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ABSTRACT

"INCREASED PROTEIN KINASE A ACTIVITY IN THE *PRKAR1A*-DEFECTIVE MOUSE IS ASSOCIATED WITH HYPERAROUSAL AND INCREASED ANXIETY"

Margaret F. Keil , M.S., CRNP

Thesis Directed By: T. John Wu, Ph.D. Associate Professor, Department of Obstetrics and Gynecology

The role of the cAMP/PKA signaling in the molecular pathways involved in fear and memory is well established. We recently reported that a *Prkar1a* heterozygote (*Prkar1a*$^{+/-}$) mouse that was developed in our lab showed tissue-specific increased PKA activity that was associated with anxiety-like behavioral phenotype. This proposal tested the following hypothesis: mice with the loss of one *Prkar1a* allele will display an anxiety-like phenotype and subsequent increased vulnerability to stress associated with increased PKA activity in brain areas involved with processing of emotional stimuli and anxiety. We measured behavioral response to stress, neural activation, and PKA activity in brain areas after exposure to predator odor or vehicle in male *Prkar1a*$^{+/-}$ and WT littermates. Significant differences were found between *Prkar1a*$^{+/-}$ and WT mice in the behavioral response to stress. The behavioral response of *Prkar1a*$^{+/-}$ mice was consistent with the 'prediction error' model of anxiety. In addition, basal and total PKA activities were independently associated with genotype and stress, and an interaction between genotype and stress was shown. Results of cFos expression in *Prkar1a*$^{+/-}$ mice showed treatment effect in basolateral amygdala only. These results suggest that the alterations in PKA signaling in *Prkar1a*$^{+/-}$ mice are not ubiquitous in the brain; tissue-specific effects of the cAMP/PKA pathway are related to the stress response and anxiety-like behavior.

"INCREASED PROTEIN KINASE A ACTIVTY IN THE *PRKAR1A*-DEFECTIVE MOUSE IS ASSOCIATED HYPERAROUSAL AND INCREASED ANXIETY"

By

Margaret F. Keil

Graduate School of Nursing

Dissertation submitted to the Faculty of the
Graduate School of Nursing
Uniformed Services University of the Health Sciences
In partial fulfillment of the requirements for the degree of
Doctor of Philosophy, 2011

DEDICATION

To my parents, who instilled in me the value of education;
and to my husband, who encouraged me to pursue my dreams.

ACKNOWLEDGEMENTS

I feel fortunate that so many people were willing to help in me various ways to achieve my goal of completing my doctoral education. I would like to acknowledge my appreciation of their support. To begin with, I thank my parents, whose example instilled in me the value of education as well as the importance of resiliency when working towards a goal. I am most grateful to my husband, Jim, for his love and unfailing support, which allowed me to complete this milestone. Your steadfast belief in me was my rock through many a storm along this journey. To our children, James and Rita, thank you for your encouragement, humor, and patience as I tried to balance full-time work, graduate school, and my most important role- your mom. To my family and friends, thank you for your love and encouragement and for being a sounding board when I needed one.

To my committee co-chair and mentor, Dr. T. John Wu, I am most grateful for your time and guidance, and the resources that you provided. Your encouragement and support gave me the confidence I needed to broaden my approach to scientific research from a clinical perspective that was familiar to me, to a more focused empirical model. I credit your skills as a mentor and scientist, which helped to cultivate and nurture my intellectual growth. Your honest criticism and caring patient manner were an immense help to me in the development and completion of this project.

To Dr. Chris Kasper, my committee co-chair, I am most appreciative of the critical guidance you offered. Your ability to impart a sense of perspective, helped keep me focused to face the many challenges of the rigorous requirements of this program.

I would like to thank Dr. Marguerite Littleton-Kearney, a member of my committee, for her support and interest in my project. Your helpful feedback, often in the form of a question, challenged me to better define my argument and for that I am appreciative. Also, I would like to thank Dr. Bruce Schoneboom, who was part of my initial committee, for his advice and support.

I would like to express my heartfelt thanks to Dr. Constantine Stratakis, a member of my committee as well as my mentor and boss, for his support and encouragement of my doctoral studies. You embody the very best in a scientist- passion, a commitment to excellence, and an

intellectual perspective that are a source of inspiration to me. I am truly appreciative of the resources you provided to facilitate my research, as well as your unwavering faith in me.

I would like to express my gratitude to my numerous co-workers and friends in Dr. Wu's lab including Omar Larco, Florencia Vasta, Madelaine Clark, Stephen Shannon, Beebee Asamoah-Mensah, Elysia Pagano, and Danette Cruthirds for your assistance with my research project and answering my many questions about lab procedures. In addition, I would like to thank Dr. Niloofar Rezvani, from Dr. Littleton-Kearney's lab and Dr. Andrei Blokhin, from Dr. Casper's lab, for their help and support of my research.

Also, I owe a debt of gratitude to my numerous co-workers and friends in Dr. Stratakis' lab including: Nirmal Gokarn, Dr. George Briassoulis, Dr. Maria Nesterova, Dr. Kitman Tsang, Nima Miraftab, Bilal Naved, Dr. Isaac Levy, Evan Ball, Dr. Kiran Nadella, Anna Batistatos, Melissa Tsang, Emily Sun, Dr. Charalampos Lysikatos, Dr. Evi Xekouki , Dr. Anelia Horvath, Dr. Monalisa Azevedo , Dr. Madson Almeida, Dr. Manolis Saloustrous, Dr. Maya Lodish, Eileen Lange, and Lucy Sierra for your help and support of my research. A special acknowledgement goes to Nirmal Gokarn for helping me get my research program up and running, and to Dr. Maria Nesterova for the endless hours she spent helping me in the lab. To Dr. George Briassoulis, my friend and colleague, I am most grateful for your hard work and dedication to our research. Your enthusiasm tempered with patience, allowed us to accomplish so much together, and your insight and support contributed a great deal to the success of this project.

In closing I would like to thank the faculty and staff of the graduate nursing program, including Dr. Ada Sue Hinshaw, Dean GSN, and Dr. Penny Pierce, Program Director, for their encouragement and support. In addition, many thanks to my classmates and colleagues for their support and friendship during the journey we shared in our graduate studies.

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LIST OF ABBREVIATIONS

ACTH	adrenocorticotropin hormone
ANOVA	analysis of variance
Bp	base pairs
BIS	behavior inhibition system
CA	California
CO2	carbon dioxide
CNC	Carney complex
C-alpha	catalytic alpha subunit
C	celsius
cAMP	cyclic adenosine monophosphate
CNS	central nervous system
CORT	corticosterone
CREB	cAMP response element binding protein
CRH	corticotropin releasing hormone
DHT	dihydrotestosterone
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immuunosorbent assay
EPM	elevated plus maze
ERK	extracellular signal-regulated kinase
GC	glucocorticoid
HZ	heterozygote
HPA	hypothalamic pituitary adrenal
Inc.	incorporated

KO	knock-out
mL	milliliter
mm	millimeter
MAPK	mitogen-activated protein kinase
MANOVA	multivariate analysis of variance
MB	marble bury
MD	Maryland
NC	North Carolina
Ns	non-significant
ng	nanogram
NICHD	National Institute of Child Health and Human Development
NIH	National Institutes of Health
PAG	periaqueductal gray
PVH	paraventricular hypothalamus
PCR	polymerase chain reaction
pg	picogram
PKA	protein kinase A
PKC	protein kinase C
PRKAR1A	protein kinase A regulatory subunit A
PTSD	posttraumatic stress disorder
R1alpha	regulatory one alpha
SAS	statistical analysis software
SEM	standard error of the mean
StAR	steroidogenic acute regulatory protein

T	testosterone
TBST	tris buffered saline tween
μl	microliter
μm	microgram
USUHS	Uniformed Services University of the Health Sciences
VEH	vehicle
VMH	ventromedial hypothalamus
Vs	versus
WT	wild type

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CHAPTER 1

INTRODUCTION

Stress has been defined in many ways, but most commonly is identified as a type of internal or external disturbance that elicits a response in the organism in order to normalize the disturbed function. An appropriate response to an acute stressor is crucial for survival, a sense of well-being, and normal functioning. Stressors above a certain magnitude or multiple stressors occurring simultaneously (traumatic stress) result in alteration of behavior and/or physiology in an organism in order to maintain homeostasis (Stratakis, Gold, & Chrousos, 1995) . Prolonged or repeated exposure to stress often results in physiological, psychological, and behavioral morbidities (i.e. anxiety, depression, post-traumatic stress disorder (PTSD)) in animals and humans. There is abundant evidence to support that vulnerability to these morbidities increases if the stress exposure occurs during periods of critical brain growth (fetal life, infancy, childhood, adolescence).

Types of traumatic stress that has been associated with maladaptive responses or psychopathology, include mass trauma, war, terrorism, natural or technological disasters, violent personal assaults, child abuse (physical, sexual, emotional), life-threatening illness, and accidents. However, not every person who is exposed to traumatic stress will develop long-lasting psychological morbidity, such as depression, anxiety, or PTSD. The development and/or severity of these conditions depends on multiple factors including genetic pre-disposition to vulnerability, exposure to adverse environmental factors, and the

timing of the stress exposure (Charmandari, Kino, Souvatzoglou, & Chrousos, 2003; Kino, De Martino, Charmandari, Mirani, & Chrousos, 2003). 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. The response to an environmental stressor involves the individual's interpretation of the threat, which is regulated by the brain. The brain and nervous system demonstrate adaptive plasticity through local neurotransmitters and systemic hormones, which interact to produce structural and functional changes (McEwen, 2002). The brain is also a target for the actions of stress hormones, in particular glucocorticoids (GC). With stress exposure, the paraventricular nucleus in the hypothalamus releases corticotrophin releasing hormone (CRH) and arginine vasopressin, which stimulate the anterior pituitary to release adrenocorticotropin hormone (ACTH), which stimulates the adrenal cortex to release GC. GC exert a negative feedback to the hypothalamus and anterior pituitary to downregulate the stress response. GC receptors in the brain are found in high density in the hippocampus, amygdala, and prefrontal cortex. Their location facilitates the formation of memories associated with strong emotions particularly during stress.

Numerous studies support that fear memories can form quickly and may be difficult to eliminate (Fanselow & Poulos, 2005; Maren & Quirk, 2004; Phelps & LeDoux, 2005). The amygdala, located in the temporal lobe of the brain, has a crucial role in the processing and expression of emotional stimuli (Davis & Shi, 2000; LeDoux, Iwata, Cicchetti, & Reis, 1988). Prior studies with humans and laboratory animals provide evidence that novelty and fear related stimuli are both processed by the amygdala (Holland & Gallagher, 1999; Knight, Nguyen, & Bandettini, 2005; Rollins, Stines, McGuire, & King, 2001; Wright, et al., 2003). Hyperactivity of the amygdala as demonstrated by functional neuroimaging studies in humans, has been identified

as a neural correlate for clinical symptoms seen in PTSD (Rauch, Shin, & Phelps, 2006; Shin, Rauch, & Pitman, 2006), which suggests that amygdala dysfunction may be a risk factor for development of affective stress-related disorders (Yang, et al., 2008). The basolateral amygdala is identified as a hub through which sensory information is relayed either directly or indirectly via the basal nucleus to the central amygdala, which is the major efferent source that directs fear-related behavioral response (Fanselow & LeDoux, 1999; Maren & Quirk, 2004). (Figure 1)

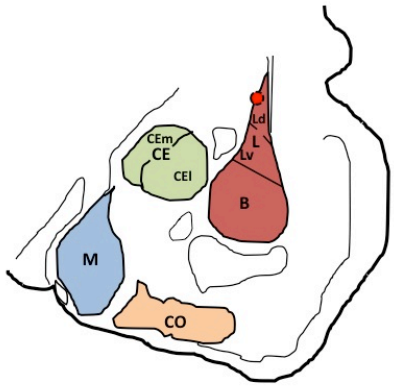

Figure 1: Representations of nuclei and subdivisions of the rodent amygdala (derived from cFos studies) (adapted from Knapska, Radwanska, Werka, & Kaczmarek, 2007)

A maladaptive response to stress, such as PTSD is associated with the inability to inhibit or suppress fear under safe or ambiguous situations as well as with hypersensitivity of the HPA axis response. Recently, Jovanovic (Norrholm, et al., 2010) reported a study of patients with PTSD, which showed that impaired fear inhibition was associated with hypersensitive feedback of the HPA axis, and suggests this may be related to amygdala hyperactivity. The hippocampus, amygdala, and prefrontal cortex play key roles in the formation of memories of stressful stimuli, including fear (Feng, et al., 2002). PTSD has been associated with hyperarousal and an

exaggerated implicit fear memory, which is thought to result from associative fear conditioning and non-associative sensitization processes (Antelman, et al., 1988; Charney, Deutch, Krystal, Southwick, & Davis, 1993; Foa, Zinbarg, & Rothbaum, 1992; Siegmund & Wotjak, 2006; Sorg & Kalivas, 1993). A better understanding of the neurobiological processes involved in the response to unconditioned fear is relevant to the outcomes of either resilience versus pathological responses to stress, such as anxiety and post traumatic stress disorders.

Researchers have invested a great deal of time and effort to develop animal models for human anxiety, depression, and post-traumatic stress in an attempt to define the mechanisms involved with these disorders in order to develop more effective diagnostic criteria and treatment. Animal models of PTSD study symptoms of the disorder that can be divided into those that relate to the memory of the trauma (re-experiencing, avoidance of and enhanced response to cues reminiscent of the trauma) or others that lack an association to the trauma (hyperarousal, irritability, hypervigilance, enhanced startle, emotional blunting, or social withdrawal). These processes are thought to involve different neurobiological substrates (Siegmund & Wotjak, 2006). Yehuha and Antelman (Yehuda & Antelman, 1993) proposed criteria for evaluation of animal models of PTSD in order to develop a systematic approach to assess the relevance of a model in research. These criteria include: the stressor should be capable of inducing biological and behavioral sequelae, the stressor should be capable of inducing sequelae in a dose-dependent manner, the biological alterations produced should last over time (or become more pronounced), the biobehavioral alterations induced should have the potential of bidirectional expression, and inter-individual variability in response to a stressor should be expressed either as a function of experience (e.g. prior stress history) or genetics, or an interaction of the two.

Models that are frequently used to study the effects of stress exposure and subsequent vulnerability to PTSD include: physical restraint, inescapable foot shock, isolation, underwater-holding, predator/predator odor exposure, social defeat, and environmental stressors (e.g. crowding, noise). Restraint stress and inescapable foot shock have been successfully used in experiments designed to assess the effect of chronic or inescapable stress within a learned helplessness paradigm, which has established validity as a model of depression. Some of these models may have ethological validity, but can only be varied in intensity by repetition or prolongation of exposure, which introduces the confounder of habituation (Siegmund & Wotjak, 2007). A predator odor stress model is a relevant paradigm to evaluate neural pathways involved in the processing of response to unconditioned fear, fear memory, and risk factors related to the development of PTSD. A predator odor stress model provides a relevant means to investigate the neural mechanisms involved in the modulation of fear. Predator odor stress is fear provoking and stressful and has been shown to increase anxiety-like behaviors (R. E. Adamec & Shallow, 1993; Berton, Vogel, & Belzung, 1998; D. C. Blanchard, Griebel, Rodgers, & Blanchard, 1998; R. J. Blanchard, M. A. Hebert, et al., 1998; Calvo-Torrent, Brain, & Martinez, 1999; Dielenberg & McGregor, 2001; Kavaliers, Wiebe, & Galea, 1994; Zangrossi & File, 1992) and activate the hypothalamic- pituitary- adrenal-axis (increase corticosterone ACTH) in rodents/small mammals (R. Adamec, Kent, Anisman, Shallow, & Merali, 1998; Belzung, El Hage, Moindrot, & Griebel, 2001; R. J. Blanchard, J. N. Nikulina, et al., 1998; Dielenberg, Hunt, & McGregor, 2001; Hayley, Borowski, Merali, & Anisman, 2001; McGregor, Schrama, Ambermoon, & Dielenberg, 2002). Since predator odor stress has been shown to induce short and long-term changes in behavior, it has been suggested as a model of hyperarousal and generalized anxiety aspects of PTSD (R. Adamec, Muir, Grimes, & Pearcey, 2007; R. Adamec, Strasser, Blundell, Burton, &

McKay, 2006; R. E. Adamec, 1998; R. E. Adamec & McKay, 1993; R. E. Adamec & Shallow, 1993; Cohen, Kaplan, & Kotler, 1999; Cohen, Zohar, & Matar, 2003).

Rodent response to predator odor is a useful model to examine an innate defensive behavior that is elicited by specific predatory cues, which has relevant clinical applications, for example, the preclinical testing of anxiolytic drugs. In addition, predator odor induced anxiety is correlated with human anxiety disorders manifested by retreat, scanning, and risk assessment (Dielenberg, et al., 2001; Dielenberg & McGregor, 2001). Studies utilizing fox urine as a model of predator odor stress have demonstrated stimulation of the HPA axis and neuronal activation as well as behavioral changes (Anisman, Hayley, Kelly, Borowski, & Merali, 2001; Campbell, Lin, DeVries, & Lambert, 2003; Deschamps, Woodside, & Walker, 2003; Funk & Amir, 2000; Marinelli, Quirion, & Gianoulakis, 2004). (Figure 2)

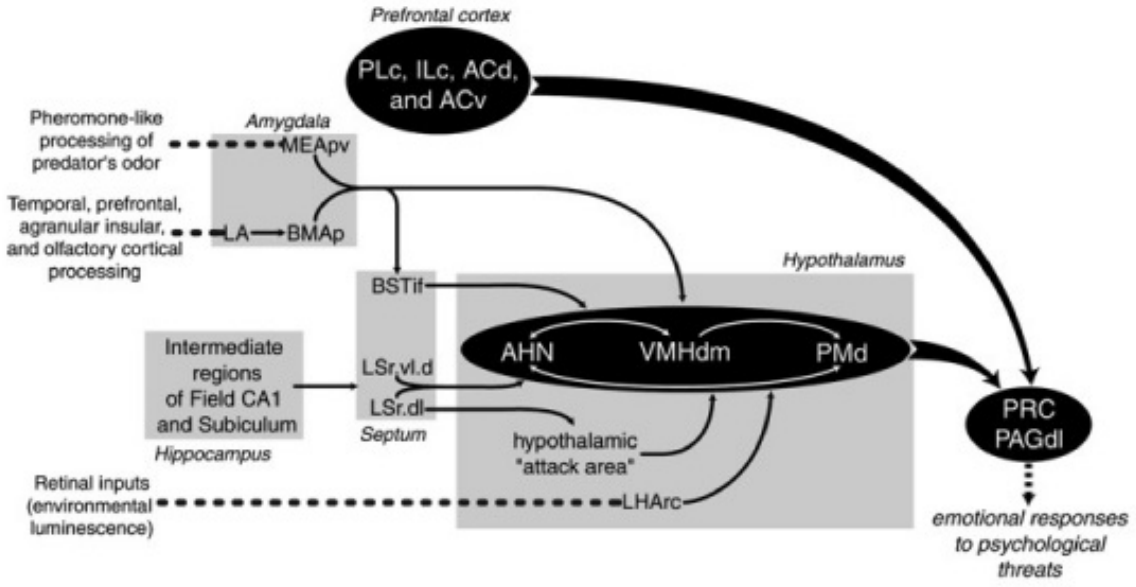

Figure 1. Summary diagram showing the organization of major parallel prosencephalic pathways involved in the control of innate fear responses to psychological threats. Abbreviations – Acd: anterior cingulate area, dorsal part; Acv: anterior cingulate area, ventral part; AHN: anterior hypothalamic nucleus; BMAp: basomedial amygdalar nucleus, posterior part; BSTif: bed nuclei of the stria terminalis, interfascicular nucleus; Ilc: imfralimbic area, caudal part; LA: lateral amygdalar nucleus; LHArc: lateral hypothalamic area, retinoceptive region; LSr.dl: lateral septal nucleus, rostral part, dorsolateral zone; LSr.vl.d: lateral septal nucleus, rostral part, ventrolateral zone, dorsal region; MEApv: medial amygdalar nucleus, posteroventral part; PAGdl: periaqueductal gray, dorsolateral part; PLc: prelimbic area, caudal part; PMd: dorsal premammillary nucleus; PRC: precommissural nucleus; VMHdm: ventromedial hypothalamic nucleus, dorsomedial part. This diagram was originally published in the Hanbook of anxiety and fear, R. J. Blanchard, D. C. Blanchard, G. Griebel & D. J. Nutt (Eds.), Neural systems activated in response to predators and partial predator stimuli, Canteras, N. S. (2008). Amsterdam: Elsevier academic press.

Figure 2. Diagram of major neural pathways involved in the processing of predator odor/psychological threats (Martinez, Carvalho-Netto, Amaral, Nunes-de-Souza, & Canteras, 2008)

Results of these studies provide evidence that fox urine is a stimulus capable of eliciting defensive responses, with quantifiable neurogenic, endocrine, and behavioral changes in rodents. The recent upsurge of research on olfaction and defensive emotions highlights the importance of olfaction in the study of response to unconditioned fear, risk assessment, anxiety, fear sensitization, fear conditioning and extinction, and the testing of pharmacologic agents to treat anxiety (R. Adamec, Burton, Blundell, Murphy, & Holmes, 2006; R. Adamec, Shallow, & Burton, 2005; Apfelbach, Blanchard, Blanchard, Hayes, & McGregor, 2005; Campbell, et al., 2003); however no standard methodology for fox urine as a model of predator odor stress exists.

McNaughton & Corr (McNaughton & Corr, 2004) propose a two-dimensional hierarchal view of defensive behavior and defensive distance (based on Gray's Neuropsychology of Anxiety, 1982), which 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), and the neural control is more elaborated at higher levels of the neural system (prefrontal cortex), and is sensitive to anxiolytics. In addition, anxiety involves inhibitory behavior and increased risk assessment (vigilance). McNaughton and Corr's model defines neural areas involved with fear and anxiety into dual hierarchical levels, both of which include the prefrontal cortex, anterior and posterior cingulated, septo-hippocampal system, amygdala, medial hypothalamus, and periaqueductal gray (McNaughton & Corr, 2004).

The model proposes that overactivity (or hyper-reactivity) of a neural structure is associated with symptoms and pathological syndromes. In the defensive approach hierarchy of the model, the arousal associated with anxiety is controlled by the amygdala (Davis, 1992), and the model predicts that pathologically increased arousal may result in a generalized anxiety disorder. In support of this, the authors cite evidence for direct effects of anxiolytic medications eliciting distinct effects on the two neural targets of anxiety disorders related to arousal (McNaughton & Gray, 2000). The symptoms associated with the activation of distinct neural areas may be a normal adaptive reaction to stimuli, or involve a maladaptive reaction as a result of enhanced sensitivity or activation to stimuli. Both serotonergic and noradrenergic systems modulate the entire defense system, which is supported by the substantial body of evidence from

clinical and preclinical testing of drugs used to treat various aspects of anxiety-related disorders(McNaughton & Corr, 2004). (Figure 3)

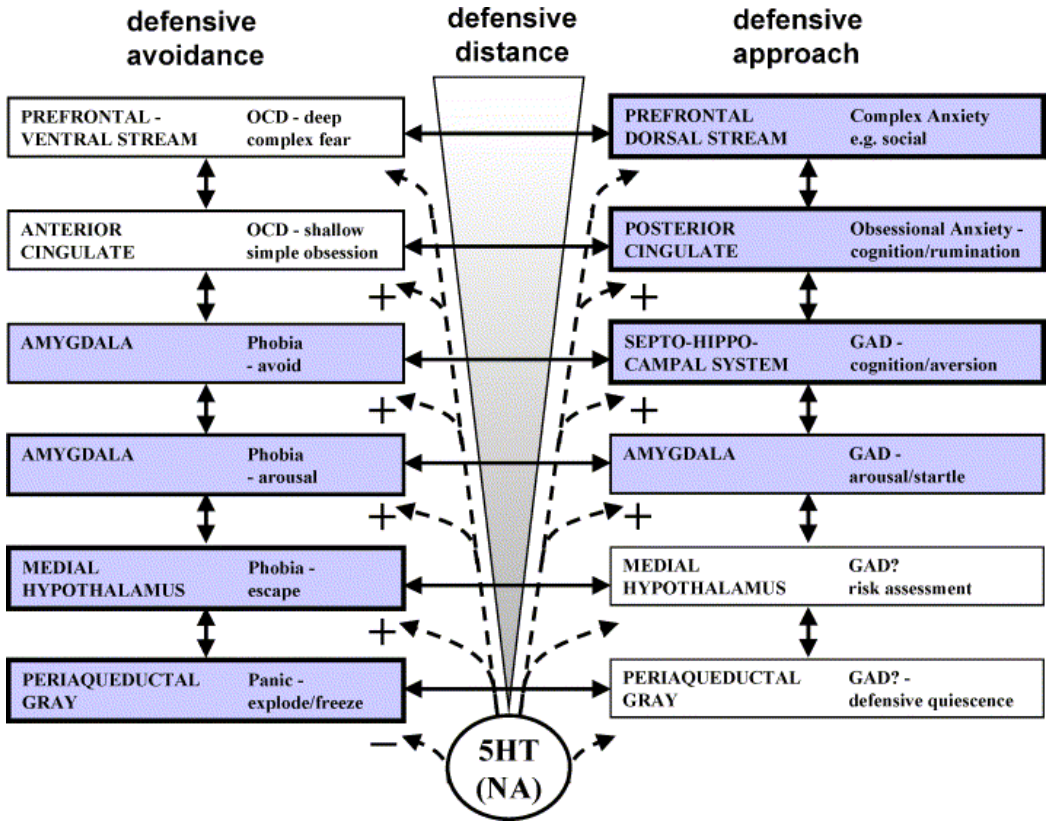

Figure 3. Diagram of two-dimensional defense system proposed by McNaughton and Corr (2004). Categorically, the model is divided into two, with the left side represents defense avoidance, and the right side represents defense approach. Each side has a hierarchal order, high to low (top to bottom), in reference to neural level (a cytoarchitectonic) and functional level, with each level associated with specific classes of behavior/symptoms. The authors propose that syndromes are associated with hyper-reactivity of a structure and symptoms with high activity in the relevant structure(McNaughton & Corr, 2004).

The defense avoidance system (fear) involves fight/flight/freezing behavior, with a goal of movement away from danger/threat, and is insensitive to anxiolytic drugs. However, anxiety functions to move toward danger in approach-avoidance conflict situations, and incorporates inhibition of prepotent behaviors and increased risk assessment, which are sensitive to anxiolytic

compounds. McNaughton and Corr's theory proposes that anxiety is elicited in situations when fear and approach systems are activated simultaneously, and this conflict activates the behavioral inhibition system (BIS), which functions to increase risk assessment risk aversion behaviors. The BIS inhibits prepotent behavior and enhances behavior directed towards conflict resolution. Activation of the BIS is associated with an increased state of arousal and amygdala activation. 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. For example, defensive burying is a behavior in rodents that involves approach to a potential threat and studies have shown that standard anxiolytic compounds suppress this behavior and the associated HPA axis activation(McNaughton & Corr, 2004; McNaughton & Gray, 2000).

Although significant advances have been made in our understanding the neurobiology of fear learning, in particular as it relates to traumatic stress, significant gaps exist in our understanding of the neurobiology of unconditioned fear, including the interaction of risk factors such as abnormal development and/or function of neuronal networks due to genetic or environmental factors. A better understanding of the neural mechanisms involved in the neurobiological response to unconditioned fear, which is an important component of the individual's short and long-term response to stress (i.e. fear sensitization), will lead to the development of more effective treatments for psychological morbidities associated with traumatic stress. Protein Kinase A (PKA) has been identified as having a critical role in the formation of fear memory and alterations in PKA activity have been implicated in psychopathologies, including anxiety and depression. However little is known about the

interaction of the PKA pathway with other risk factors such as abnormal development and/or function of neuronal networks due to genetic or environmental factors (Ghirardi, et al., 1992; Kelly, et al., 2007).

Robinson-White and Stratakis (Robinson-White & Stratakis, 2002) describe PKA signaling as a "central hub," which interacts with various other signaling pathways in endocrine cells. PKA functions as a mediator and communicator of cAMP effects to MAPK, and protein kinase C and B pathways (Robinson-White & Stratakis, 2002). The regulation of the HPA and ANS via signal transduction pathways such as Protein Kinase A (PKA) and Protein Kinase C (PKC), may be important in the expression of genes that contain cAMP in their promoters, which include key proteins that regulate the stress response in the brain (e.g. brain derived neurotrophic factor, glucocorticoid receptor) (Shelton, 2007). These kinase pathways have a time-dependent activation profile in relation to the learning process involving fear memories. In addition, studies using inhibitors or activators of PKA provide evidence to show that it is a crucial intracellular regulator of neuroplasticity in the amygdala (Tronson, Wiseman, Olausson, & Taylor, 2006). The mechanisms of PKA involved in fear memory consolidation and neural plasticity include a wide range of cellular processes, including the activation of cAMP response-element-binding protein (CREB) and other transcription factors involved in the regulation of de novo protein synthesis required for long-term memory formation, and interaction with various intracellular signaling cascades and receptors(Tronson, et al., 2006).

The role of PKA in fear memory formation has been characterized in different processes in several species (Aplysia, Drosophila, mouse, chick, and rat). Numerous experiments were performed with transgenic animals that were unable to express cAMP response-element-binding protein (CREB) or PKA normally, or with PKA inhibitors, and established the essential role of

PKA and CREB for memory formation. In addition, research with pharmacologic agents that interfered with the activity of PKA or CREB helped to clarify the precise timing of the intervention of cAMP, PKA, or CREB in the process of fear memory formation (e.g. short or long term memory). Recent research with transgenic mouse models with inhibitors of protein synthesis or PKA activity demonstrate that inhibition of PKA activity blocks long-term potentiation in the hippocampus and interfere with memory consolidation for fear in the amygdala. Studies with transgenic mice provide evidence to support that increased signaling in the cAMP is associated with an anxiety phenotype and provide indirect evidence that an increase in PKA activity is associated with an increased risk for anxiety.

A recently developed transgenic mouse model with a down-regulation of the Protein kinase AR1α (*Prkar1a*) gene (knock-out) provides a unique opportunity to investigate the effects of increased PKA activity Functionally, a loss of Prkar1a is associated with excess PKA signaling. Using a similar transgenic mouse model, Batista (Batista et al., 2006) reported that mice with inactivating mutations of the 1- α regulatory subunit of the PKA exhibited behavioral abnormalities, including anxiety and depression, which is consistent with the importance of cAMP/PKA signaling in the brain. A transgenic mouse model with down-regulation of Prkar1a provides an opportunity to test the affect of altered PKA expression on the acquisition or expression of learned fear. A better understanding of the neurobiological substrates that are affected by early life stress may lead to the development of more effective treatments. (Figure 4)

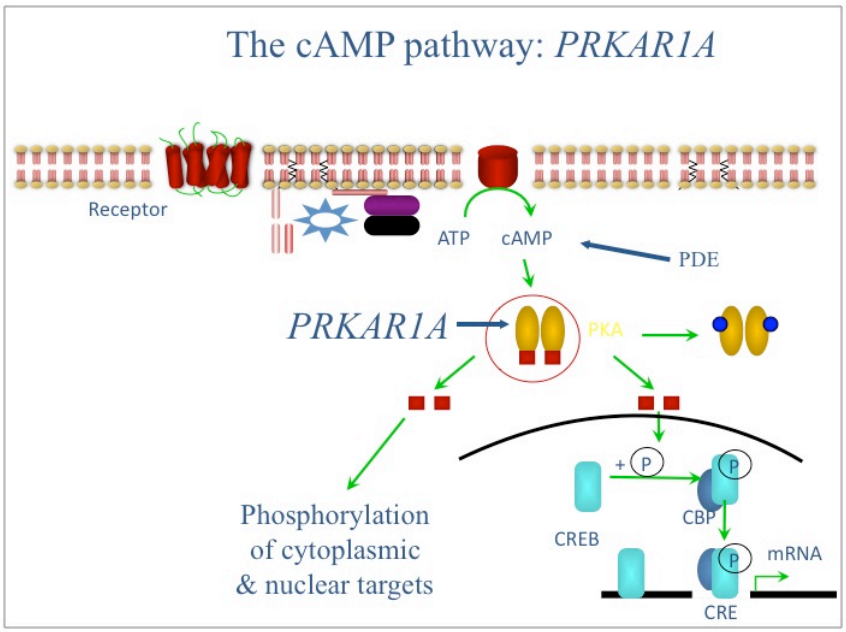

Figure 4. Diagram representing major components of cAMP pathway, including gene responsible for inactivating mutation of the regulatory subunit of (R1a) of PKA. (Adapted with permission from slide of Dr.Constantine Stratakis).

The primary objective of this proposal is to test for the first time the *direct* effect of altered PKA expression on anxiety-like behavior and subsequent response to acute stress. 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 investigate genetic mechanisms underlying neural pathways associated with anxiety and fear-related behaviors and stress vulnerability.

The significance of this study is that a better understanding of the neural mechanisms involved in the formation of fear memories, such as alteration of a neural pathway known to have a crucial role in the processing of fear memory and the stress response, will provide insight regarding differences in vulnerability to psychopathology and/or adaptive or maladaptive responses after exposure to stress. A transgenic mouse with a down regulation of *Prkar1a*

provides an excellent neurodevelopmental model to examine the link between fear sensitization and vulnerability to stress. In accord with McNaughton and Corr's model, it is predicted that *Prkar1a*$^{+/-}$ will exhibit behaviors (symptoms) associated with the activation of distinct neural areas associated with anxiety and will demonstrate a maladaptive reaction to stress as a result of enhanced sensitivity or activation to stimuli(McNaughton & Corr, 2004).

This proposal will test the following overall **hypothesis**:

Mice with a loss of one *Prkar1a* allele, which is associated with an increase in cAMP/PKA activity, will display an anxiety-like behavioral phenotype and increased vulnerability to stress.

Specific aims:

a. To determine the effect of up-regulated PKA expression on anxiety-like behavior in a transgenic mouse model with down-regulation of *Prkar1a.* This will be accomplished by examining the role of increased PKA signaling on anxiety-like behaviors using behavioral assays known to be sensitive to anxiolytics (i.e. defensive marble bury test, elevated plus maze) in *Prkar1a*$^{+/-}$ mice compared to wild-type (WT) littermates. In addition, the expression of PKA activity (basal and cAMP stimulated) in various brain areas will be measured for comparison between *Prkar1a*$^{+/-}$ mice and their WT littermates to investigate any association with the behavioral phenotype.
b. To establish the efficacy of fox urine as a stimulant of the hypothalamic adrenal pituitary (HPA) response and investigate whether there is a dose dependent effect of fox urine to elicit HPA-axis activation. Olfactory stimuli are widely utilized in rodent studies to investigate

behavioral and neuroendocrine responses to stress as well as a model of hyperarousal and generalized anxiety aspects of PTSD. However, a standard methodology for fox urine in a predator odor stress model has not been established. This will be addressed by performance of a dose-reponse study of fox urine (predator odor) compared to vehicle (normal saline) in adult male C57BL/6J mice.

c. To determine the effects of increased PKA signaling on hyperarousal and fear sensitivity and the response to predator odor stress in a transgenic mouse model with the loss of one *Prkar1a* allele. This will be addressed by experiments designed to investigate behavioral and neuroendocrine response to acute stress (fox urine versus vehicle) in a transgenic mouse model with the loss of one *Prkar1a* allele compared to WT controls. In addition, to gain a better understanding of the neural mechanisms involved in the formation of fear memories, the stress induced expression of PKA activity and neural activation (cFos expression) in the amygdala will be measured after stress exposure in male mice with loss of one *Prkar1a* allele and wild-type littermates.

The first manuscript describes the behavioral phenotype of mice with an inactivating mutation of Prkar1a and establishes this as an appropriate mouse model to investigate the direct effect of increased PKA activity on anxiety-like behavior and neural pathways involved in the processing of emotional memory. The second manuscript describes a methodology for fox urine as a predator odor stressor for mice using a dose-response experiment in order to establish a specific dose of fox urine for investigation of behavioral and neuroendocrine response to unconditioned fear. The third manuscript investigates the behavioral response to predator odor stress in Prkar1a heterozygote male mice compared to WT male littermates and evaluates

whether differences in PKA activity and cFos expression in the amygdala are associated with behavioral response to predator odor stress.

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CHAPTER 2

Title: Anxiety phenotype in mice that overexpress protein kinase A

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Short title: Anxiety phenotype in *Prkar1a*$^{+/-}$ mice

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Keywords: Protein kinase A, regulatory subunit, anxiety

Abstract

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 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.

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, which 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 the 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. This 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 (Roa, 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., 1999, Burton et al., 1997).

Studies with Gsalpha transgenic mice have shown that increased cAMP signaling is associated with an anxiety-like phenotype (Favilla et al., 2008). Zhang (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) (Griffin et al., 2004a) exhibited behavioral abnormalities, including anxiety (Batista, 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). 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 compared the expression in PKA activity (basal and cAMP stimulated) in various brain areas in HZ mice and their WT control littermates and possible to the behavioral phenotype.

Methods

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 0600h). 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.

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 two 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. *Prkar1a* $^{+/-}$ mice (which contain one null allele of *Prkar1a*$^{\Delta 2}$) were previously generated in our laboratory (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., 2004b, Amieux et al., 1997, Griffin et al., 2004a, 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.

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 base pair (bp) fragment and the null allele generated an 180 bp product (Kirschner et al., 2005).

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 two hours prior to acclimate to the room prior to the experiment. Standard rodent sawdust bedding was placed in standard mouse cages (38x22x16cm) 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 for 30-min and then the number of marbles buried 2/3 or greater 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 two hours prior to acclimate prior to testing. The EPM consists of two open arms (30 x 5 cm) and two enclosed arms (30 x 5cm), with end and side- walls (15cm height), and a center platform (5 x 5 cm). The maze was raised to a height of 38cm 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). 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.

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 one to two hours 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 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.

5. PKA assay

Prkar1a $^{+/-}$ and WT littermates were moved to testing room two hours prior to euthanization by CO_2. The brains were removed and immediately frozen (-80 ^{o}C) until cyrosectioning. Cyrosections of 250 μm and punch biopsies (0.5mm diameter stainless steel punch) of tissue from the following brain regions were obtained using the punch method of Palkovits (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 (Paxinos, 2001) was used to guide the dissections.

PKA enzymatic activity was measured following the protocol described earlier by Nesterova (Nesterova et al., 1975, Nesterova et al., 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), 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.

6. Statistical analysis

Data were analyzed for effect of genotype and sex by ANOVA and Bonferroni posthoc 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.

Results

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. ANOVA analysis showed an interaction of genotype and sex, with increased marble burying for females and *Prkar1a*$^{+/-}$ mice compared to males and WT littermates (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) (Figure 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 sec., vs. *Prkar1a*$^{+/-}$ 10.1 ± 4.2; $p< 0.05$) (Figure 2) and open to total arm ratio (WT 0.1 ± 0.02 vs. *Prkar1a*$^{+/-}$ 0.04 ± 0.02; $p < 0.05$) (Figure 3). 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 to 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.

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 seconds vs. females: WT 32.6 ±1.9, *Prkar1a*$^{+/-}$ 32.6 ±1.7 seconds; $p<0.05$; n= 15-22 per group) (Figure 4). 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.

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/1mcg, $p<0.03$) and central amygdala (WT 3196 ± 693 vs. *Prkar1a*$^{+/-}$ 10923 ± 3097 –cAMP/1mcg, $p<0.05$) (Figure 5a) and cAMP-stimulated kinase activity (WT 16717 ± 2938 vs. *Prkar1a*$^{+/-}$ 510230 ± 14301 +cAMP/1mcg, $p<0.03$; WT 21448.1 ± 6380 vs. *Prkar1a*$^{+/-}$ 68329 ± 16916 +cAMP/1mcg; basolateral and central amygdala respectively, $p<0.02$) (Figure 5b) 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 (Figures 5a, 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 (Table 1). In both genotypes, however, basal and cAMP-stimulated kinase differed significantly ($p<0.05$) in all studied brain areas.

Discussion

Behavioral responses to novel stressors

The present findings demonstrate that mice with a downregulation 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, 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, Adamec et al., 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 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 & Corr (McNaughton and Corr, 2004) propose 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. 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.

Behavioral response to nocioceptive stimuli

Since the amygdala is known to have a key role in the emotional –affective dimension of pain, the inclusion of a nocioceptive assessment was important to include in the behavioral phenotype of the *Prkar1a* knock-out mouse. Specifically the central nucleus 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 venteromedial 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., 2009, Griffin et al., 2004c, Kirschner et al., 2005, 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.

1. 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 affect 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.

Table

Area	Basal PKA activity (- cAMP Normalized PKA/1 mcg) mean±SEM		p-value	Total PKA activity (+ cAMP Normalized PKA/1 mcg) mean±SEM		p-value
	WT	R1a		WT	R1a	
Ventromedial Hypothalamus	3521± 977	6046±1827	ns	14766±3417	28079±9304	ns
Cerebellum	406±166	301±77	ns	1129±294	616±217	ns
Olfactory	288±235	463±81	ns	6994±6493	6165±2423	ns
Eyes	844 ± 235	762 ± 230	ns	5232±870	4225 ±545	ns

Table 1. Comparison of basal and cAMP-stimulated PKA activity between wild type mice and mice with loss of one *Prkar1a* allele in a control (non-stressed) situation in various brain areas.

Figures

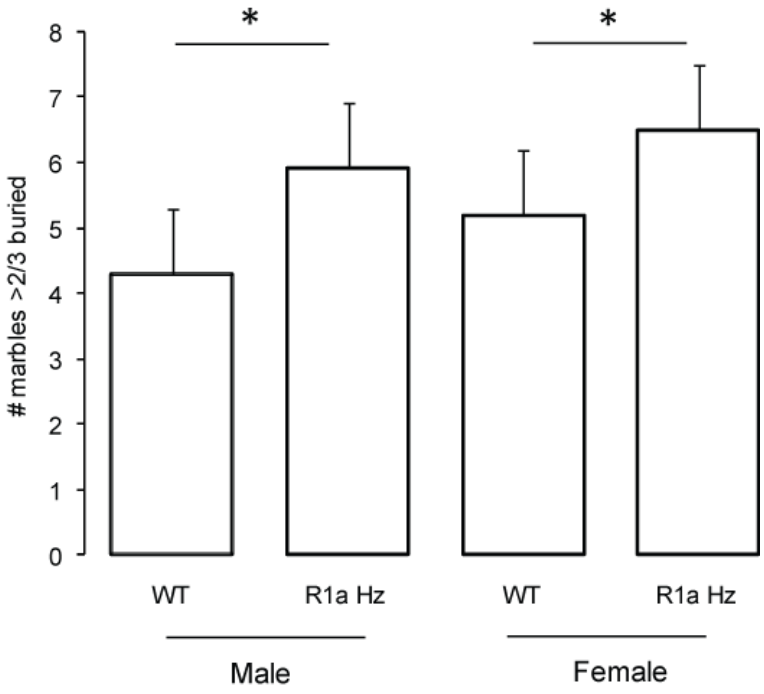


Figure 1. Marble Bury Test. Mean (± SEM) number of marbles buried 2/3 or greater after 30 minutes. *Prkar1a*$^{+/-}$ mice buried significantly more marbles than WT littermates ($p<0.05$).

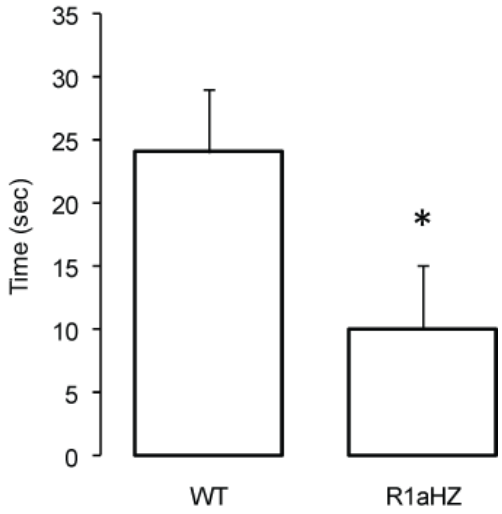


Figure 2. Elevated plus maze: open arm time. Mean (± SEM) time in seconds of time mouse spent in open arm of elevated plus maze test (total test time 5min.) *Prkar1a*$^{+/-}$ mice spent significantly less time in open arm than WT littermates ($p<0.05$).

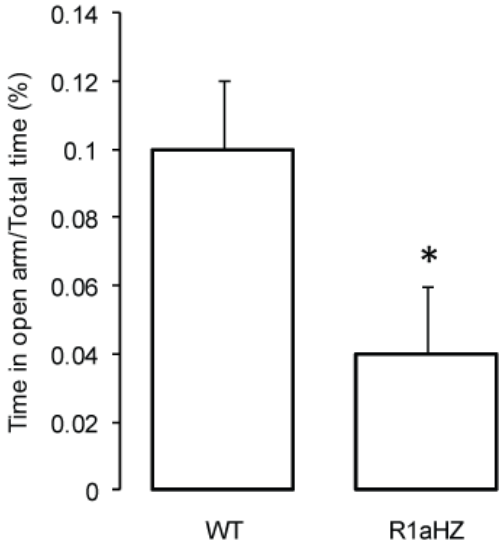


Figure 3. Elevated plus maze: ratio of open arm time to total time. Mean (± SEM) percentage of time that mouse spent in open arm of elevated plus maze test (i.e. open arm time/(open arm + closed arm time). *Prkar1a*$^{+/-}$ mice had significantly lower percentage of open to total time than WT littermates ($p<0.05$).

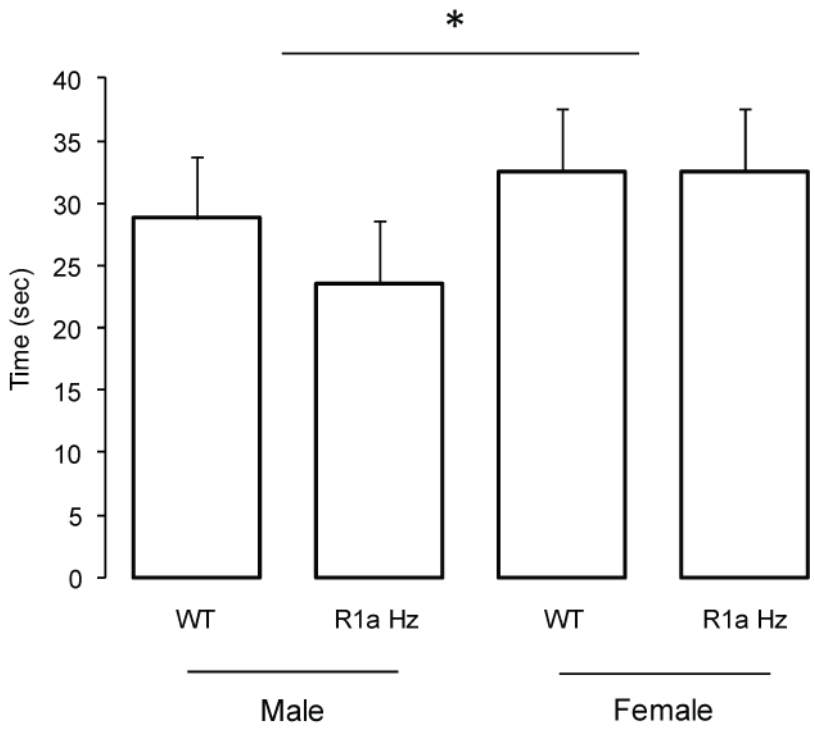
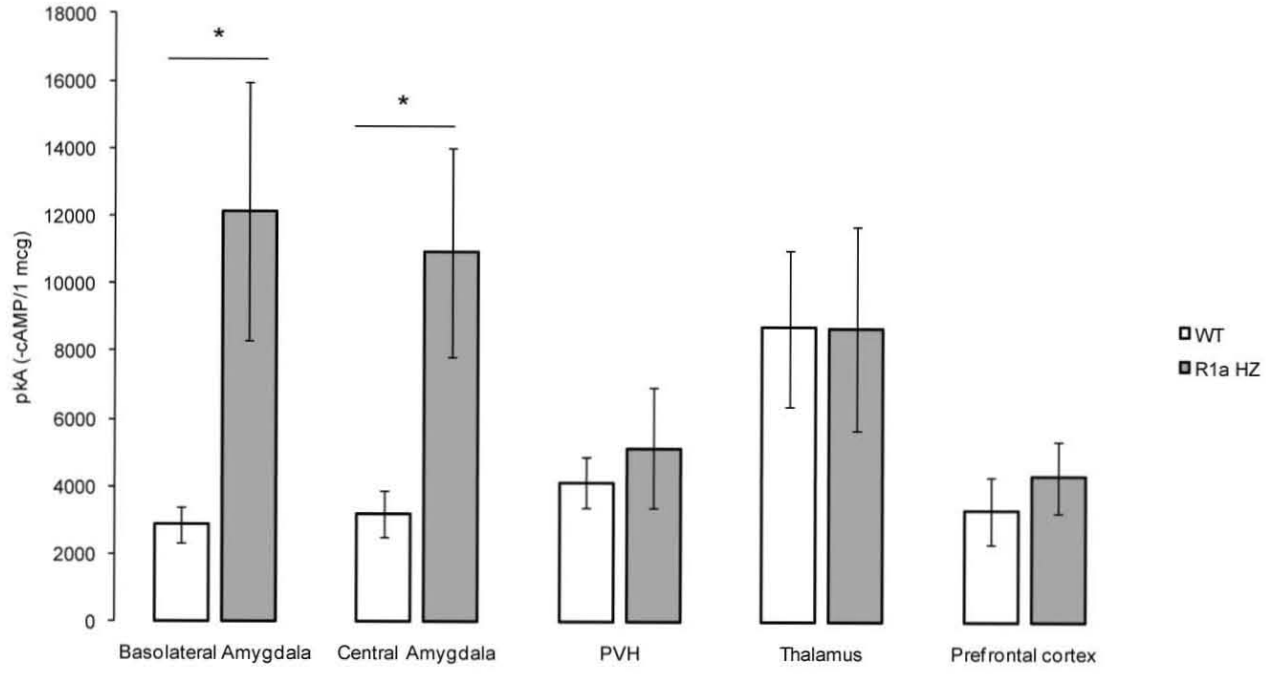


Figure 4. Hotplate test. Mean (± SEM) latency (# seconds) to hindpaw lick for mice during hotplate test. Longer latency associated with higher thermal pain threshold. Female *Prkar1a*$^{+/-}$ and WT mice had significantly longer latency ($p<0.05$).

A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Basolateral Amygdala
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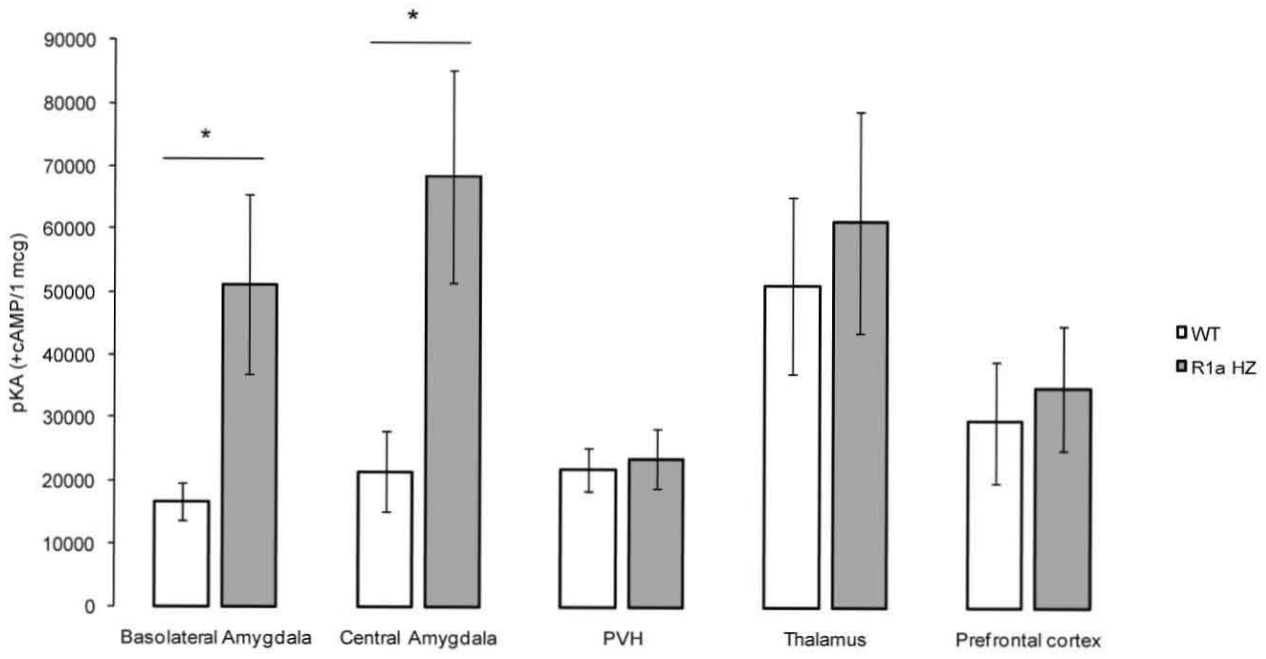
Thalamus
Prefrontal cortex
WT
R1a HZ

B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Basolateral Amygdala
Central Amygdala
PVH
Thalamus
Prefrontal cortex
WT
R1a HZ

Figure 5 a, 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 ($p<0.05$).

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CHAPTER 3

Title: Androgen dependent effects on HPA axis activation in a fox urine stress model in male mice

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Short title: Androgen effects on HPA axis response to predator odor stress

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Keywords: predator odor stress, androgen, hypothalamic pituitary adrenal axis, dose response, fox urine

Abstract

Prior studies document behavioral and neurobiological responses to predator odor in rodents using fox urine; however no standard methodology exists. We evaluated a dose-dependent response of fox urine (predator odor) as a stimulant of the hypothalamic-pituitary-adrenal (HPA)- gonadotropin axis in adult male mice (C57BL/6J). Corticosterone response showed significant interactions for dose and time. Post-hoc comparisons showed a significant difference between the 300µl group and all other groups at all times. There was a significant main effect of time for adrenocorticotropin (ACTH) secretion, but not for dose. Testosterone response showed a main effect for dose, time, and significant interaction for dose and time. Results of basal in vitro androgenesis in the testes showed a significant difference for treatment versus vehicle, with higher in vitro androgenesis in fox urine exposed mice. There was no significant main effect for dose or time for progesterone levels, suggesting a non-adrenal source of androgen response. In conclusion, exposure to predator odor resulted in significant and prolonged activation of serum corticosterone, ACTH, and androgens. The finding of a robust HPA axis response in the vehicle group is consistent with the lack of attenuation, since androgen levels were not altered in this group. Prior studies support the role of androgens in suppression of the CORT response to acute physical or psychological stressors. Evidence for a testicular origin of androgen response is suggested by elevation of in vitro androgenesis in fox urine exposed mice only. Our results suggest that androgen levels may be useful to help differentiate a stress response to novelty versus defense/threat.

Introduction

Ethologically based paradigms such as predator odor exposure are based on an unconditioned response to fear stimuli. Predator odor paradigms are aversive without involving pain and may be used to assess the neuroendocrine response in rodents to threat or danger to elucidate the neurobiological mechanisms underlying fear (45). Prior studies using fox urine as a predator stimulus have reported mixed results with regard to endocrine, neural, and behavioral responses. It is difficult to generalize results from prior studies using fox urine as an aversive stimulus, due to differences in methodologies, including amount of fox urine, presentation of stimuli, and diurnal variation. Therefore, it is not surprising to note that various studies of the endocrine effects of fox urine exposure report either no difference (20, 30), lower (30), or a significant increase in corticosterone (CORT) in rodents exposed to fox urine versus control (7, 23, 38).

Olfactory stimuli are widely utilized in rodent studies to investigate behavioral and neuroendocrine responses to stress as well as learning and avoidance behavior. Psychogenic stressors, such as predator odor, offer an advantage over neurogenic stressors, which has a confounder of physical versus psychological effects. The seminal work of Blanchards (15, 16) using a visible burrow system provided the first detailed description of the response of rodents to cat odor. Predator odor stress is fear provoking and stressful and has been shown to increase anxiety-like behaviors (6, 12, 14, 17, 19, 25, 31, 53) and activate the hypothalamic- pituitary-adrenal-axis (increase CORT, adrenocorticotropin hormone (ACTH) in rodents/small mammals (1, 11, 18, 24, 29, 41). Since predator odor stress has been shown to induce short and long-term changes in behavior, it has been suggested as a model of hyperarousal and generalized anxiety aspects of post -traumatic stress disorder (PTSD) (2-6, 21, 22).

A substantial number of studies have assessed the behavioral and/or neurobiological response to predator odor in rodents using fox urine; however no standard methodology exists. Studies utilizing fox urine as a model of predator odor stress have demonstrated stimulation of the HPA axis and neuronal activation as well as behavioral changes (7, 20, 23, 26, 37). Results of these studies provide evidence that fox urine is a stimulus capable of eliciting defensive responses, with quantifiable neurogenic, endocrine, and behavioral changes in rodents. However, prior studies that used fox urine as a predator odor have presented the odor using different types,

methods, and amounts, making it difficult to compare results across studies. It is likely that differences in intensity or duration of stressors result in qualitative or quantitative differences in behavioral and/or neural responses (14). Studies of acute and chronic restraint stress provide evidence for the role of androgens in the regulation of ACTH and CORT response in rodents (13, 27, 28, 34, 40, 51); however there is a paucity of data regarding the effect of predator odor stress on serum androgen levels and the interaction with the HPA response (8, 9). Although there is a wide body of evidence to support the role of sex hormones in the regulation of the HPA axis, with an excitatory effect of estrogen and inhibitory effect of androgen; little is known about the effect of fox urine and androgen response and the interaction with the HPA axis response.

The purpose of this study was to establish the efficacy of fox urine as a stimulant of the hypothalamic pituitary adrenal gonadotropin axis in mice and investigate whether there is a dose dependent response to various amounts of fox urine, in order to determine the minimum and maximum amount of fox urine required to elicit a significant defensive stress response in mice. The hypothesis is that exposure to predator odor will stimulate the hypothalamic- adrenal- pituitary-gonadotropin axis as demonstrated by an increase in corticosterone, ACTH, and androgens compared to vehicle. The specific aims of this study are to: 1) establish the efficacy of fox urine as a stimulant of the hypothalamic adrenal pituitary-gonadotropin-axis; and 2) investigate whether there is a dose dependent response of fox urine to elicit an HPA axis response.

Materials and methods

1. Animals

Young adult male mice (C57BL/6J, Jackson Laboratories), six weeks age were housed in our animal facility for 14 days after purchase in order to acclimate to the facility. The C57BL/6J strain was selected because they are commonly used for stress studies (Blanchard 1995; Bullock 1997; Anisman 2001). Mice were housed 5 per a standard mouse cage and received *ad libitum* rodent chow and water. The mice were kept on a 12:12 light: dark cycle (light on at 1800). All animals were post-pubertal at the time of testing (8 weeks age). 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.

2. Procedures

2.1 Stress exposure and blood sampling

Mice were placed in the testing room 1.5 h prior to start of the experiment to acclimate to the testing room. A total of 80 mice were used for this experiment. Mice were divided into 4 groups by home cage (n=5 per cage; for a total n=20 per dose group) and a 4 x 4 factorial analysis comparing the effect of dose (vehicle, 30µl, 300µl, 5ml) X time (+ 5, +10, +25, +90 minutes post- start of stress exposure). Each cage of mice was exposed to one of the four doses for 5 minutes after which the swab was removed from the cage. For each dose group, 20 mice (5 per cage) were exposed to cotton swabs with either: vehicle, 30µl, 300µl, or 5mL of fox urine (Buck Stop Lure Co., Stanton, MI) for 5 minutes in the home cage (standard size mouse cage), then at the specified time point one cage (5 mice) were euthanized with CO2, and trunk blood collected (timepoint refers to number of minutes post- start of exposure). The 30µl dose was selected based on Marinelli's (2004) study, which showed differences in beta-endorphin levels in the arcuate nucleus in rodents after exposure. The 300µl was selected based on results of a pilot study in our laboratory, which showed activation of the HPA axis (corticosterone level) in exposed mice. The 5mL dose was selected based on results of studies that showed differences in behavior and HPA –axis after exposure to saturated cottonswabs (20, 43). Samples were

centrifuged at 4° C and plasma removed and stored in -80° freezers until the assays were performed.

2.2 In Vitro incubation and Testosterone and Androgen Assays

Testes from stressed and control mice were quickly removed, decapsulated and placed individually in 24-well plates kept on ice and containing 1 ml medium 199 with Earle's salts, L-glutamine, Hepes and antibiotics, pH 7.4 enriched with 0.1% BSA (M199-0.1% BSA). At the end of isolation, all testes were quickly transferred in new 24-well plates with fresh media M199-0.1% BSA without (left testis from each animal) or with 50 ng/ml hCG (right testis from each animal). Following incubation for 2 h at 34^{o}C in a shaking water bath oscillating at 100 cycles/min under an atmosphere of 95% O_2-5% CO_2, medium and testicular tissue from each well were transferred in sterile 1.5 ml tubes and centrifuged for 10 min at 200 x *g* at 4 C. Individual samples of supernatants were stored at -70° C prior to measurement of androgens levels by enzyme-linked blah following manufacturer's instructions by ELISA (Cayman Chemicals, Ann Arbor, MI, USA) (intra-assay variation 4.4- 19.1%).

Individual testicular tissues were weighed at the end of the experiment and androgen values were expressed as pg/mg tissue. Because the antitestosterone serum has a high cross-reactivity with dihydrotestosterone, assay values are referred to as testosterone + dihydrotestosterone (T+DHT) levels. The same assay was used to estimate serum androgen levels, which were expressed as ng/ml.

2.3 Corticosterone, Progesterone, and Adrenocorticotropin hormone assays

Concentrations of corticosterone (CORT) in mouse plasma were also measured by ELISA (Cayman Chemicals; intra-assay variation 5.6-20.3%) Plasma samples were thawed from -80ºC in ice bath. The plasma samples were diluted 1:1000 concentration (1.5µL minimum volume of plasma used for dilution) using ultrapure water. The samples were heated in a water bath (preheated to 60ºC) for 60 minutes. Progesterone levels were measured by the ELISA (Cayman (interassay variation 4.9- 54.5%) following manufacturer's instructions (1:100 dilution).

Concentrations of adrenocorticotropin hormone (ACTH) in mice plasma were measured by the ACTH Ultrasensitive lumELisa kit (calculated sensitivity <1pg/mL; intra-assay variation 5.6-6.7%). Following manufacturer's instructions, specimens were diluted 1:100 with dilutent supplied by the manufacturer (Calbiotech, Spring Valley, CA, USA).

Results

Data were analyzed using SAS software v 9.2 (SAS Institute, Inc., Carey, NC). Data were assessed for normality, outliers, and homogeneity of variances, and were log transformed as appropriate. Two-way analysis of variance (ANOVA) was carried out by generalized linear modeling, and post-hoc comparisons were corrected by the Bonferroni-Dunn's method. A two-sided alpha of 0.05 was used for statistical significance.

1. Corticosterone

Two-way ANOVA analysis revealed main effect of dose ($p<0.0003$; $F=7.04$) and time ($p<0.0001$;$F=45.3$) and a significant interaction for dose and time ($p<0.0023$; $F=3.23$). Post-hoc comparisons showed statistically significant differences between the 300µl group and all other groups at all timepoints. For all dose groups, the corticosterone level at 90 minutes was significantly lower than all other timepoints ($p<0.05$). (Figure 1)

2. ACTH

Two-way ANOVA indicated a statistically significant main effect for time ($p<0.0001$; $F=13.67$), but not for dose. Post-hoc comparisons showed statistically significant higher values between all timepoints within groups, except for 5min versus each of 10 and 25 min. (Figure 2)

3. Androgens

Results from the two-way ANOVA analysis results revealed a statistically significant main effect for dose ($p<0.0001$; $F=9.26$), time ($p<0.0001$;$F=10.3$), and a significant interaction for dose and time ($p<0.0001$; $F=5.37$). Based on post-hoc comparisons, all fox urine doses versus vehicle were statistically different ($p<0.05$). Additionally, statistically significant differences between the 5min timepoint compared to 10 or 25min ($p<0.05$) were also observed. (Figure 3)

Results of basal in vitro androgenesis in the testes showed significant difference for dose (vehicle vs. 300µl and 5mL, $p<0.05$), with higher in vitro androgenesis (T + DHT pg/mL) in fox

exposed compared to vehicle. No difference was found between timepoints (25 vs 90 min). (Figure 4)

4. Progesterone

There was no significant main effect for dose or time for progesterone levels in any of the groups. (Figure 5)

Discussion

1. Overall effect

Consistent with prior studies, our results demonstrate that brief exposure to fox urine stimulates a robust HPA axis response in adult male mice. Congruent with our hypothesis, fox urine exposure resulted in significant and prolonged activation of CORT, ACTH, and serum androgens compared to vehicle. An increase in CORT and ACTH was found in the vehicle group, demonstrating the ability of novelty to elicit activation of the HPA axis. A robust CORT response was noted in all groups at 5 and 10 minutes (post-start of exposure to odor), and recovery noted by 90 minutes. ACTH levels peaked for all groups at 25min., and decreased thereafter for all groups except the 5mL group, in which the ACTH level remained elevated. A main objective of the present study was to determine if there was a dose effect for HPA activation in response to fox urine and our results showed a significant difference with the 300µl dose group (enhanced CORT response) compared to other doses and vehicle. In contrast, no dose effect was found with ACTH response, but an expected difference between timepoints was confirmed. The CORT response in the30µl and 5mL groups was attenuated compared to vehicle.

2. Androgen effect on HPA axis response

In accord with prior studies, results of our experiment provide support for the role of androgens in attenuation of the stress reactivity of adult males (27, 28, 35, 39, 40, 43, 47-49, 52). Consistent with the known effect of androgens in regulation of the HPA response, the 5mL-dose group showed the lowest CORT levels, with prolonged elevation of ACTH, and significantly elevated serum androgens, compared to the vehicle group. Importantly, the finding of a robust HPA axis stimulation (CORT and ACTH increase) in the vehicle group is consistent with the lack of inhibition of the HPA response, since no change in androgen levels was found. Evidence for a testicular origin of androgen response is demonstrated by elevation of basal (+5min after start of exposure) in vitro androgenesis in fox-stressed mice compared to the vehicle group. In our study no dose or time effect was found for progesterone levels, which suggests a non-adrenal source of the increased androgens seen in the fox-urine exposed groups. There is ample evidence in the literature to support the role of androgens in suppression of the CORT response to acute

physical or psychological stressors; however the mechanism by which androgens inhibit HPA activity is not well defined (27, 28, 35, 39, 40, 47, 50).

Although a number of studies have examined the effects of acute and chronic restraint stress in rodents on the HPA axis including the gonadotropin response there is a paucity of data regarding effect of predator odor stress and interaction of gonadotropin, CORT, and ACTH response to stress. Acute restraint stress (30 minutes) typically results in reduced testosterone levels in males, likely related to inhibition of activity of steroidogenic enzymes (36, 42, 46). In addition, acute immobilization stress has been shown to inhibit testicular steroidogenesis (44). Recently, Kostic (34) reported that Leydig cells develop an adaptive response to chronic restraint stress compared to acute restraint stress in rats, via upregulation of PKA and StAR protein expression. Studies of adult male rodents that manipulated the gonadal axis by castration and testosterone replacement provide evidence to support the significant role of testosterone in attenuating the HPA response under acute stress conditions (13, 48, 49, 51). Future studies are needed to confirm the mechanism of action for the androgen response to acute predator odor stress

Our data suggest that serum androgen levels may be useful to help differentiate a stress response to novelty versus defense/threat. In addition, variations in androgen and testosterone levels are an important factor in the evaluation of HPA response to predator stress. It is possible that the mechanism for the pronounced response of CORT in the vehicle group is related to the timing of the experiment, since this group was tested first and had the least amount of time to acclimate to the stress of the move to the testing room (approximately 1.5 hours). Timing may also have contributed to the mild increase in ACTH response at 90 minutes in all groups, and in particular with the 5mL dose, since this group was tested after all others, and the results could be confounded by the proximity in time to the normal diurnal rise in ACTH. The vehicle group provides an important control, and demonstrates the ability of exposure to a novel object to elicit an increase in CORT and ACTH, without an effect on serum androgen (8-10).

Data from this study suggest that the dose of fox urine is an important variable in the design of experiments to investigate defensive response to unconditioned fear. A dose of 30µl of fox urine was sufficient to elicit a rise a CORT and ACTH response as well as an increase in serum androgen, consistent with a defensive response to threat. However, only the 300µl dose of

fox urine elicited a significant difference in CORT response post-stressor, compared to the other groups. The HPA axis response in the 5mL group indicates that this dose exceeds the threshold, since the results do not significantly differ from the 30µl group. At 90 minutes post-stressor, there was no difference in CORT and androgen levels between groups, consistent with a return to non-stress levels. Inclusion of androgen measurement in predator odor stress paradigms can help to differentiate a stress response to novelty from a defensive response to fear or threat.

In summary, results of our study provide support for the inhibitory effect of androgens on HPA function after exposure to fox urine stress. Future studies are needed to determine the effect of fox urine stress on Leydig cell function, specifically whether the mechanism of action during predator stress involves up-regulation of PKA and StAR protein expression, similar to what has been reported during chronic restraint stress (32, 33).

Acknowledgements

This study was supported in part by intramural graduate student grant from the Uniformed Services University of the Health Sciences (MFK) and Department of Defense grant CO85DP (TJW). It was also supported in part, by the *Eunice Kennedy Shriver* National Institutes of Child Health & Human Development (NICHD) (N.Gokarn, G Briassoulis, S Stojilkovic, CA Stratakis).

We thank Ms. Florencia Vasta, Mr. Nima Miraftab, Mr. Stephen Shannon, Ms.Beebee Asamoah-Mensah for their assistance with this project.

Figures

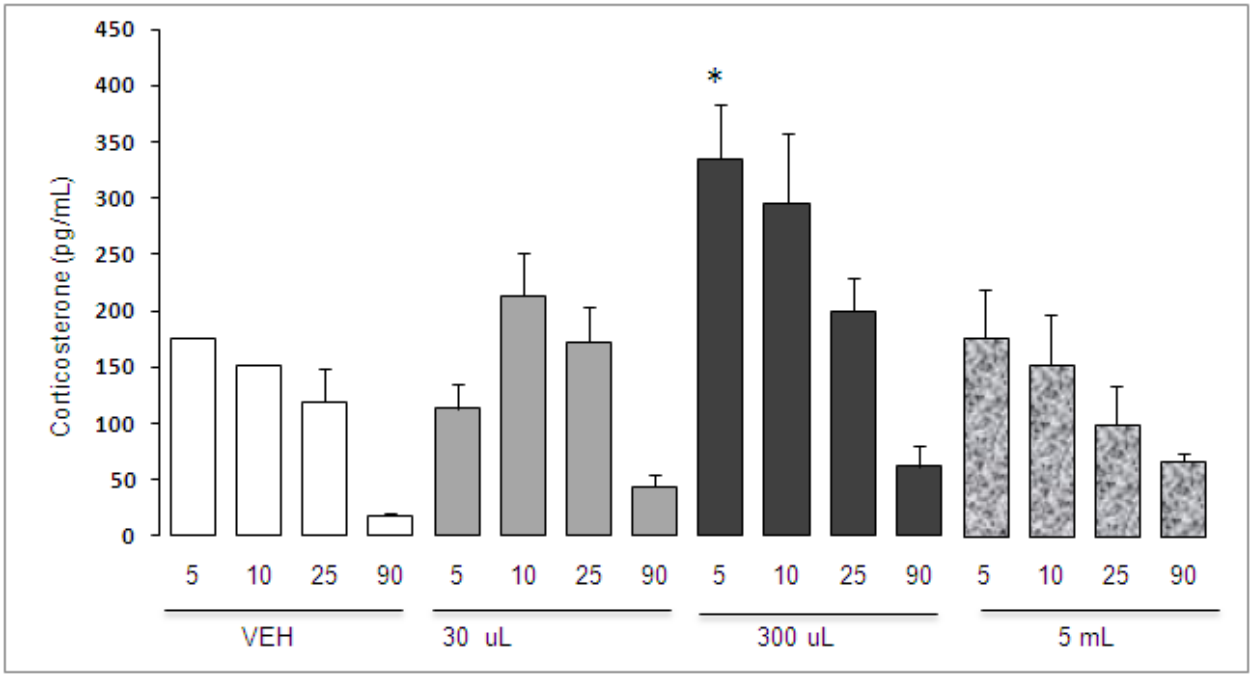

Figure 1. Corticosterone. Means (±SEM) for corticosterone measured at specified timepoints post- odor exposure (+5, +15, + 25, + 90min) grouped by dose (vehicle, 30µl, 300µl, 5mL). A significant main effect was shown for dose, time, and interaction of dose and time ($p<0.05$). The 300µl dose group showed an enhanced response compared to vehicle ($p<0.05$).

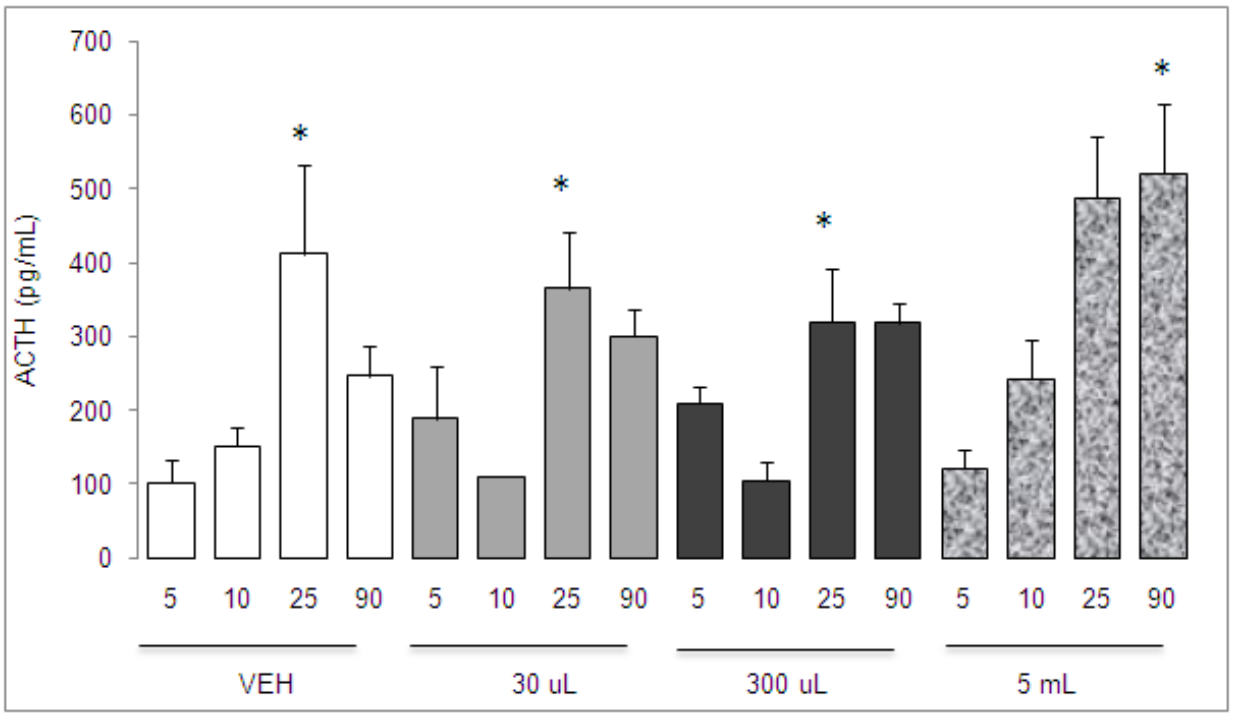

Figure 2. ACTH. Means (±SEM) for ACTH measured at specified timepoints post- odor exposure (+5, +15, + 25, + 90min) grouped by dose (vehicle, 30µl, 300µl, 5mL). A significant main effect was shown for time ($p<0.05$), but not for dose.

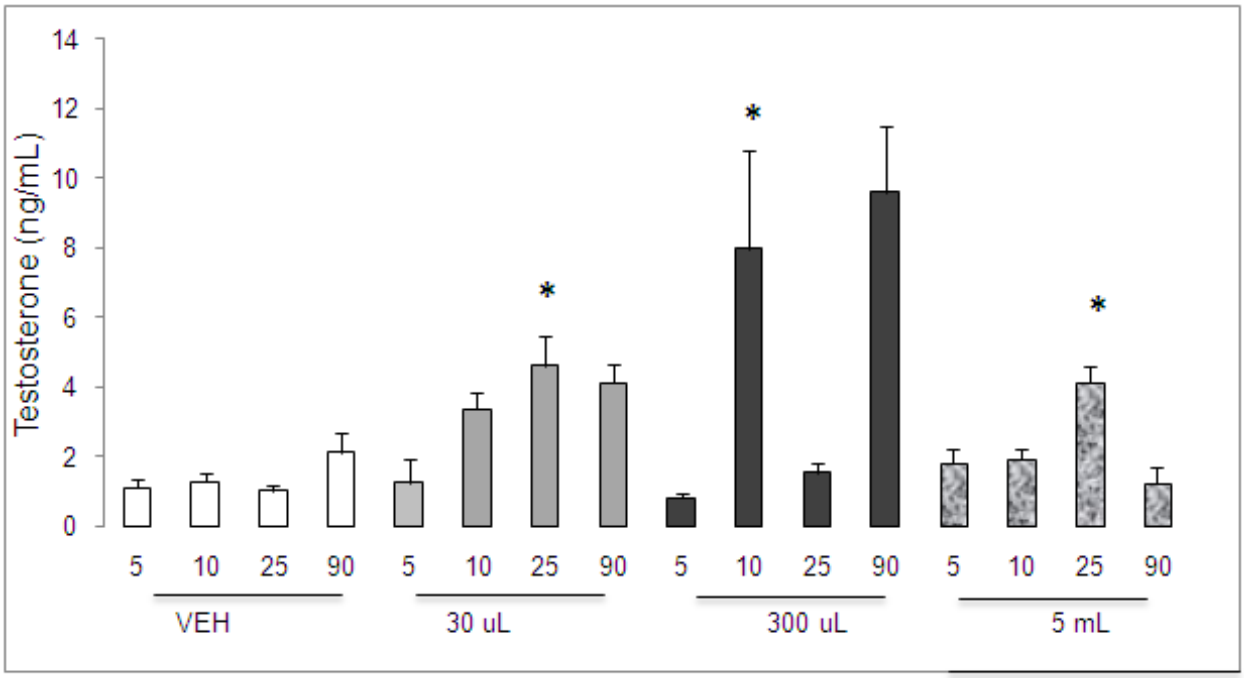

Figure 3. Testosterone (T+DHT). Means (±SEM) for testosterone measured at specified timepoints post- odor exposure (+5, +15, + 25, + 90min) grouped by dose (vehicle, 30µl, 300µl, 5mL). Comparison of testosterone levels showed a significant main effect for dose, time, and an interaction for dose time ($p<0.05$). An increase in testosterone was found in all groups exposed to predator odor, while no change was found in vehicle group.

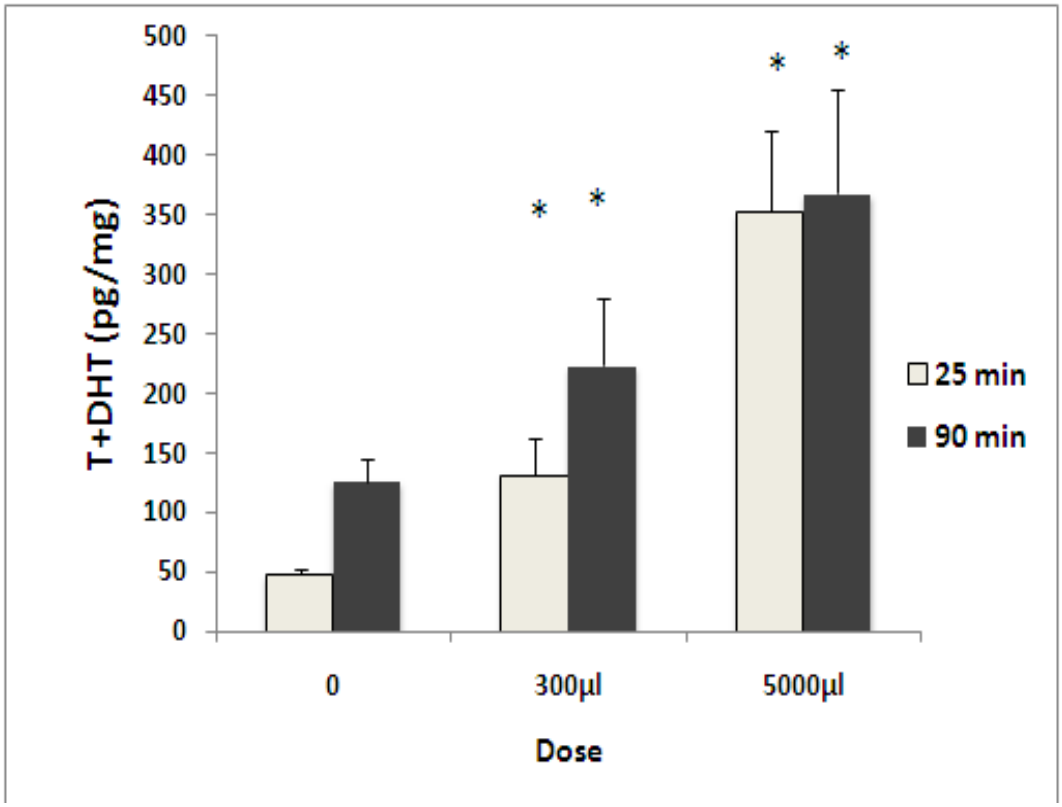

Figure 4. In vitro androgenesis in testes. Means (±SEM) of vitro androgen levels in testes showed a significant effect for dose ($p<0.05$), with higher levels in predator odor exposed compared to vehicle.

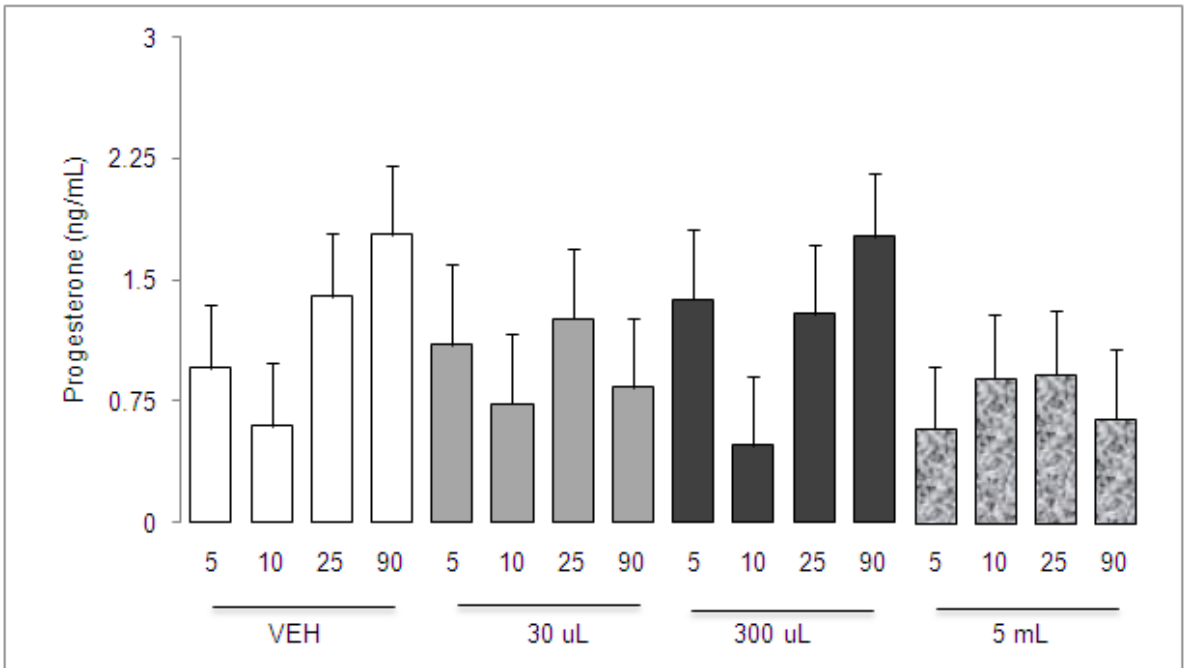

Figure 5. Progesterone. Means (±SEM) for progesterone measured at specified timepoints post- odor exposure (+5, +15, + 25, + 90min) grouped by dose (vehicle, 30µl, 300µl, 5mL).Comparison of progesterone levels showed no difference for dose or time.

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CHAPTER 4

Title: Stress Response in Mice with Inactivating Mutations of *Prkar1a*

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Short title: Predator odor stress in *Prkar1a*$^{+/-}$ mice

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Keywords: Protein kinase A, amygdala, anxiety, Prkar1a, cFos, hyperarousal

Abstract

The role of the cAMP/PKA signaling in molecular pathways involved in fear and memory is well established. We recently reported that a *Prkar1a* heterozygote (*Prkar1a*$^{+/-}$) mouse that was developed in our lab to investigate Carney complex, the disease caused by *PRKAR1A* mutations, exhibits an anxiety-like behavioral phenotype. We measured behavior, neural activation, and PKA activity in brain areas after exposure to predator odor or vehicle in male *Prkar1a*$^{+/-}$ and WT littermates. There were significant differences between *Prkar1a*$^{+/-}$ and WT in the behavioral response to stress. WT mice showed the expected response of decrease in exploratory behavior and an increase in defensive behavior during predator odor *vs*. vehicle. However, *Prkar1a*$^{+/-}$ mice did not show alterations in behavior with exposure to different stressors. Results showed that basal and total PKA activity was independently associated with genotype and stress, with an interaction between genotype and stress. *Prkar1a*$^{+/-}$ mice had higher PKA activity in amygdala and ventromedial hypothalamus in response to predator odor, while PKA activity in orbitofrontal cortex did not differ between stressors. In contrast, WT mice had higher PKA activity in amygdala and orbitofrontal cortex after exposure to vehicle, whereas PKA activity in the ventromedial hypothalamus was higher after predator odor exposure. Results of cFos expression in *Prkar1a*$^{+/-}$ mice showed treatment effect in basolateral amygdala only. 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 stress responses and fear sensitization.

Introduction

The role of the cAMP/PKA signaling in molecular pathways involved in fear and memory is well established (Abel et al., 1997, Bernabeu et al., 1997b, Bernabeu et al., 1997a, Bourtchouladze et al., 1998, Isiegas et al., 2006, Kelly et al., 2007, Huang and Kandel, 1996, Mayford et al., 1996). PKA is a naturally occurred holoenzyme known to have a critical role in anxiety and formation of fear memories. This 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 (Roa, 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).

Prior studies have investigated the role of the cAMP pathway in anxiety with transgenic mice with alterations in cAMP signaling. Studies with Gsalpha transgenic mice have shown that increased cAMP signaling is associated with an anxiety-like phenotype (Favilla et al., 2008). Zhang (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 cited 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.

We recently reported that a *Prkar1a* heterozygote knock-out mouse that was developed in our lab to investigate Carney complex (CNC), the disease caused by *PRKAR1A* mutations, exhibits an anxiety-like behavioral phenotype (Keil, 2010). *Prkar1a*$^{+/-}$ mice showed increased anxiety-like behavior and increased PKA activity in the amygdala compared to wild type littermates. Also, prior studies in our lab reported that transgenic mice with a down-regulated *Prkar1a* gene (Griffin et al., 2004) exhibited behavioral abnormalities including anxiety (Batista, 2005) and depression (Batista, 2006). A transgenic (KO) mouse with an inactivating mutation of *Prkar1a* that was recently developed in our lab provides an opportunity to investigate for the first

time the direct effect of excess PKA signaling on anxiety-like behaviors and fear sensitivity (Kirschner et al., 2005). We focused on anxiety-related behaviors in this mouse model and examined the extent to which predator odor exposure elicits an anxiety-like behavioral response in *Prkar1a*$^{+/-}$ mice compared to WT littermates. In addition we measured PKA activity and neural pathway activation (cFos expression) to determine any association with behavioral response to predator odor stress in male *Prkar1a*$^{+/-}$ mice and WT mice. Predator stress has been used as a model of hyperarousal and generalized anxiety aspects of PTSD and represents a form of psychosocial stress with a principal target of the amygdala (Adamec, 1997, Adamec, 1998, Adamec et al., 2006b, Dielenberg et al., 2001a, Dielenberg et al., 2001b, Figueiredo et al., 2003, LeDoux, 1996, Roseboom et al., 2007, McGregor et al., 2005).

Methods

Animals

Adult male *Prkar1a*$^{+/-}$ and wild-type (WT) mice 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 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 by the Animal Care and Use Committee of NICHD, NIH.

Initial genotyping of founders was by polymerase chain reaction (PCR) 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 base pair (bp) fragment and the null allele generated an 180 bp product.

Procedures

Mice were randomly assigned to one of three treatment groups: (n= 5-15 per group). The groups were (1) handled controls (2) vehicle (cotton swab with physiological saline), and (3) predator odor stress (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 two hours prior to experiment to acclimate to the room change. In a clean cage with 3 cm of sawdust bedding, two cotton- tipped swabs containing either 300ul of fox urine or physiological saline (vehicle) 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 minutes with standard room lighting (overhead fluorescent). The mouse was then removed from the testing cage and placed in a clean cage with water and kept in a quiet area of the test room prior to euthanization.

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Experiment 1

Presentation of fox urine or vehicle was performed as described above. After 5 minutes of exposure to fox urine or saline, the mouse was removed from the cage and placed in a clean standard housing cage with free access to water. The cage was covered and kept in a quiet area of the test room until ninety minutes after the initial presentation of the odor (fox or vehicle), when the mice were euthanized by CO2. The time point of 90 minutes was chosen to coincide with the expected second peak of PKA activity post-stress. Mice in the control group were moved to the testing room in the home cage and kept in a quiet area for 2 hours to acclimate to the room change prior to euthanization. The brains were removed and immediately frozen (-80 C) until cryosectioning.

Experiment 2

After 5 minutes of exposure to fox urine or vehicle, the *Prkar1a*$^{+/-}$ mouse was removed from the cage and placed in a clean standard housing cage with free access to water. The cage was covered and kept in a quiet area of the test room until two hours after the initial presentation of the odor (fox or vehicle), when mice were deeply anesthesized by isofluorane, and perfused transcardially with 4% paraformaldehyde in 0.1M phosphate buffer. The time point of 120 minutes was chosen to coincide with the expected second peak of early immediate gene activation (cFos) post-stress. Mice in the control group were moved to the testing room in the home cage and kept in a quiet area for 2 h to acclimate to the room change prior to euthanization (deeply anesthesized by isofluorane, and perfused transcardially). Brains were processed by immunocytochemistry for cFos expression (see below).

Behavioral response

Four behaviors were scored and summated for the 5-minute time spent in the testing cage. The four behaviors quantified were: 1) number of times the animal reared with weight bearing on hind legs only, with or without upper extremity weight bearing on the side wall of the cage (vertical position), (2) number of digging episodes, (3) number of grooming episodes, (4) number of times the mouse touched the beaker containing the swabs. The behavioral parameters

selected were based on the work of Blanchard and Blanchard as components of behavior that typically change in response to exposure to a predator or predator odor. Predator odor is associated with a reduction of overall locomotor activity (exploratory) as well as suppression of non-defensive behaviors (grooming, foraging, and reproduction) (Dielenberg and McGregor, 2001, Blanchard and Blanchard, 1989). Stretch-attend (approach of stimuli) and defensive burying (fling cage bedding toward object) behaviors are considered reliable measures of animal fear and anxiety.

Histological procedures

Whole brains were sectioned at 250 μm and punch biopsies (0.5mm diameter stainless steel punch) of tissue from the following brain regions were micro-punched based on outlines in the Allen brain atlas of the mouse (Dong, 2008) as follows: basolateral, central, and medial amygdala, bilateral punches corresponding to Figs. 64-70; ventromedial and paraventricular hypothalamus, and thalamus, bilateral punches corresponding to Figs.64 and 70 (Bregma -0.955 to -1.555mm), and orbitofrontal cortex, bilateral punches corresponding to Figs. 64 and 70 (Bregma -0.955 to -1.555mm). Olfactory bulb and cerebellum were dissected in entirety and stored in cryotubes at -80 prior to homogenization and measurement of PKA activity. The mouse brain atlas of Allen (Dong, 2008) was used to guide the dissections.

Immunocytochemistry

Brains were sent to Histoserv Inc. (Germantown, MD) for immunohistochemistry processing. Briefly, brains were dehydrated and cleared with xylene and infiltrated with paraffin. Coronal slices of 10um thickness were mounted on slides and the procedure for IHC consisted of: deparaffinization, distilled water wash, pre-treatment of slides at 70˚C for 40 min, distilled water wash, hydrogen peroxidase blocking, distilled water wash, bovine serum albumin blocking, tris buffered saline-tween (TBST) wash, primary antibody (c-Fos from abCam #7963) @ 1:100, TBST wash, secondary antibody (Biotinylated Rabbit IG Vector #BA1000), TBST wash, Streptavidin-HRP, tris buffered saline-tween wash, develop slides with DAB (Diaminobenidine), dehydrate, clear, and coverslip (Dilutent for Primary & Secondary antibody is 1% BSA).

Quantification of cFos-positive neurons

The mouse brain atlas of Allen brain atlas of the mouse (Dong, 2008) was used to identify corresponding Bregma location for all cFos stained slides. Slides were coded prior to the analysis in order to blind the experimenters to the treatment condition. The basolateral, central, and medial amygdala areas were identified by following the published coordinates in the Allen brain atlas (Dong, 2008) Figs.64 and 70 (Bregma -0.955 to -1.555mm). Three to four slides per brain containing the amygdala regions were examined. For each section, slides were photographed at 100x magnification with a Leica microscope/camera (DC500) and files created by Image J (NIH) for personal computer (Rasband, 1997-2009). For the central, and medial amygdala, a 100x 300μm circular area, and for the basolateral amygdala a 280 x 365μm oval area was created with Image J and the number of cFos positive neurons in each area was hand counted and recorded.

PKA assay

PKA enzymatic activity was measured following the protocol described earlier by Nesterova (Nesterova et al., 1975, Nesterova et al., 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), 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.

Statistical analysis

The results were analyzed using SPSS software. 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 *posthoc* comparisons where appropriate. All values are reported as means ± SEM. Behavioral measures during the stress exposures were analyzed by a multifactorial ANOVA, with between subject factors of genotype and treatment.

For PKA and cFos data to confirm any differences between groups of conditions, a two (group: WT, *Prkar1a*$^{+/-}$) x three (treatment: predator odor (fox urine), vehicle) ANOVA was performed, and post-hoc comparisons with Bonferroni correction where appropriate. Significance was determined at $p < 0.05$.

Results

Behavioral response: fox urine versus novelty stress (Figure 1).

There were significant differences in the behavioral response of male *Prkar1a*$^{+/-}$ and WT mice to predator odor (fox) vs. novelty (vehicle) stress conditions. *Prkar1a*$^{+/-}$ mice did not show alterations in behavior (exploratory or defensive) with exposure to different stressors (predator vs. vehicle) compared to WT littermates. WT mice showed the expected response of decrease in exploratory behavior (vertical postures) during predator (fox urine) *vs*. novelty (vehicle) stress ($p<0.02$). ANOVA analysis showed genotype ($p<0.001$); and stressor ($p<0.03$) effects for digging, with *Prkar1a*$^{+/-}$ engaging in more digging episodes compared to WT mice ($p<0.001$). In addition, *Prkar1a*$^{+/-}$ mice did not alter defensive behavior (digging) with exposure to different stressor types, while WT mice showed the expected decrease in defensive behavior during exposure to vehicle stress ($p<0.03$). ANOVA analysis showed a similar trend for decreased latency to contact swabs and an increase in number of contacts with the cotton swabs during predator odor compared to vehicle exposure for both WT and *Prkar1a*$^{+/-}$ mice ($p<0.08$). Neither *Prkar1a*$^{+/-}$ nor WT mice showed a change in grooming behavior (non-defensive behavior) during predator vs. novelty (saline) stress. Overall locomotion was not measured, however little, if any stationary/freezing behavior was observed with WT or *Prkar1a*$^{+/-}$ mice during the time in the testing cage, which is consistent with prior studies of threat exposure (Blanchard and Blanchard, 1989, Blanchard et al., 2000).

Differences between WT and Prkar1a$^{+/-}$ *in control condition (Table 1).*

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 control (non-stressed) situation. The loss of one *Prkar1a* allele led to an increase in basal kinase activity in the central amygdala (WT 100 ± 54 vs. *Prkar1a*$^{+/-}$ 702 ± 144 –cAMP/1mcg; $p<0.003$,$F=22.5$), basolateral (WT 171 ± 82 vs. *Prkar1a*$^{+/-}$ 1017± 417 –cAMP/1mcg; $p<0.04$,$F=6.8$), and thalamus (WT 52 ± 11 vs. *Prkar1a*$^{+/-}$ 507 ±87–cAMP/1mcg; $P<0.001$, $F=38.4$) compared to the WT mice. In the controls, the loss of one *Prkar1a* allele also led to an increase in increase in cAMP-stimulated kinase activity in the central amygdala (WT 2039 ± 536 vs. *Prkar1a*$^{+/-}$ 6712 ± 679 +cAMP/1mcg; $p<0.002$, $F=28.8$), basolateral amygdala (WT 2639 ± 819 vs. *Prkar1a*$^{+/-}$ 7757 ±

1289 +cAMP/1mcg; $p<0.012$, $F=12.6$), and thalamus (WT 1235 ± 591 vs. *Prkar1a*$^{+/-}$ 5491 ±725–cAMP/1mcg; $p<0.001$, $F=20.4$) compared to the WT mice (n=5-12 per group). No genotype effect was seen for the other brain or sensory areas measured.

Differences in PKA activity in amygdala areas of WT and Prkar1a$^{+/-}$ *mice (Figures 2a, 2b: Central amygdala; Figures 2c, 2d: Basolateral amygdala).*

Multivariate AVOVA analysis of basal and total PKA activity in amygdala areas (basolateral, central, and medial) revealed a main effect for genotype ($p<0.001$; $F=14.37$), treatment ($p<0.005$; $F=3.213$), and a significant interaction for genotype and treatment ($p<0.014$; $F=2.73$). Post-hoc comparisons showed significant differences in central amygdala ($p<0.05$), and basolateral amygdala ($p<0.05$). In the medial amygdala, a significant main effect was shown for genotype in basal PKA activity ($p<0.01$;$F=7.894$); however no treatment effect was shown.

In WT mice, post-hoc comparisons showed a significant difference in central amygdala (basal PKA activity: predator odor vs. controls; total PKA activity: vehicle vs. control; $p<0.05$); basolateral amygdala (basal and total PKA activity: vehicle vs. control; $p<0.05$). In *Prkar1a*$^{+/-}$ mice, post-hoc comparisons showed a significant difference in central amygdala (basal and total PKA activity: predator odor vs. control; $p<0.05$); and basolateral amygdala (basal and total PKA activity: predator odor vs. control; $p<0.05$).

Differences in PKA activity in hypothalamic and thalamic nuclei nuclei in WT and Prkar1a$^{+/-}$ *mice (Figures 3a, 3b: Thalamus; Figures 3c, 3d: Ventromedial hypothalamus).*

Results of multivariate ANOVA analysis of PKA activity in thalamus and hypothalamus areas (VMH, PVH) revealed a statistically significant main effect for treatment group ($p<0.006$; $F=6.562$); a trend for genotype ($p<0.066$; $F=7.295$); and a significant interaction for genotype and treatment ($p<0.016$; $F=4.906$). Genotype and treatment effect was shown for total PKA activity in thalamus and VMH ($p<0.05$) and an interaction of genotype and treatment was noted for total PKA activity in VMH ($p<0.05$). There was no significant main effect for paraventricular hypothalamus.

In wild type mice, post-hoc comparisons showed a significant difference in thalamus (basal PKA activity: predator odor vs. control; total PKA activity: predator odor vs. vehicle and control,

$p<0.05$); ventromedial hypothalamus (basal PKA activity: treatment groups vs. control, $p<0.05$; total PKA activity: predator odor vs. vehicle and control $p<0.05$). In *Prkar1a*$^{+/-}$ mice, post-hoc comparisons showed a significant difference in thalamus (total PKA activity: treatment groups vs. control, $p<0.05$); and ventromedial hypothalamus (basal PKA activity: predator odor vs. control, $p<0.05$; total PKA activity: treatment groups vs. control, $p<0.05$).

Differences in PKA activity in other brain areas in WT and Prkar1a$^{+/-}$ *mice (Tables 2, 3).*

ANOVA analysis showed a significant main effect for treatment ($p<0.001$; $F=8.057$) and genotype ($p< 0.013$; $F=6.86$) in basal and total PKA activity in the orbitofrontal cortex. In wild type mice, significant differences were shown in basal PKA activity (predator odor vs. control, $p<0.05$) and total PKA activity (vehicle vs. control, $p<0.05$) in the orbitofrontal cortex; whereas in *Prkar1a*$^{+/-}$ mice there was no significant difference between treatment groups. ANOVA analysis showed a significant main effect for treatment in basal and total PKA activity in olfactory bulb ($p<0.0014$, $F=7.288$; $p<0.001$, $F=12.161$, respectively). Post-hoc comparisons showed a significant difference for basal and total PKA activity in olfactory bulb between predator odor vs. control groups in both wild type and *Prkar1a*$^{+/-}$ mice ($p<0.05$). There was no significant main effect for PKA activity levels in cerebellum.

cFos activity in amygdala areas of Prkar1a$^{+/-}$ *mice: treatment vs. controls (Figures 4 & 5a, 5b).*

We compared cFos expression in amydala regions of *Prkar1a*$^{+/-}$ mice after stress exposure (predator odor, vehicle) versus handled controls. ANOVA analysis showed an overall significant difference for amygdala area ($p<0.001$; $F=30.5$), treatment group ($p<0.04$; $F=5.16$), and an interaction between amygdala area and treatment group ($p<0.001$;$F=7.86$). Post-hoc comparisons showed significantly higher cFos expression in the basolateral amydgala after predator odor ($p<0.001$) and vehicle ($p<0.001$) exposure, compared to the control groups (predator odor 41.2 ± 2.5, VEH 32.1 ± 2.3, control 23.9± 2.5; $p<0.004$) A significant difference (higher) was found in medial versus central amygdala cFos expression ($p<0.001$), although there was no difference between treatment groups.

Discussion

Based on findings of laboratory and field studies it was expected that exposure to predator odor would reduce exploratory and non-defensive behavior, which the behavioral response of the WT mice in this study confirmed (Blanchard and Blanchard, 1989, Dielenberg and McGregor, 2001, Farmer-Dougan et al., 2005, Blanchard et al., 2000). However, we detected a behavioral effect of the loss one *Prkar1a* allele as observed by the failure of *Prkar1a*$^{+/-}$*mice* to exhibit behavioral changes (exploratory or defensive) to distinguish between predator odor vs. novelty stress conditions. 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 threatening situation. These findings are consistent with results of prior studies in our lab that reported an anxiety-like behavioral phenotype of *Prkar1a*$^{+/-}$*mice,* which was associated with up-regulated PKA activity in the amygdala compared to WT littermates (Keil, 2010).

As expected, PKA activity increased after exposure to a stressor (vehicle or predator odor) for both WT and *Prkar1a*$^{+/-}$ mice in the basolateral and central amygdala, thalamus, ventromedial hypothalamus, orbitofrontal cortex, and olfactory bulb. However, an interesting difference in the pattern of PKA activity response was found in various brain areas between WT and *Prkar1a*$^{+/-}$ mice. For WT mice, basal and total PKA activity was higher in the amygdala and orbitofrontal cortex after exposure to vehicle, whereas total PKA activity was higher in the ventromedial hypothalamus and thalamus after predator odor. In contrast, *Prkar1a*$^{+/-}$ mice showed higher PKA activity in the amygdala after predator odor exposure compared to vehicle, and no difference in PKA activity in orbitofrontal cortex between predator odor vs. vehicle groups (although levels in treatment groups were higher than controls). Also, in *Prkar1a*$^{+/-}$ mice total PKA activity in the ventromedial hypothalamus was higher in vehicle compared to the predator odor group, which is contrast to the findings with the WT mice. These findings suggest that these brain areas are differentially affected by the loss of one *Prkar1a* allele and subsequently may have a role in mediating the behavioral response to stress. The finding of a significant difference in the pattern of response in PKA activity between WT and *Prkar1a*$^{+/-}$ mice in the orbitofrontal cortex, and ventromedial hypothalamus is congruent with the crucial

roles of both the ventromedial hypothalamus in defensive response and the prefrontal cortex control of the amygdala

Results from our study support finding of prior studies, which endorse the importance of the basolateral amygdala in mediating the response predator odor stress, shown by an increase in cFos expression after treatment exposure (predator odor or vehicle), compared to controls. Overall, the higher expression in cFos expression in basolateral and medial amygdala versus central amygdala, supports data from prior studies that the behavioral response to uninhibited fear is mediated by the basolateral and medial amygdala, not the central amygdala. However, the finding of increased cFos activity in *Prkar1a*$^{+/-}$ *mice* in the basolateral amygdala after fox exposure does not parallel with the behavioral data that showed no differences in responses between stress conditions, but is consistent with increased sympathetic tone and hyperarousal. The finding of a difference in cFos activation in the basolateral amygdala in conjunction with a lack of distinction in the behavioral response between treatment groups in *Prkar1a*$^{+/-}$ mice, suggests that neural mechanism(s) downstream from the basolateral amygdala also contribute to the response to unconditioned fear. The posterior part of the basolateral amygdala projects to the bed nucleus stria terminalis, as well as to the medial hypothalamic defense circuit (Martinez et al., 2011, Sah et al., 2003). In addition, the orbitofrontal cortex also contributes to the regulation of defensive behavior through connections to the periaqueductal gray (PAG) and basolateral amygdala (LeDoux, 2003, Davis and Whalen, 2001, Mota-Ortiz et al., 2009). Alterations in the medial hypothalamic defense circuit, orbitofrontal cortex, precommissural nucleus, and/or PAG due to chronic upregulation of PKA are likely candidates involved with the atypical response to stress shown in *Prkar1a*$^{+/-}$ mice. Unlike results of lesion studies of both medial and basolateral amygdala that show reduction or ablation of defensive behaviors; results of our study indicate that up-regulation of PKA activity in the amygdala and other brain areas associated with processing of emotional stimuli, results in hyperarousal behavior.

In this study, it is interesting to note that the results of PKA activity show a significant difference between genotype and treatment group in the basolateral amygdala that parallel the cFos differences shown in *Prkar1a*$^{+/-}$ mice. These results suggest that the alteration in PKA signaling in *Prkar1a*$^{+/-}$ mice has tissue-specific effects in the cAMP/PKA pathway, which are likely related to the altered response to stress and hyperarousal behavior. These findings are

consistent with results of prior studies, which have shown increased amygdala activity during certain type of emotional stimulation is associated with maladaptive response to stress (Goosens and Maren, 2004, Fecteau et al., 2007, Phelps and LeDoux, 2005). Possible mechanisms mediating the behavioral response of *Prkar1a*$^{+/-}$ mice include structural and or functional variations in amygdala circuitry, compensatory changes in the PKA-pCREB pathway, alterations in neurotransmitter signaling, and/or interaction with other brain areas involved in inhibition of the amygdala related to chronic up-regulation of PKA activity. Results of electrophysiology studies suggest that changes in 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., 2005, Adamec et al., 2006a, Adamec et al., 2004). Alterations in PKA activity in the orbitofrontal cortex may also contribute to the atypical response to stress in *Prkar1a* heterozygote mice since a key function of the orbitofrontal cortex is to inhibit prepotent behaviors and promote task relevant operations (i.e. top-down regulation). 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 stress conditions, which was seen in WT littermates.

In conclusion, 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 basolateral and central amygdala, ventromedial hypothalamus, and orbitofrontal cortex as evidenced by up-regulation and altered pattern of PKA activity in these areas in *Prkar1a*$^{+/-}$ mice compared to WT littermates. These results support our prior findings that increased PKA activity noted in *Prkar1a*$^{+/}$ controls is related to a behavioral phenotype of anxiety leading to a dysregulated response to stress. An amygdala-localized effect on anxiety-like behavior associated with the loss of one *Prkar1a* allele is consistent with extensive data on the role of the amygdala as a mediator of the effects of stress and emotional arousal (Blanchard and Blanchard, 1972, Amaral et al., 2003, Davis and Whalen, 2001, LeDoux, 2000, Kalin et al., 2004). However, the loss of one *Prkar1a* allele was not a ubiquitous effect since PKA activity in other areas such as the paraventricular hypothalamus, olfactory, and cerebellum was similar between WT and *Prkar1a*$^{+/-}$controls. In addition, results of cFos expression support findings of

other studies for the role of the basolateral amygdala in mediating the response to predator unconditioned fear.

The neurobiological mechanisms that underlie the behavioral response to unconditioned fear to predator stimuli are not well defined. There is ample evidence to support that the formation of a strong aversive memory tract is an important pathogenic mechanism for the development of anxiety disorders including post-traumatic stress disorder (de Quervain et al., 2009, Roozendaal et al., 2009). Our findings suggest that the effect of increased PKA signaling in *Prkar1a* mice is associated with anxiety and hyperarousal symptoms. These results are consistent with prior studies of transgenic mice with increased cAMP signaling that report an association with anxiety-like behavior (Kelly et al., 2008, Favilla et al., 2008, Zhang et al., 2008). In addition, these 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, Bourdeau et al., 2005). 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 post-traumatic stress disorder.

	WT	*Prkar1a*$^{+/-}$	p-value	WT	*Prkar1a*$^{+/-}$	p-value
Central amygdala	100±54	702±144	p<0.003	2039±536	6712±679	p<0.002
Basolateral amygdala	171±82	1017±417	p<0.04	2639±819	7757±1289	p<0.012
Medial amygdala	532±216	2285±867	NS	7522±1789	11991±1502	NS
Ventromedial hypothalamus	383±81	720±195	NS	2205±403	3386±1058	NS
Paraventricular hypothalamus	211±92	293±72	NS	681±349	2434±987	NS
Thalamus	52±11	507±87	p<0.0001	1235±591	5491±725	p<0.001
Orbitofrontal cortex	91±30	117±25	NS	522±110	897±174	NS
Cerebellum	375±131	158±44	NS	743±244	644±128	NS
Olfactory	194±53	192±39	NS	2211±598	1098±123	NS

Tables

Table 1. Comparison of basal (-cAMP) and total (+cAMP)-stimulated PKA activity between WT and *Prkar1a*$^{+/-}$ mice in various brain/sensory areas in a control (non-stressed) state (ANOVA).

	Basal PKA activity (- cAMP Normalized PKA/1 mcg) Mean ± SEM				Total PKA activity (+ cAMP Normalized PKA/1 mcg) Mean ± SEM			
	Control	Novelty	POS	p-value	Control	Novelty	POS	p-value
Central amygdala	100±54	2689±697	3091±752	0.01	2039±536	14302±4075	9971±1522	0.013
Basolateral amygdala	171±82	3241±601	2397±765	0.013	2639±819	19344±4827	12814±3313	0.014
Medial amygdala	532±216	1047±198	942±157	NS	7522±1789	21100±1958	13762±2156	NS
Ventromedial hypothalamus	383±81	2421±275	2211±417	0.0001	2205±403	3438±1598	16922±1903	0.0001
Paraventricular hypothalamus	211±92	2585±501	2398±630	0.04	681±349	15795±4042	2398±3034	0.03
Thalamus	52±11	1039±77	2498±1038	0.024	1235±591	10806±2469	31820±8226	0.001
Orbitofrontal cortex	91±30	921±336	1075±421	0.013	522±110	14451±4921	8907±3526	0.004
Cerebellum	375±131	173±47	391±77	NS	743±244	469±127	1199±249	NS
Olfactory	194±53	424±98	1356±552	0.004	2211±598	4562±1555	7893±2009	0.008

Table 2. Comparison of basal (-cAMP) and total (+cAMP)-stimulated PKA activity in **WT mice** in various brain/sensory areas: controls (non-stressed) vs vehicle vs. predator odor exposed (ANOVA post-hoc comparisons).

	Basal PKA activity (- cAMP Normalized PKA/1 mcg) Mean ± SEM				Total PKA activity (+ cAMP Normalized PKA/1 mcg) Mean ± SEM			
	Control	Novelty	POS	p-value	Control	Novelty	POS	p-value
Central amygdala	702±144	7771±1812	17285±3830	0.024	6712±679	62773±19142	106565±18383	0.02
Basolateral amygdala	1017±417	7184±1042	17550±3198	0.006	7757±1289	44739±7985	73862±12554	0.012
Medial amygdala	2285±867	1495±336	2412±821	NS	11991±1502	25231±7308	16443±3972	NS
Ventromedial hypothalamus	720±195	4585±1612	5703±1442	0.05	3386±1058	49189±4622	36546±3783	0.0001
Paraventricular hypothalamus	293±72	3879±1907	6173±2069	NS	2434±987	28089±11441	25221±4208	NS
Thalamus	507±87	2700±1324	8760±3459	0.05	5491±725	55101±6252	69656±13023	0.001
Orbitofrontal cortex	117±25	4987±2620	4680±1255	NS	897±174	29659±14148	33944±9654	0.05
Cerebellum	158±44	254±22	295±41	NS	644±128	744±18	733±260	NS
Olfactory	192±39	653±151	590±42	0.001	1098±123	3748±549	7876±2707	0.006

Table 3. Comparison of basal (-cAMP) and total (+cAMP)-stimulated PKA activity in ***Prkar1a***$^{+/-}$ **mice** in various brain/sensory areas: controls (non-stressed) vs vehicle vs. predator odor exposed (ANOVA post-hoc comparisons).

Figures

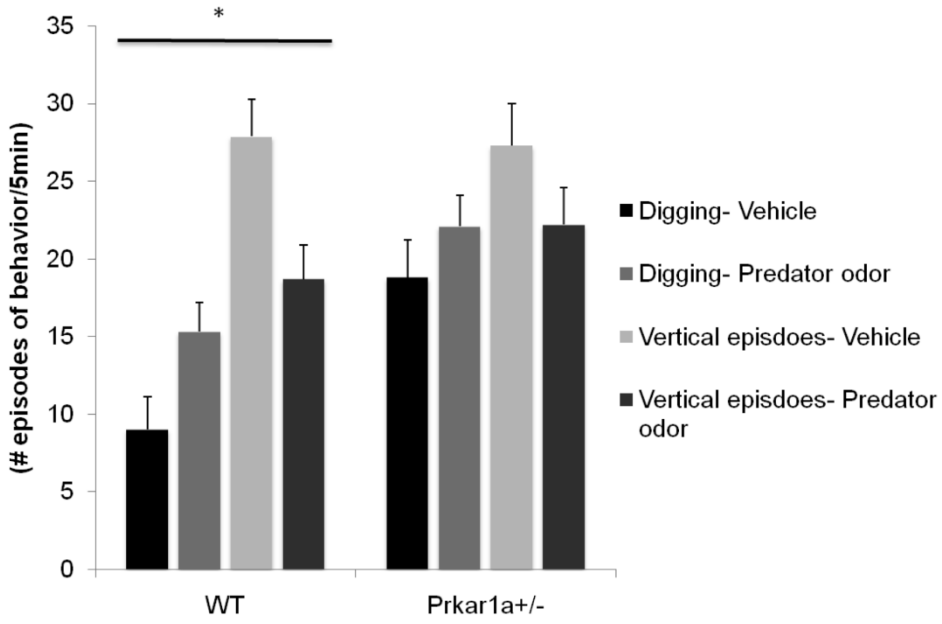
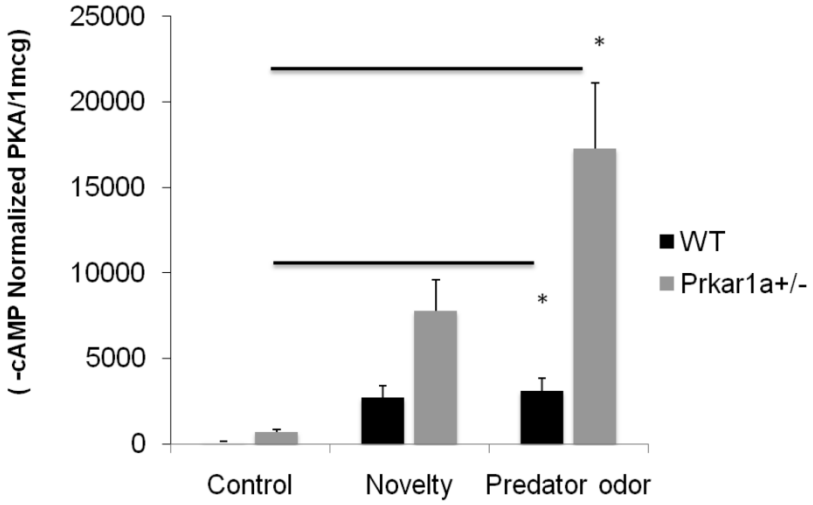


Figure 1. Behavioral responses of WT and *Prkar1a*$^{+/-}$ mice. Comparison of number of exploratory (vertical postures) vs. defensive (digging) episodes in WT and *Prkar1a*$^{+/-}$ mice during predator odor versus vehicle exposure. There were significant differences in the behavioral response of male Prkar1a+/- Hz and WT mice to predator odor (fox) vs. novelty (vehicle) stress conditions. *Prkar1a*$^{+/-}$ mice did not show alterations in behavior (exploratory or defensive) with exposure to different stressors (predator vs. vehicle) compared to WT littermates. WT mice showed the expected response of decrease in exploratory behavior (vertical postures) during predator (fox urine) vs. novelty (vehicle) stress ($p<0.02$).

A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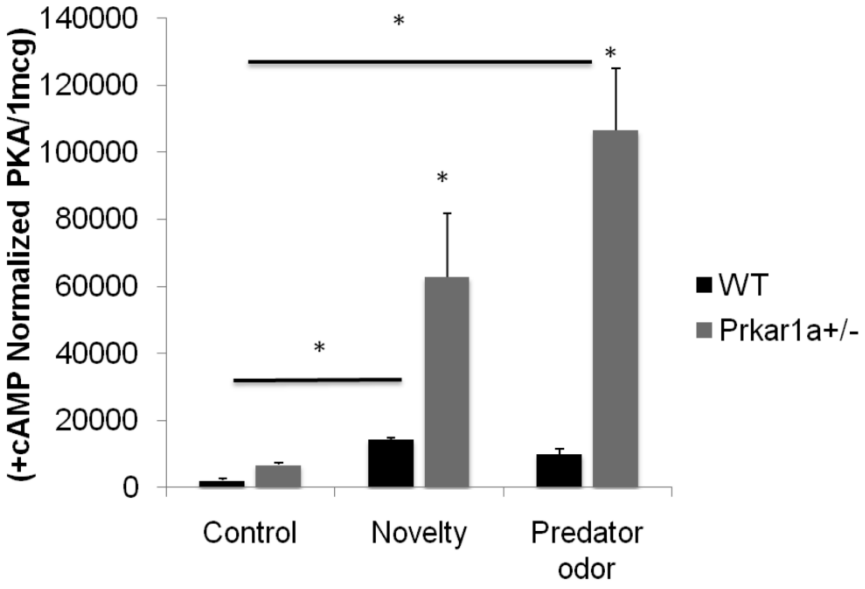
B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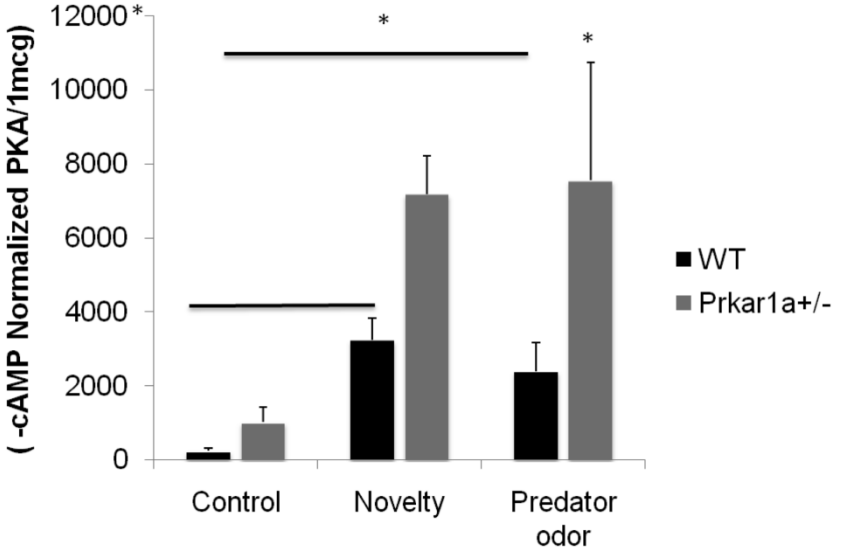

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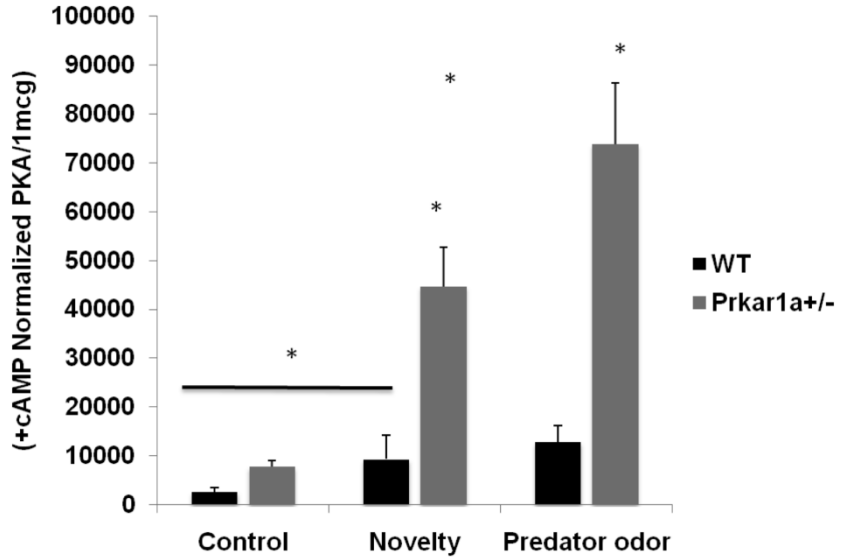
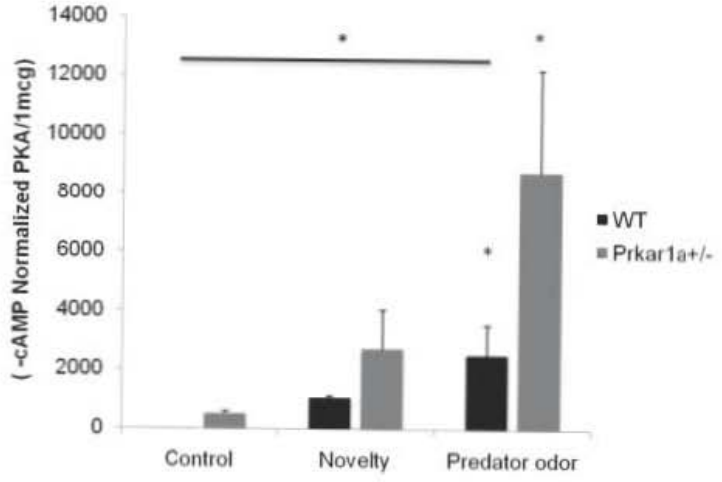


Figure 2. PKA activity in amygdala areas of WT and *Prkar1a*$^{+/-}$ mice. Comparison of basal (-cAMP) and total (+cAMP stimulated) activity in central amygdala (Figures 2a, 2b) and basolateral amygdala (Figures 2c, 2d) of WT and *Prkar1a*$^{+/-}$ mice: control (non-stressed) vs. vehicle (NOV) vs. predator odor exposed.

A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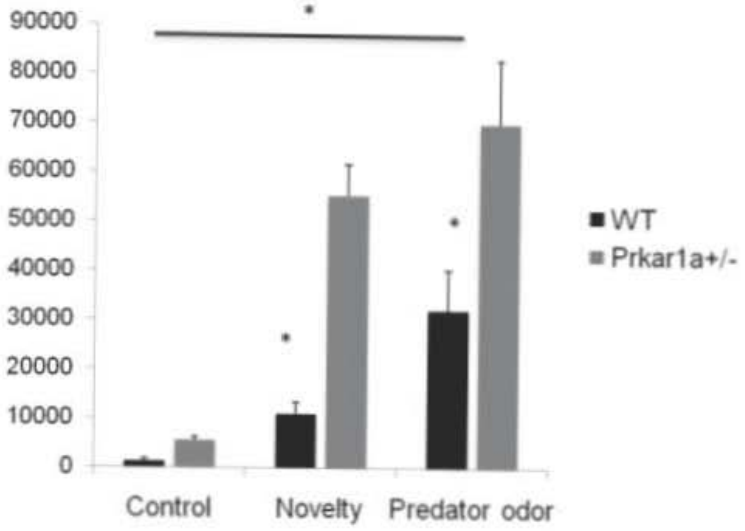
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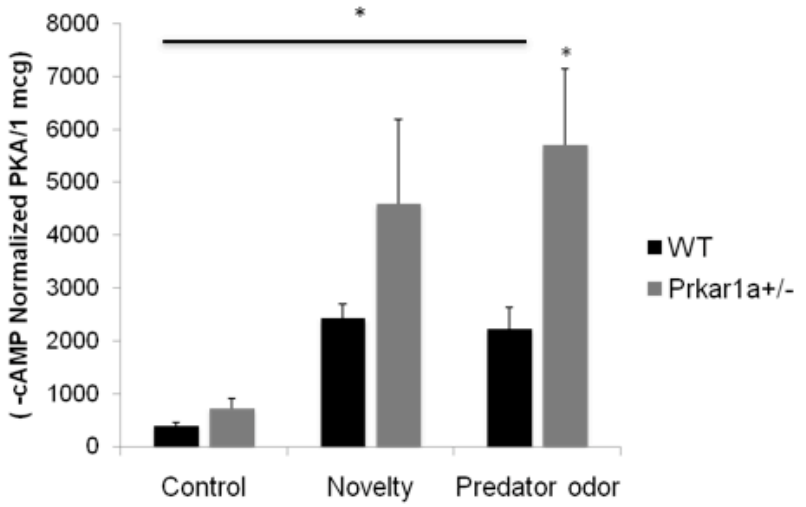

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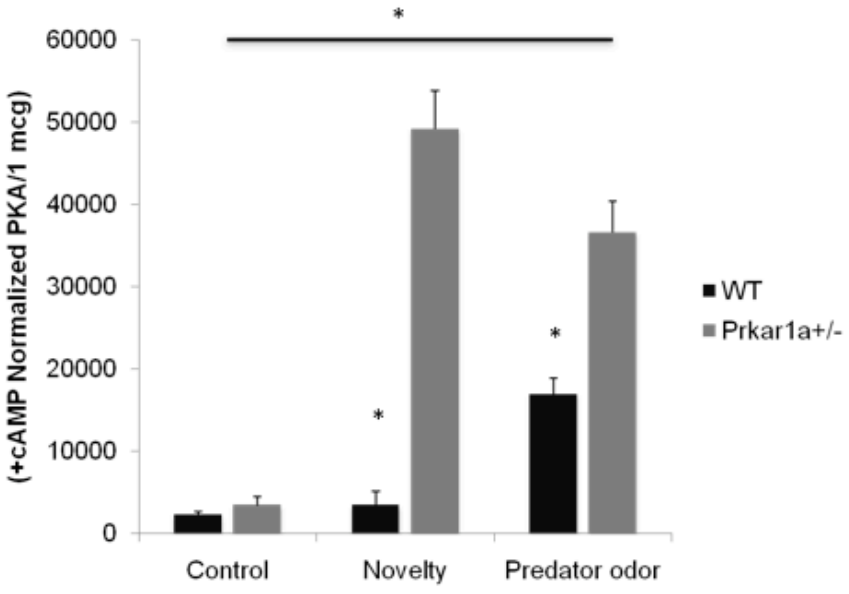

Figure 3. PKA activity in thalamus and ventromedial hypothalamus. Means (±SEM) of basal (-cAMP) and total (+cAMP stimulated) PKA activity in thalamus (Figures 3a, 3b) and ventromedial hypothalamus (Figures 3c, 3d) of WT and *Prkar1a*$^{+/-}$ mice: control (non-stressed) vs. vehicle vs. predator odor exposed. . In WT mice, post-hoc comparisons showed significantly higher PKA activity after predator odor (vs. control) for basal and total PKA in thalamus; and significantly higher basal (treatment groups vs. control) and total (predator vs. vehicle and

control) PKA activity in ventromedial hypothalamus ($p<0.05$). In *Prkar1a*$^{+/-}$ mice post-hoc comparisons revealed significantly higher total PKA activity in thalamus (treatment groups vs. control); and significantly higher basal (predator vs. control) and total (treatment groups vs. control) PKA activity in the ventromedial hypothalamus ($p<0.05$).

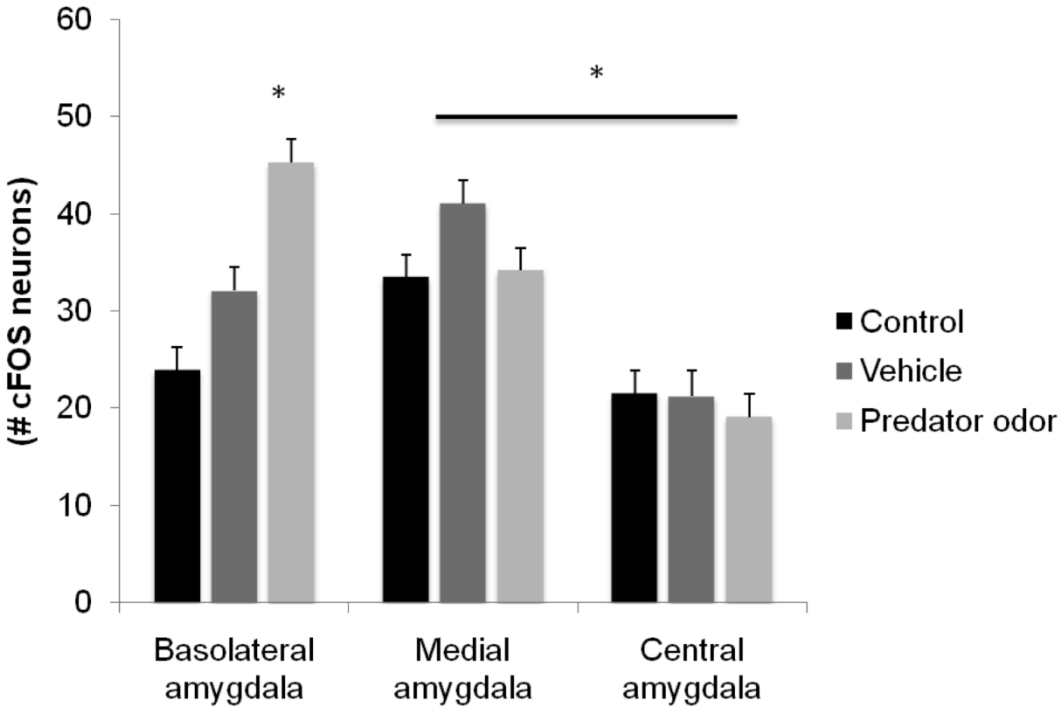

Figure 4. Comparison of mean (±SEM) number of cFos positive neurons in basolateral, central, and medial amygdala nuclei by treatment group (control, vehicle, predator odor). cFos expression was significantly higher in the basolateral amygdala in predator odor group, compared to vehicle or control ($p<0.05$). No significant difference was found between treatment groups in central or medial amygdala.

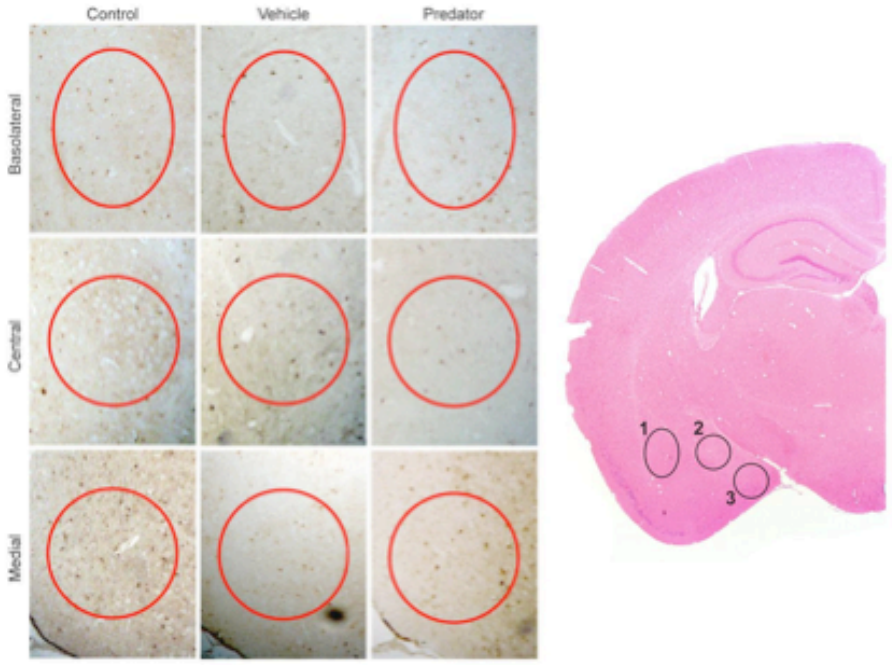

Figure 5. cFos expression in *Prkar1a*$^{+/-}$ mice. cFos expression in *Prkar1a*$^{+/-}$ mice. (5a) cFos expression *Prkar1a*$^{+/-}$ mice: control (non-stressed) vs. vehicle vs. predator odor exposed (100x magnification); (5b)Hematoxylin eosin stained reference slide for nuclei of amygdala (1=basolateral; 2=central; 3=medial amygdala).

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Acknowledgements

This work was supported by NICHD, NIH intramural project Z01-HD-000642-04 to Dr.C.A.Stratakis. . It was also supported in part by the Department of Pediatrics, University of Crete, Heraklion, Crete, Greece (G.Briassoulis).

This study was supported in part by intramural graduate student grant from the Uniformed Services University of the Health Sciences (MFK) and Department of Defense grant CO85DP (TJW).

We thank Dr. Kitman Tsang, Dr. Niloofar Rezvani, Dr. Andrei Mr. Bilal Naved, Ms.Florencia Vasta, Ms Beebee Asamoah-Mensah, Ms. Anna Batistatos, Ms. Melissa Tang, Ms. Emily Sun, Dr.Charalampos Lyssikatos, and Dr. Isaac Levy for their assistance with this project.

CHAPTER 5

CONCLUSIONS

The first objective of this proposal was to test for the first time the direct effect of increased PKA on anxiety-like behavior the behavioral in mice with the loss of one *Prkar1a* allele. The present findings demonstrate that mice with a down-regulation of the regulatory subunit of PKA, exhibit behavioral changes in behavioral assays of anxiety (elevated plus maze and marble bury test) suggesting a key role of PKA in modulating anxiety-related behaviors In addition, 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. Results of this study suggest that the effect of down regulation of the regulatory subunit of PKA is tissue-specific for the amygdala, since *Prkar1a*$^{+/-}$ mice showed increased anxiety-like behaviors and increased PKA activity in the amygdala, but not all brain areas.

After determining that the loss of one *Prkar1a* allele is associated with an anxiety-like behavioral phenotype the next objective was to investigate the effect of chronically increased PKA signaling on the neurobehavioral response to stress. Olfactory stimuli are widely utilized in rodent studies to investigate behavioral and neuroendocrine responses to stress as well as a model of hyperarousal and generalized anxiety aspects of PTSD; however no standardized methodology exists (Adamec & Shallow, 1993; Anisman, Hayley, Kelly, Borowski, & Merali, 2001; Dielenberg & McGregor, 2001). The second objective of this study was to establish the efficacy of fox urine as a stimulant of the hypothalamic pituitary adrenal gonadotropin axis in mice and investigate whether there is a dose dependent response to various amounts of fox urine,

in order to determine the minimum and maximum amount of fox urine required to elicit a significant defensive stress response in mice. Data from this study suggest that the dose of fox urine is an important variable in the design of experiments to investigate defensive response to unconditioned fear. A dose of 30µl of fox urine was sufficient to elicit a rise a corticosterone and ACTH response as well as an increase in serum androgen, consistent with a defensive response to threat. However, only the 300µl dose of fox urine elicited a significant difference in corticosterone response at 5 minutes post-stressor, compared to the other groups. The HPA axis response (corticosterone) in the 5mL group indicates that this dose exceeds the threshold, since the results do not significantly differ from the 30µl group. At 90 minutes post-stressor, there was no difference in corticosterone and androgen levels between groups, consistent with a return to non-stress levels. Importantly, data from this study support the inclusion of androgen measurement in predator odor stress paradigms to differentiate a stress response to novelty from a defensive response to fear or threat.

The results of the dose response study reviewed above, support the use of exposure to 300µl of fox urine as a model of unconditioned fear. Therefore the third objective of this proposal was to evaluate the effects of increased PKA signaling on the behavioral response to an ethologically valid stressor in a transgenic mouse model with the loss of one *Prkar1a* allele. The present findings demonstrate that mice with a down-regulation of the regulatory subunit of PKA, fail to exhibit behavioral changes (exploratory or defensive) to distinguish the response to predator odor (fox) vs. novelty (vehicle) stress conditions, suggesting a key role of PKA in modulating anxiety-related behaviors. In this study, the behavioral response of WT mice is consistent with prior laboratory and field studies; however the behavioral response of *Prkar1a*$^{+/-}$ *mice* indicate that the loss of one *Prkar1a* allele is associated with atypical behavior, specifically

a deficit in the ability to discriminate between an ambiguous versus threatening situation, consistent with the 'prediction error' hypothesis of anxiety disorders.

Results of prior studies in our lab reported an anxiety-like behavioral phenotype of *Prkar1a*$^{+/-}$*mice,* which was associated with up-regulated PKA activity in the amygdala compared to WT littermates (Keil, 2010). Similarly, in this study male *Prkar1a*$^{+/-}$ mice in a control (non-stressed) state showed higher basal and stimulated (cAMP) PKA activity levels in the central and basolateral amygdala, and thalamus compared to male WT littermates. The up-regulated PKA activity *Prkar1a*$^{+/-}$ mice in brain areas known to have a critical role in the processing of sensory information related to anxiety and emotion, as well as the regulation of arousal levels, likely contributes to the anxiety-like behavior and atypical response to unconditioned fear shown in *Prkar1a*$^{+/-}$ mice. In response to stress, interesting differences were found in PKA activity between WT and *Prkar1a*$^{+/-}$ mice. In WT mice, PKA activity was higher in basolateral and central amygdala, and orbitofrontal cortex after vehicle exposure, but after predator odor PKA activity was higher in ventromedial hypothalamus and thalamus. In contrast, PKA activity in *Prkar1a*$^{+/-}$ mice was higher in basolateral and central amygdala after predator odor, while PKA activity in the ventromedial hypothalamus was higher after vehicle, and PKA activity in the orbitofrontal cortex did not differ between treatments. There are conflicting reports in the literature regarding which subnuclei of the amygdala have a key role in mediating the response to predator odor. Results of this study endorse the importance of the basolateral amygdala, but not the central amygdala, in mediating the response predator odor stress.

In summary, these studies provide evidence to support the hypothesis of the proposal that increased PKA activity is associated with increased anxiety-like behavior. In addition, the findings of tissue-specific effects of increased PKA in the amygdala, and altered response to a

hyperarousal model of stress (predator odor) suggests that the *Prkar1a* mouse may be a useful model for investigation of stress vulnerability, such as PTSD. The alteration of PKA activity in response to stress shown in *Prkar1a*$^{+/-}$ mice is an interesting parallel to a 'prediction error' type of behavioral response. It is likely the dysregulated behavioral response to stress resulted from up-regulated PKA activity, which affected the normal function of the amygdala and/or lack of inhibition ('toning down') of the orbitofrontal cortex.

Future research

There is strong evidence to support the role of PKA in fear memory formation, so future studies should investigate fear conditioning in *Prkar1a*$^{+/-}$ mouse model. Studies have shown that PKA (and protein synthesis) in the amygdala is required for fear memory consolidation, but is a constraint for fear extinction (Abel, et al., 1997; Bourtchouladze, et al., 1998; Isiegas, Park, Kandel, Abel, & Lattal, 2006; Schafe & LeDoux, 2000). Therefore it would be useful to validate these findings in a *Prkar1a*$^{+/-}$ model to define the effects increased PKA on behavior and memory with fear conditioning and extinction. There is debate in the literature regarding the role of downstream targets of PKA (phosphates and kinases) that regulate learning and memory, and this model likely would contribute to a better understanding of these processes (Isiegas, et al., 2006).

Results from this study suggest that chronic up-regulation of PKA has tissue-specific effects in the amygdala, therefore future research is needed to elucidate possible alterations in the amygdala including: structural variation in amygdala circuitry, changes in dendritic morphology, compensatory changes in the PKA-CREB pathway, alterations in neurotransmitter signaling, and/or interaction with other brain areas involved in inhibition of the amygdala (i.e. prefrontal

cortex). A model of anxiety proposed in the literature emphasizes the importance of molecular cascades that support cellular plasticity, such as PKA, which is consistent with findings of this study (Li, Nair, & Quirk, 2009; Rogan, Staubli, & LeDoux, 1997). Recent studies in animal models have demonstrated that inhibitors of protein synthesis or PKA activity block long-term potentiation in the hippocampus and interfere with memory consolidation for fear in the amygdala (Schafe, Nader, Blair, & LeDoux, 2001), which emphasizes the significant role of PKA in plasticity in the amygdala. However, a limitation with the use of inhibitors of PKA or protein synthesis is the non-specificity and cross-reactivity with other proteins. Studies of *Prkar1a* heterozygote mice would add to our understanding of the role of PKA in neural mechanisms of amygdala plasticity.

Alterations in PKA activity in the orbitofrontal cortex may also contribute to the atypical response to stress in *Prkar1a* heterozygote mice since a key function of the orbitofrontal cortex is to inhibit prepotent behaviors and promote task relevant operations (known as top-down regulation). During stress, the amygdala is activated and the subsequent release of hormones (i.e. corticosterone, noradrenaline, and dopamine) function to enhance amygdala function while impairing orbitofrontal function (known as bottom-up regulation). During acute stress the predominant influence of the amygdala results in a tendency towards rapid, reflexive, habitual motor responding, rather than an orbitofrontal dominant response of inhibition of prepotent behavior and flexible regulation (Arnsten, 2009; LeDoux, 2003, 2007). PKA activity likely has a role in the regulation of orbitofrontal cortex as suggested by results of study using an activator of PKA (SpcAMPs) into the prefrontal cortex of rats showed a dose-dependent impairment in delayed alternation performance, and a perseverative pattern of behavior (Taylor, Birnbaum,

Ubriani, & Arnsten, 1999). Future studies are needed to elucidate the specific effects (i.e. structure and function) of increased PKA activity in the orbitofrontal cortex.

Once a better understanding of the effect of increased PKA activity in the *Prkar1a*$^{+/-}$ mouse on structure and function of the amygdala as well as the effect on fear learning and extinction; then future studies should be targeted to pharmacological and behavioral interventions to alleviate anxiety and related morbidities. In addition, experiments are underway in our lab using transgenic mice with downregulation of other components of the PKA molecule, including mice with loss of one allele for C-alpha, and mice with loss of an allele for both R1-alpha and C-alpha ("double-heterozygous"). Preliminary results of the behavioral phenotype of C-alpha mice indicate no increase in anxiety; that is, their behavior is similar to WT mice. This suggests that the increase in anxiety-like behavior in *Prkar1a*$^{+/-}$ mice may be related to actions of the increase in catalytic subunits due to the downregulation of the R1 alpha subunit. Studies of mice with the loss of one allele for C-alpha and C-alpha/R1-alpha mice will also help to increase our understanding of the PKA pathway in fear and anxiety.

Human neuroimaging studies also report that plasticity of the amygdala is associated with the ability to habituate or tone down amygdala activity while maintaining vigilance for subsequent threat. Amygdala sensitization can be thought of as a 'habituation failure,' which contributes to heightened anxiety (Britton, Lissek, Grillon, Norcross, & Pine, 2011). Presented with a novel ethologically relevant stressor *Prkar1a*$^{+/-}$ mice failed to distinguish between ambiguous versus threatening emotional stimuli, which was also associated with specific alterations in PKA activity in brain areas crucial in the interpretation, processing and regulation of response to stress. The unique features of structural and functional plasticity in the amygdala highlight the importance of this brain area in the study of memory of emotionally arousing and

fearful experiences as well as the pathological consequences. Future studies should incorporate neuroimaging of the amygdala of *Prkar1a*$^{+/-}$ to determine if PKA activity is correlated with functional MRI findings, which may provide a useful biomarker for preclinical treatment studies in this mouse model.

Implications for clinical practice

Anxiety disorders affect approximately 18% of American adults in a given year and the lifetime prevalence of severe anxiety is 5.9% of 13 to 18 year olds. In addition, in children, anxiety disorders accounts for 32% of mental health service use (National Institutes of Health, 2011). The significance of anxiety disorders occurring in childhood, a vulnerable growth period for the brain, is underscored by the finding that they predict a 2- 3 – fold increased risk for adult mental health disorders, including anxiety (Britton, et al., 2011). There is an extensive body of research on the significant impact of the child's early life environment on his/her future health. Therefore during the health care reform that is underway in the United States, it is important to support programs that prioritize children's health as an investment in the future.

Results from this study support findings from studies with humans, which show that factors affecting functional amygdala reactivity are likely to have a critical role in vulnerability to stress. In this study, increased basal and cAMP-stimulated PKA activity in the amygdala of mice with loss of one *Prkar1a* allele was associated with atypical response to non-threatening stimuli, which provides an interesting parallel to the literature on studies of patients with PTSD that report enhanced right amygdala activity in response to both trauma and ambiguous negative stimuli (Rauch, Morales, Zubritsky, Knott, & Oslin, 2006; Shin, et al., 2005). These results are also consistent with McNaughton and Corr's model, which proposes that overactivity (or hyper-

reactivity) of a neural structure is associated with symptoms and pathological syndromes. In the defensive approach hierarchy of the model, the arousal associated with anxiety is controlled by the amygdala, and the model predicts that pathologically increased arousal may result in a generalized anxiety disorder (Davis, 1992; McNaughton & Corr, 2004). Implications for clinical practice include an awareness of factors (i.e. trauma, violence, history of anxiety disorder, medications, etc) that may negatively effect amygdala activation in a patient to prioritize initiation of early treatment(s) (i.e. cognitive behavioral therapy, propanolol, etc) to reduce associated long-term morbidity. In addition, a better understanding of the role of PKA in the neurobiology of anxiety will help to identify potential targets for pharmacologic treatment. As the neural mechanisms involved in the development of anxiety and PTSD are elucidated, it is imperative that funding for research focused on prevention and treatment of chronic PTSD remains a priority. In addition, support of policies and funding for accessible, affordable mental health resources is a sound investment to reduce the morbidities associated with this condition.

The findings of this study support an extensive literature that exposure to atypical steroid levels or other neuromodulators during the critical windows of vulnerability for brain growth and development (prenatal, childhood, adolescence) can disrupt normal function of various brain areas, with lifelong consequences (i.e. cognitive, behavioral, social morbidity). Implications of this study for the healthcare community, educators, and policy makers are to recognize the long-term 'footprint' of early life exposures (i.e. persistent fear and anxiety due to violence, abuse, or poverty) that alter normal brain development and function, to improve preventative and early interventions measures for children, adolescents, and pregnant women. Anxiety sensitivity has both heritable & developmental origins and results of this study suggest the a transgenic mouse with the loss of one *Prkar1a* allele is a relevant model to investigate the neurobiological

mechanisms of anxiety in order to develop novel targets for treatment of anxiety and prevention of associated disorders, such as PTSD.

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