their home cages to the testing room two hours prior to acclimate prior to testing. The EPM consists of two open arms (30 × 5 cm) and two enclosed arms (30 × 5 cm), with end and side- walls (15 cm height), and a center platform (5 × 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 minutes. 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 minutes, 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) [39]. 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.

Hotplate test of nociception

This test was performed as described previously [24, 36]. Mice were transported in their home cages to the testing room one to two hours prior to the experiment. The mouse was placed on a hotplate inside a clear plastic cylinder, with the temperature of the hotplate set to 50 degrees Celsius. Latency to lick the hind-paw was recorded. If no response was observed after 45 seconds, the mouse was removed from the hotplate, to avoid any tissue injury.

PKA assays

WT, *Prkar1a*^{+/-}, *Prkaca*^{+/-}, and *Prkar1a*^{+/-}/*Prkaca*^{+/-} littermates were moved to the testing room 2 h prior to euthanization by CO₂ inhalation. Mice were 6- 10 months age at time of tissue procurement, after a minimum of two months from last behavioral testing. The brains were removed and immediately frozen in liquid nitrogen and stored at -80°C until processing. Punch biopsies (0.5 mm diameter stainless steel punch) of specific tissues were obtained from 250 µm sections from the following brain regions [40] : BLA, CeA, (corresponding to Figs. 64-70, Bregma -0.955 to -1.555 mm); ventromedial (VMH) and paraventricular hypothalamus (PVH) (corresponding to Figs. 64 and 70, Bregma -0.955 to -1.555 mm). Olfactory bulb (Olf), eyes, prefrontal cortex (PFC), cerebellum (Cb), hippocampus (Hipp), and thalamus (Thal) were dissected in entirety. All dissected tissues were stored in cryotubes at -80 C prior to homogenization and PKA activity assay. The mouse brain atlas of Allen [41] was used to guide the dissections.

PKA enzymatic activity was measured following our previously described protocol [24, 27, 35, 42]. The assays were carried out in a total volume of 50 μ L 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 μ mol/L Kemptide (a phosphate acceptor peptide; Leu-Arg-Arg-Ala-Ser-Leu-Gly), 20 μ mol/L [γ -³²P] ATP (25 Ci/mmol), with or without 5 μ mol/L cAMP and 10 μ L 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.

Immunohistochemistry

Perfused brains were sent to Histoserv Inc. (Germantown, MD) for immunohistochemistry (IHC) processing. Briefly, brains were dehydrated and cleared with xylene and infiltrated with paraffin. Coronal slices of 10 μ m thickness were mounted on slides and processed for IHC with the following steps: deparaffinization with xylene, distilled water wash, pre-treatment of slides at 70°C for 40 min, hydrogen peroxidase treatment to remove endogenous peroxidase activity, bovine serum albumin blocking, tris buffered saline-tween (TBST) wash, incubation with the phosphodiesterase 4 (PDE4) antibody (1:100; Cat #ab14628, abCam, Cambridge, MA). TBST wash, secondary antibody (Biotinylated Anti-Rabbit IgG; #BA1000, Vector, Burlingame, CA), TBST wash, Streptavidin-HRP, tris buffered saline-tween wash, develop slides with DAB (3,3'-Diaminobenzidine; Sigma, St Louis, MO), dehydrate in graded ethanol, treated with xylene to remove ethanol, and coverslipped. This anti-PDE4 antibody detects all known PDE4 A and D variants.

The Allen brain atlas of the mouse was used to identify corresponding nuclei location for all PDE4 antibody stained slides. Slides were coded and analyzed by two independent experimenters were conducted blinded to genotype. Three to four slides per brain (3 brains per genotype) containing the amygdala and hippocampus were assessed at approximately -1.8 to -3.30 Bregma. For each section, slides were photographed at 5 $\times$ magnification with a Leica microscope/camera (Leica DMRX; Olympus DP72) and files created by CellSens software.

Statistical Analyses

Data were analyzed for effect of genotype and sex by ANOVA and Bonferroni *posthoc* comparisons among the four groups using SPSS statistical software. A paired samples *t*-test was done for the paired (basal and total PKA activity) experiments. Significance was determined at $p < 0.05$; all values are reported as means $\pm$ SEM. Behavioral measures in the EPM were analyzed by a multifactorial ANOVA, with between subjects' factors of sex and genotype.

Results

Measurement of anxiety-like behaviors

Marble bury (MB) test—*Prkar1a*^{+/-}, *Prkaca*^{+/-}, and *Prkar1a*^{+/-}/*Prkaca*^{+/-} were compared to *WT* littermates. ANOVA analysis showed a significant difference ($F 5.6$, $df 3$, $p < 0.001$), with increased marble burying for *Prkar1a*^{+/-} (6.3 ± 0.3) and *Prkar1a*^{+/-}/*Prkaca*^{+/-} (5.9 ± 0.3) compared to *WT* (4.5 ± 0.3 , $p < 0.001$, $p < 0.03$, respectively) (Figure 1). *Prkaca*^{+/-} mice showed no difference in marble burying behavior (5.2 ± 0.3) compared to *WT* mice. No sex difference was observed within each genotype ($N = 11$ -24 per sex/genotype).

Elevated Plus Maze (EPM)—ANOVA showed differences among groups in the center of EPM ($F 2.6$, $df 3$, $p < 0.05$), closed arm time ($F 2.7$, $df 3$, $p < 0.05$), open arm time ($F 3.3$, $df 3$, $p < 0.03$), and open to total time ratio ($F 2.9$, $df 3$, $p < 0.04$). Bonferroni post hoc analysis showed that *Prkaca*^{+/-} spent significantly more time in the center (63.75 ± 10.7 vs. 28 ± 8.2 sec; $p < 0.05$) and less in the closed arms (220.5 ± 13.4 vs. 259.45 ± 5.8 sec; $p < 0.05$) than *Prkar1a*^{+/-} mice (Figure 2a and 2d), while *Prkar1a*^{+/-}/*Prkaca*^{+/-} and *WT* mice had similar amount of center time. *Prkar1a*^{+/-} spent less time (6.8 ± 1.7 sec) in the open arm than *WT* mice (21.4 ± 3.9 sec, $p < 0.02$) (Figure 2b), scoring half open to total time ratio (0.03 ± 0.01 , $F 2.9$, $df 3$, $p < 0.04$) compared to *WT* (0.09 ± 0.02), *Prkaca*^{+/-} (1.04 ± 0.05) or *Prkar1a*^{+/-}/*Prkaca*^{+/-} mice (0.08 ± 0.02). No other differences between genotypes *Prkaca*^{+/-} (18.3 ± 6.8 sec) or *Prkar1a*^{+/-}/*Prkaca*^{+/-} (16.4 ± 4.2 sec) were detected. Similarly, no difference was found between heterozygote groups and *WT* for risk assessment. Interestingly, *Prkaca*^{+/-} and *Prkar1a*^{+/-}/*Prkaca*^{+/-} mice displayed significantly less exploratory behavior compared to *WT* mice (total head dips: 3.7 ± 0.9 , 4.2 ± 0.9 , respectively vs. 7.3 ± 0.6 ; $F 4.9$, $df 3$, $p < 0.03$) ($n = 49$ to 57 per group) (Figure 2c). The number of closed arm entries was used as an indicator of locomotor activity and no genotype differences were found (#closed arm entries: *WT* 8 ± 0.6 ; *Prkar1a*^{+/-} 7.5 ± 0.7 ; *Prkaca*^{+/-} 8 ± 1.3 ; *Prkar1a*^{+/-}/*Prkaca*^{+/-} 7.9 ± 1.3 , $p < 0.9$), which suggests that locomotion did not influence the measures of anxiety-like behavior. No genotype effect was found for percentage of open arm entries (% open/closed arm entries: *WT* 17%; *Prkar1a*^{+/-} 13%; *Prkaca*^{+/-} 6%; *Prkar1a*^{+/-}/*Prkaca*^{+/-} 22%, $p < 0.74$). No sex effects on various EPM measures of unconditioned response to novelty were found (data not shown).

Measurement of nociception—Since the amygdala is known to have a key role in the emotional –affective dimension of pain, the inclusion of a nociceptive assessment was important to include in the behavioral phenotype of the *Prkar1a*, *Prkaca* knock-out mice. To investigate the potential role of the R1 α subunit in nociception 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 noted for *Prkar1a*^{+/-} and *WT*, with a longer latency in females compared to males (males: *WT* 28.8 ± 1.6 , *Prkar1a*^{+/-} 24.9 ± 1 seconds vs. females: *WT* 31.7 ± 1.2 , *Prkar1a*^{+/-} 31.4 ± 1.7 seconds; $p < 0.05$; $n = 20$ -21 per group); but no significant gender effect for *Prkaca*^{+/-} or *Prkar1a*^{+/-}/*Prkaca*^{+/-} mice (Figure 3). The finding of expected sex differences in *Prkar1a*^{+/-} and *WT*, but not *Prkaca*^{+/-} or *Prkar1a*^{+/-}/*Prkaca*^{+/-} mice, suggests that the alteration in PKA signaling is not a ubiquitous effect.

PKA activity—To investigate possible anatomical sites associated with changes in anxiety-like behavior we determined the PKA activity in the brain of *WT*, *Prkar1a^{+/-}*, *Prkaca^{+/-}*, and *Prkar1a^{+/-}/Prkaca^{+/-}* mice. Only BLA, CeA, and VMH PKA activity differed among genotypes (Table 1). Bonferroni post-hoc tests revealed differences among the following brain regions: BLA and CeA basal PKA activity was higher in the *Prkar1a^{+/-}* mice compared to *WT* ($p<0.03$) and *Prkaca^{+/-}* mice ($p<0.01$) (Figure 4a,c); BLA and CeA total PKA activity was higher in *Prkar1a^{+/-}* compared to *WT* mice ($p<0.02$). (Figure 4b,d). VMH basal PKA activity was significantly higher in *Prkar1a^{+/-}* compared to *WT* ($p<0.01$) and *Prkaca^{+/-}* mice ($p<0.04$). VMH and thalamus total PKA activity was significantly higher in *Prkar1a^{+/-}* compared to *WT* mice ($p<0.01$) (Figures 4e,f). No genotype differences in basal or cAMP-stimulated PKA activity were found in PVH, hippocampus, PFC, cerebellum, olfactory bulb, or eyes (Table 1). However, all four genotypes, without exception, showed an increase in their cAMP-stimulation of kinase activity ($p<0.0001$) compared to basal PKA activity.

PDE4 expression—To investigate possible compensatory mechanisms of PKA-catalytic subunits we examined PDE4 expression in the hippocampus and amygdala, which comprise the two independent memory systems of the temporal lobe that have a crucial role during emotional arousal. Photomicrographs were qualitatively analyzed by two observers (blinded) and scored (-, +, ++, +++, +++++). Photomicrographs demonstrate qualitative differences in expression of PDE4 in brain areas across genotypes (Figure 5).

In the dorsal dentate gyrus (DG) PDE4 expression is attenuated in *Prkar1a^{+/-}* compared to *WT*. PDE4 expression in *Prkaca^{+/-}* and *Prkar1a^{+/-}/Prkaca^{+/-}* is diminished compared to *WT* and less than the expression in *Prkar1a^{+/-}*. PDE4 expression in the CA-subfields of the hippocampus shows a similar pattern as the DG, with less expression in *Prkar1a^{+/-}*, *Prkaca^{+/-}*, and *Prkar1a^{+/-}/Prkaca^{+/-}* compared to *WT*. The differences in PDE4 expression in the basolateral amygdala between genotypes is less pronounced; however a pattern of attenuated expression is noted in *Prkar1a^{+/-}*, *Prkaca^{+/-}*, and *Prkar1a^{+/-}/Prkaca^{+/-}* compared to *WT*. There are minor observed differences in PDE4 expression in the cortex between genotypes; a pattern of attenuated expression is noted in *Prkar1a^{+/-}* compared to *WT* and *Prkaca^{+/-}*, and *Prkar1a^{+/-}/Prkaca^{+/-}* (Table 2).

Discussion

The results of the present study suggest first and not surprisingly, that the relative availability of the R1 α and C α subunit in various parts of the brain is important for regulating the PKA activity-related effects on anxiety-like behaviors. Second and interestingly, by halving the amount of the main PKA catalytic subunit (*Prkaca^{+/-}*) in the previously described *Prkar1a^{+/-}* mice, the anxiety phenotype of the latter was attenuated but not eliminated. Third, our data support the importance of the BLA, CeA, and DG in mediating PKA effects on anxiety-related behaviors.

Results of the MB test, a test of unconditioned defensive behavior (that involves approach-avoidance behavior) showed a clear effect of the genotype: *Prkar1a^{+/-}* and *Prkar1a^{+/-}/Prkaca^{+/-}* mice buried significantly more marbles than *WT* or *Prkaca^{+/-}* mice, consistent with higher anxiety-like behavior. Interestingly, the behavioral response of *Prkaca^{+/-}* mice was

similar to the *WT* mice, which suggests that the finding of increased anxiety-like behavior noted in the *Prkar1a*^{+/-} and *Prkar1a*^{+/-}/*Prkaca*^{+/-} mice is most likely related to attenuated inhibition from knockdown of the regulatory subunit. Recent studies propose that marble bury behavior in mice may reflect repetitive and perseverative behavior that is sensitive to anxiolytic and 5-HT1A compounds that are regulated by cAMP/PKA signaling [43, 44].

However, *Prkar1a*^{+/-}/*Prkaca*^{+/-} mice did not demonstrate increased anxiety-like behavior in the spatiotemporal measures of the EPM, suggesting that alterations in catalytic subunit activity independently affect neural pathways associated with various aspects of anxiety behavior [24, 45]. Although *Prkar1a*^{+/-} mice spent less time in the open arms of the maze compared to *WT* and *Prkaca*^{+/-} littermates, no difference was found in open arm time between *Prkaca*^{+/-} or *Prkar1a*^{+/-}/*Prkaca*^{+/-} and *WT* mice, implying that *Cα* subunit activity is an important modulator of anxiety behavior. This is further supported by the finding that both genotypes with down-regulated *Cα* exhibited less exploratory behavior (total head dips), compared to *WT* mice [46] since exploratory behavior loads as a distinct dimension from anxiety in factor analysis of spatiotemporal measures of the EPM [45]. *Prkaca*^{+/-} mice spent significantly more time in the center area and less time in the closed arms than *Prkar1a*^{+/-} mice (a factor related to decision making behavior in factor analysis of EPM behavior), which also illustrates the behaviorally selective effects of down-regulation of the catalytic subunit in the EPM.

It is interesting there was no apparent effect of the genotypes in risk assessment in the EPM [46-48], which implies that arousal/emotional reactivity behavior [49, 50] was similar among the four genotypes. Recent studies report that risk assessment behavior is a highly sensitive index of anxiety, based on ethological and pharmacological manipulations while head dipping is as an index of exploratory behavior (head dip over side arm of the maze). Although exposure to the open arm area functioned as a stressor that was sufficient to elicit increased anxiety-like (avoidance) behavior in *Prkar1a*^{+/-} mice, the similarities in risk assessment in all four groups suggest that alteration in PKA activity discriminated between anxiety and sedation by independently affecting neural pathways associated with various aspects of anxiety behaviors [51]. Importantly, these behaviors were observed in the absence of changes to total arm entries, suggesting that the observed changes in spatiotemporal-related measures in the EPM were behaviorally selective [52-54].

Studies on the neural mechanisms of anxiety and threat processing propose two mechanisms: trait anxiety significantly increases prefrontal cortex-amygdala connectivity during threat processing; or that trait anxiety may be the result of failed top-down inhibition of the prefrontal cortex resulting in attenuated inhibition of the amygdala aversive response [55-59]. In this study there was no genotype difference in PKA activity in the PFC, an area known to have extensive reciprocal connections with the amygdala suggesting that the alteration of PKA activity in the amygdala of the *Prkar1a*^{+/-} mice has a role in the inhibition of approach in situations of potential threat (results of MB test) and unconditioned response to novelty (i.e. EPM).

Measurement of PKA activity in various brain areas revealed increased PKA activity in the amygdala in *Prkar1a*^{+/-} compared to *Prkaca*^{+/-} or *WT*, and in part compared to *Prkar1a*^{+/-}/

Prkaca^{+/-} mice. The alteration in PKA activity in these transgenic mice was not a ubiquitous effect, since PKA activity was found to be similar between heterozygotes and WT mice in other brain (orbitofrontal cortex, hippocampus, cerebellum, paraventricular hypothalamus) and neural sensory (olfactory bulb, eyes) areas. The data presented here also suggest that the effect of down regulation of the catalytic subunit of PKA is localized to the amygdala, since *Prkaca*^{+/-} and *Prkar1a*^{+/-}/*Prkaca*^{+/-} mice did not differ from WT mice in PKA activity in the BLA and spatiotemporal measures of the EPM. However, *Prkar1a*^{+/-}/*Prkaca*^{+/-} and *Prkar1a*^{+/-} mice showed increased anxiety-like behavior in the MB test, which also correlated with higher CeA- PKA activity compared to WT and *Prkaca*^{+/-} mice. These data indicate that the partial restoration of the amygdala PKA activity is associated with a parallel increase in the anxietylike behavior in the *Prkar1a*^{+/-}/*Prkaca*^{+/-} mice, further supporting the previously established role of the amygdala in the processing of sensory information related to anxiety and emotion as well as regulation of arousal level [19, 24]. In addition, the anxiety-like behavior noted in the open arm time in the EPM of *Prkar1a*^{+/-} but not the *Prkar1a*^{+/-}/*Prkaca*^{+/-} mice (compared to the WT) further supports the role of the catalytic subunit activity in ameliorating the anxiety-like behavior in the open arm.

Prior studies provide evidence that the CeA, which is the major output and the anatomical link between the amygdala complex and motor regions in the brain stem, is responsible for exploratory behavior in the EPM [60]. The BLA, strongly involved in emotional processing and the first to process an aversive stimulus [61], mediates anxiety possibly through hypothalamic modulation of glucocorticoid release [62, 63]. Importantly, John et al. [51], reported that the intra-BLA infusion of an anxiolytic increased the time spent in the open arms as well as the number of open arm entries. The higher PKA activity of *Prkar1a*^{+/-} mice in the VMH and the overall higher PKA activity in both *Prkar1a*^{+/-} and *Prkar1a*^{+/-}/*Prkaca*^{+/-} mice may account for the differences in exploratory or marble burying behavior of the two genotypes bearing one (alone or combined) *Prkar1a* allele loss in our study. These findings also support the hypothesis of discrete regions of the hypothalamus in mediating anxiogenic effects through the major catalytic subunit of PKA [49].

In parallel studies of generalized anxiety disorder patients, the BLA and CeA connectivity patterns with the midbrain, thalamus, and cerebellum measured during functional magnetic resonance imaging were significantly less distinct than in healthy controls, and increased gray matter volume was noted primarily in the CeA [59, 64]. Similarly, adolescents with generalized anxiety disorder, but not controls, showed greater activation to fearful faces than to happy faces in a distributed network including the amygdala, ventral prefrontal cortex, and anterior cingulate cortex [65]. These results are in accordance with findings of this study showing a significantly higher PKA activity difference between amygdala and other brain areas (thalamus, cerebellum, olfactory bulb or eyes) in the *Prkar1a*^{+/-} and even *Prkar1a*^{+/-}/*Prkaca*^{+/-} mice as compared to the WT and *Prkaca*^{+/-} mice. Recent imaging studies on aversion highlighted the recruitment of thalamus, midbrain and orbitofrontal regions that remained independent of sensory modality [66], a finding that supports our results of indifferent eye and olfactory PKA activity between genotypes.

Compensatory mechanisms in remaining PKA subunits and PDE4 may also play a role in areas not showing any differences in PKA activity between *Prkar1a*^{+/-} and *Prkar1a*^{+/-}/*Prkaca*^{+/-}

Prkaca^{+/-} and *WT* or *Prkaca*^{+/-} mice. In some brain areas, such as in the PVH, *Prkaca*^{+/-} mice appeared to have more PKA activity as compared to the *WT* mice, probably indicating a compensatory mechanism. Prior studies report that compensatory increases in C β levels occurred in brain of Ca knockout mice [67]. Also, it has previously been shown that, although accounting for just 5–10% of total PKA activity in mouse cells, C β compensates not only states of Ca deficiency [29] but also in the presence of strain-specific genetic modifiers [68] and dysregulated PKA activity [26]. Similarly, RI compensation has been shown in the hippocampus and cerebral cortex of RII^{-/-} mice, resulting in a PKA holoenzyme with a significantly increased basal activity, probably caused by a lower threshold of activation associated with the RI holoenzyme [69, 70].

The dentate gyrus and hippocampus have a significant role in affective processing via extensive connections from the amygdala and hypothalamus and have been implicated in anxiety disorders. The dorsal axis of the hippocampus has an important role in spatial processing and the ventral axis is important in anxiety and olfactory processes. PDE4 is highly expressed in brain regions involved with the regulation of memory and anxiety. PDE4 activity is enhanced through PKA phosphorylation, leading to an abrupt termination of the signal [71], and has a crucial role in the regulation of its intracellular concentration. An increase in PDE4 activity may be a compensatory mechanism to accelerate cAMP degradation.

Our data show that the pattern of PDE4 expression in neural areas involved with emotional arousal and anxiety is not ubiquitous across genotypes similar to the variance in PKA activity noted, and is consistent with a lack of compensatory mechanism. In the dentate gyrus and CA-subfields of the hippocampus and to a lesser extent in the amygdala and cortex the pattern of PDE4 expression parallels the differences in behavioral and PKA activity noted among genotypes suggesting that compensatory mechanisms in PKA-catalytic or regulatory subunits may mediate various aspects of anxiety behavior. The increase in PKA activity noted in various neural areas parallels the attenuated PDE4 expression across genotypes. Since PDE4 accelerates cAMP degradation, the attenuated expression noted across genotypes is consistent with a lack of a compensatory mechanism. These findings are consistent with what is known about PDE4 expression and the regulation of anxiety and depression [72-75] and provide additional support that the relative availability of the R1 α and Ca subunit in various parts of the brain is important for regulating the PKA activity-related effects on anxiety-like behaviors.

It appears that *PRKARIA* inhibition in humans and *Prkar1a*-down-regulation in mice is associated with increased risk for psychopathology, consistent with the importance of this protein in cAMP/PKA signaling in brain function. We recently reported an increased incidence of psychiatric disorders in adults and children with *PRKARIA* mutations. The most frequent psychiatric diagnosis in adults were anxiety, depression, and bipolar disorder (in that order), while for children learning difficulties, attention deficit hyperactivity disorder, anxiety, and depression (in that order) [76]. The amygdala and prefrontal cortex are key structures in the pathophysiology of anxiety disorders, and a putative target for anxiolytic treatments [77, 78]. Liu et al [79] recently reported the essential role of protein kinase C in anxiety-related behaviors. Using a mouse model they showed that activation of

glutamatergic kainate-type receptors led to anxiety-related behaviors and glutamergic synapse up-regulation through protein kinase C located in the dendritic spines of the prelimbic cortical excitatory neurons, which suggests that PKC inhibitors may have a role in pharmacologic management of anxiety. Our results suggest that 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.

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Highlights

- Alterations of total PKA activity in the amygdala regulate the anxiety response.
- Both R1 α and C α subunit activity regulate PKA signaling in the amygdala and anxiety.
- C α activity is not the sole determinant of PKA's cAMP signaling effects.

Figure 1. Marble bury test

Comparison of *wild type*, *Prkar1a*^{+/-} and *Prkaca*^{+/-} heterozygotes, and *Prkar1a*^{+/-}/*Prkaca*^{+/-} double heterozygous for number of marbles 2/3 or more buried after 30 min; Bonferroni significance: *, $p < 0.05$ vs. WT (data combined for gender by genotype).

Figure 2. Elevated plus maze

a) Closed arm time and b) Open arm time. Comparison of *wild type*, *Prkar1a*^{+/-} and *Prkar1a*^{+/-} heterozygotes, and *Prkar1a*^{+/-}*Prkaca*^{+/-} double heterozygotes for amount of time spent (seconds) in closed arm of elevated plus maze during 5 min test period. *, $p < 0.05$. c) Total head dips. Comparison of *wild type*, *Prkar1a*^{+/-} and *Prkaca*^{+/-} heterozygotes, and *Prkar1a*^{+/-}*Prkaca*^{+/-} double heterozygotes for the total number of head dips in closed and open arms and center of elevated plus maze during 5 min test period. *, $p < 0.05$ vs. WT d) Center time. Comparison of *wild type*, *Prkar1a*^{+/-} and *Prkar1a*^{+/-} heterozygotes, and *Prkar1a*^{+/-}*Prkaca*^{+/-} double heterozygotes for amount of time spent (seconds) in center of elevated plus maze during 5 min test period. *, $p < 0.05$ vs. *Prkar1a*^{+/-} (data combined for gender by genotype).

Figure 3. Hotplate test

Comparison of latency to paw lick (sec) in male and female mice of all 4 genotypes. *, $p < 0.05$.

Figure 4. PKA Activity (basal and total)

Comparison of PKA activity among all four genotypes in the basolateral amygdala (BLA) (a, b), central amygdala (CeA) (c, d), and ventromedial hypothalamus (VMH) (e, f). Basal (left column (figures a, c and e)) and total (right column (figures b, d and f)) PKA activity. Bonferroni significance: * *Prkar1a*^{+/-} vs. *WT*, *Prkaca*^{+/-} (p<0.01); ** *Prkar1a*^{+/-} vs. *WT* (p<0.01).

Figure 5. PDE4 expression

Photomicrographs of PDE4 expression in brain areas among all four genotypes (WT, *Prkar1a*^{+/-} (R1α), *Prkaca*^{+/-} (C α), *Prkar1a*^{+/-}/*Prkaca*^{+/-} (DH) in the hippocampus (CA1, CA2, CA3, Dentate gyrus (DG)), amygdala, and cortex. Scale bar at the lower right corner = 100 μm.

Table 2

Comparison of PDE4 expression among WT, *Prkar1a*^{+/-}, *Prkaca*^{+/-}, and *Prkar1a*^{+/-}/*Prkaca*^{+/-} various brain areas (Dentate gyrus of hippocampus, CA-subfields of hippocampus (CA1, CA2, CA3), BLA = basolateral amygdala; LA = lateral amygdala, hippocampus; cortex).

	WT	R1α	Cα	R1α/ Cα
Dentate gyrus	++++	++	+	+
CA-subfield hippocampus (CA1, CA2, CA3)	+++	++	+	+
BLA	+++	-	++	++
LA	+++	+/-	++	+
Cortex	+++	+	++	++