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Anxiety-like behavior and other consequences of early life stress in mice with increased protein kinase A activity

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Abstract

Anxiety disorders are associated with abnormalities in fear-learning and bias to threat; early life experiences are influential to the development of an anxiety-like phenotype in adulthood. We recently reported that adult mice (*Prkar1a+/−)* with haploinsufficiency for the main regulatory subunit of the protein kinase A (PKA) exhibit an anxiety-like phenotype associated with increased PKA activity in the amygdala. PKA is the main effector of cyclic adenosine mono-phosphate signaling, a key pathway involved in the regulation of fear learning. Since anxiety has developmental and genetic components, we sought to examine the interaction of a genetic defect associated with anxiety phenotype and early life experiences. We investigated the effects of neonatal maternal separation or tactile stimulation on measures of behavior typical to adolescence as well as developmental changes in the behavioral phenotype between adolescent and adult wild-type (WT) and *Prkar1a+/−* mice. Our results showed developmental differences in assays of anxiety and novelty behavior for both genotypes. Adolescent mice showed increased exploratory and novelty seeking behavior compared to adult counterparts. However, early life experiences modulated behavior in adolescent WT differently than in adolescent *Prkar1a+/−* mice. Adolescent WT mice exposed to early life tactile stimulation showed attenuation of anxiety-like behavior, whereas an increase in exploratory behavior was found in *Prkar1a+/−* adolescent mice. The finding of behavioral differences that are apparent during adolescence in *Prkar1a*$^{+/-}$ mice suggests that long-term exposure of the brain to increased PKA activity during critical developmental periods contributes to the anxiety-like phenotype noted in the adult animals with increased PKA activity.

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Conflict of interest

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Keywords

Protein kinase A; anxiety; developmental; early life stress

1. Introduction

Approximately one in five American adults are affected by an anxiety disorder and longitudinal research indicates that approximately two-thirds of affected adults exhibit signs of a mental disorder earlier in life [1, 2]. There is ample data to support that early mechanisms (e.g. genetic or environmental) can determine the long-term trait of an organism to express anxiety in response to threatening stimuli [3, 4]. Data suggest that anxiety-related disorders are very complex and polygenic, and despite substantial progress in genetics and epigenetics, few responsible loci have been identified for these disorders [5–7]. Advances in molecular genetic approaches have identified pathways that are associated with anxiety risk; experimental and preclinical evidence provides support that anxiety disorders are associated with abnormal processing of threat-related stimuli, mediated to a large extent by the cyclic adenosine mono-phosphate (cAMP) signaling pathway [8–15].

Developmental periods of the brain such as infancy and adolescence are associated with active synaptic development and are a particularly crucial time for adjustment of anxiety circuits in response to experience-dependent stimuli consistent with a gene-environment-timing interaction [16]. Alterations in maternal care, such as early maternal separation, are associated with changes in anxiety-related behaviors and altered ability to cope with stress in adulthood [17, 18]. Alternately, brief handling of neonatal rodents is reported to induce resistance of the hypothalamic-pituitary-adrenal (HPA) axis to stress in adulthood, as shown by a decrease in anxiety-like behavior in the elevated plus maze (EPM) test [19–21]. In addition, rodent studies using neonatal tactile stimulation demonstrate an effect on stress reactivity, with an increase in curiosity and problem-solving behavior and less emotion in stressful situations [22–24]. Animal models have proven useful in elucidating cellular and molecular mechanisms underlying these effects. However, little is known about the effect of maternal separation or tactile stimulation on behavior in adolescent mice.

Genetically modified mice are a valuable tool to investigate consequences of manipulation of specific genes and environmental variables on anxiety-related behavior. Therefore, it might be possible to influence the development of a variety of stress responses and anxiety-like behaviors that are dependent on genetic factors (e.g. genetic mutations), simply with the manipulation of early environmental experiences, such as maternal deprivation and tactile stimulation. An example of such genetic factors is the inactivation of the *Prkar1a* gene (by the *tTA/X2AS* antisense transgene) that predisposed animals to the development of anxiety-like disorders and altered stress responses [10]. Mice heterozygous for a null allele of *Prkar1a* were developed as a model to investigate Carney complex, a disease that is caused by heterozygous inactivating *PRKAR1A* mutations [25], which lead to increased cAMP signaling in all cells where this gene is expressed.

cAMP-dependent protein kinase or protein kinase A (PKA) mediates most of cAMP signaling and is a tetrameric holoenzyme consisting of two catalytic (C) subunits each bound

to a regulatory (R) subunit. PKA exists in two isoforms: type I and type II (PKA-I and -II, respectively). This is due to the presence of either type-I or -II regulatory subunits; there are four such subunits (RIα and -Iβ and RIIα and -IIβ, coded by the *PRKAR1A*, *PRKAR1B*, *PRKAR2A*, and *PRKAR2B* genes, respectively) that normally form a homodimer that binds two molecules of a catalytic subunit (one of Cα, Cβ, or Cγ coded by the *PRKACA*, *PRKACB*, and *PRKACG* genes, respectively) in the PKA tetramer [26–29]. RIα, the subunit that is deficient in PPNAD and in Carney complex, is the most abundant of the four PKA regulatory subunits [29, 30]. Our laboratory has studied the consequences of R and C subunit dysregulation [25].

PKA is the principal effector for G-protein-coupled receptors (GPCRs) linked to the regulation of several behavioral responses, notably fear learning and memory [31]. Previous studies have demonstrated that PKA's main regulatory subunit, R1α, is involved in the expression of anxiety-like behaviors; the loss of one *Prkar1a* allele in mice (*Prkar1a*$^{+/-}$) led to an augmentation of anxiety-like behaviors in association with an increase in PKA activity in both the basolateral (BLA) and central amygdala (CeA) [10]. In a subsequent study, Briassoulis et al. [11] studied the effects of deleting one allele of the main catalytic subunit (Cα) and compared anxiety-related behaviors in *Prkar1a*$^{+/-}$ and *Prkar1a*$^{+/-}$/*Prkaca*$^{+/-}$ mice: the former spent significantly less time in the open arms of the elevated plus maze (EPM), while *Prkaca*$^{+/-}$ and *Prkar1a*$^{+/-}$/*Prkaca*$^{+/-}$ mice displayed less exploratory behaviors [11].

In this study, we examined the effect of RIα's deletion on early life experiences and their association with anxiety-like behaviors. We investigated the effects of neonatal maternal separation or tactile stimulation, on measures of behavior typical to adolescence, namely novelty and sensation-seeking. Also, to determine whether the anxiety-like behavior typical of adult mice with *Prkar1a* mutation was an early or a late phenotype, we investigated developmental changes in adolescent and adult mice of both genotypes [10].

2. Materials and methods

2.1 Animals

Prkar1a$^{+/-}$ heterozygous (*Prkar1a*$^{+/-}$) male mice [25] were bred with wild-type (WT) C57BL/6 females. For these experiments, we used only WT mothers to avoid confounder of possible differences in maternal care to offspring attributable to the genotype. Starting from day 15 post-coitus, the females were weighed every 2 days to recognize pregnancy and therefore separate them from the male. Around the third week post-mating, the cages were inspected once a day. The day of birth was considered day 0. The litters were composed of both WT and *Prkar1a*$^{+/-}$ mice and never exceeded 6 pups; thus, all pups from each litter were used for the experiments. The animals were weaned at 21 days, separated from the mother and transferred into different cages divided by gender and housed with siblings. Adult mice (breeder males and non-breeder females) were 3 to 8-month age at time of behavioral testing.

All mice were housed 3 – 4 per standard barrier cages on a ventilated rack in a room with a constant temperature (22+/1−°C) with same-sex littermates with ad libitum access to food and water and maintained on a 12:12 light schedule (lights on at 0600 h). All animal

procedures were conducted in accordance with the standards approved by the NIH Guide for the Care and Use of Laboratory Animals. All animal protocols received prior approval at the NIH. All behavioral testing was performed, as previously reported, between the hours of 1300–1700 h. One behavioral test per day was performed, with a span of at least two days between tests. The order of behavioral tests was randomly assigned. Two scorers performed behavioral testing and obtained scoring of all results in a blinded fashion. The timeline for experiments is depicted in Figure 1.

2.2 Genotype analysis

After weaning, all mice were genotyped. A total of 45 *Prkar1a*$^{+/-}$ mice and 51 WT were included in the study. All mice were genotyped using tail DNA by polymerase chain reaction (PCR) using primers previously validated [32]. Three primers (5'-AGCTAGCTTGGCTGGACGTA-3', 5'-AAGCAGGCGAGCTATTAGTTTAT-3' and 5'-CATCCATCTCCTATCCCCTTT-3') were used for *Prkar1a* geno-typing: the WT allele generated a 250- base pair (bp) fragment and the null allele generated an180 bp product (data not shown).

2.3 Protocol of maternal separation and tactile stimulation

Immediately after birth (Day 0) each litter was assigned to one of three experimental conditions:

1. Maternal separation group (MS): 14 *Prkr1a*$^{\pm}$ and 12 WT pups were separated from the mother 180 minutes per day from day 1 to day 14.
2. Tactile stimulation group (TS): 10 *Prkr1a*$^{\pm}$ and 17 WT were separated from their mother to receive tactile stimulation (gentle massage by hand) once a day for 15 minutes from day 1 to day 14.
3. Control group (CNT): 21 *Prkr1a*$^{\pm}$ and 22 WT received routine facility care with their mother in the cage.

Each litter was separated from the mother and moved to a different cage from day 1 to day 14. The cages that accommodated the pups during this period were identical to the home cages both in size and material. The cages were left without lid, food or water and were heated by a lamp to maintain the temperature at ~ 32 °C for the first week and ~ 30 °C for the second week of the experiment. Pups in the maternal separation condition were left undisturbed for 180 min in the above conditions in the hosting cage [33]. Pups in the tactile stimulation group were individually subjected to tactile stimulation protocol during the separation period. At the end of each session, the pups were reunited with their mother. The tactile stimulation protocol consisted in gently caressing and massaging the puppies by hand imitating the maternal care. Every animal received 15 minutes of tactile stimulation with a speed of about 3 cm/sec using three fingers of the preferred hand of the experimenter. The experimenter wore latex gloves for single use, thus reducing the human smell, but maintaining contact, i.e. heat and pressure. The mice were caressed on the back dorsal thorax always in the same direction, from head to tail. Two different experimenters trained to perform the same procedure carried out the experiments. Behavioral testing was performed during postnatal day 40–50, which is considered late-adolescence. The treatment group mice

(tested in adolescence) were not re-tested as adults as they were used for a separate experiment.

2.4 Behavioral tests

2.4.1 Elevated Plus Maze Test—Mice from the three groups (MS, TS, CNT) were tested on the Elevated Plus Maze Test (EPM) during the life period considered as adolescence (postnatal day 40–50). Adult mice were 3 to eight months old at the time of testing. EPM testing was performed as previously described [10, 34]. A video system tracking software© (Stoelting Co., Wood Dale, IL, USA) was used to automatically acquire the behavioral data. In addition, hand scoring was performed to validate time and entries into arms, as well as risk assessment behavior (calculated by dividing the number of protected stretch attend postures by total closed arm time) and exploratory behavior (head dips).

The procedure consisted of placing the animals at the junction between the arms open and closed, arms (center of the maze), facing the open arm opposite to the position of the experimenter. Behavioral testing was performed from 09.00 to 12.00, after a 2-hour period of acclimation of the animals to the behavioral room in which they were transported. Measures scored in the EPM included: (1) *Open arm time:* total amount of time (seconds) the mouse spent in the open arms; (2) *Center time:* total amount of time (seconds) the mouse spent in the center area of the maze; (3) *Unprotected head dip* (index of exploratory behavior): total amount of time the mouse spent with head and shoulders pointing down and all the elongated body in a point of not protected maze, both in the center and in the open arms; (4) *Risk assessment:* number of protected stretch attend postures/amount of closed arm time.

2.4.2 Open-field test—The Open field test (OF) was used to measure locomotor activity and exploratory behavior based in the mouse's drive to explore unfamiliar environments. Mice were tested in the OFT during the late adolescence (around postnatal day 40–50). The Open Field apparatus consisted of a square arena (60×60 cm), with a floor divided into 36 squares (10×10 cm), enclosed by a continuous, 25-cm-high walls made of black Plexiglas. The 20 squares cm adjacent to the wall represent a protected field (periphery of the arena), while the square in the middle of the arena (center of the arena) represents the most exposed area of the field. The animals were tested during the first half of the dark phase of their light/dark cycle. The test was initiated by placing a mouse in one of the arena corners and allowing it to explore freely for 20 min. The mouse behavior was continuously recorded by a video camera placed over the arena and then encoded using a continuous sampling method. Test session videotapes were scored with Anymaze software®. After 20 minutes a novel object (blue cap from a 30mL test tube) was placed in the center of the open field and the mouse behavior was recorded by a video camera for 5 minutes. The arena was carefully cleaned with 70% alcohol and rinsed with water after every test.

To test for spontaneous locomotor activity as well as response to novelty in the open field we measured the following variables: (1) *Central area time*: the total amount of time the mouse spent in the more aversive center of arena; (2) *Peripheral area time*: the total amount of time the mouse spent in the more protective outer zone near the walls (thigmotaxic behavior

associated with anxiety); (3) *Central ambulation distance*: the total amount of distance (cm) the mouse traveled in the center the arena center; and (4) *Peripheral ambulation distance* the total amount of distance (cm) the mouse traveled in the arena periphery; (5) *Center latency*: the amount of time the mouse spent from the beginning of the test before entering the first time in the center arena . (6) Response to novelty was recorded for 5 min after a novel object was placed in the center arena and the following variables were measured as described above: (1) central area time, (2) peripheral area time, (3) central ambulation distance, (4) peripheral ambulation distance, and latency to explore novel object (amount of time in seconds from beginning of novel object introduction to when mouse touched novel object.

2.4.3 Defensive Marble burying—Prior studies suggest that the defensive marble burying test (MB), which involves the selective suppression of marble burying, correlates with anxiolytic behavior. The MB test is widely used to evaluate anxiolytic compounds [35–37] and has also been suggested as a model of compulsive or perseverative behavior [37–42]. Mice were transported in their home cages to the testing room two hours prior to acclimate prior to testing. Five centimeters of 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- minute test period. Lights were turned off in the room for 30 minutes and then the number of marbles buried 2/3 or greater was recorded [39].

2.5 Statistical analysis

Two-way ANOVA analysis (age x genotype) was performed to assess developmental differences between adolescent CNT group and adult mice with Bonferroni or LSD comparisons where appropriate using SPSS software. Two-way ANOVA analysis was performed to assess treatment group and genotype differences for adolescent mice with Bonferroni or LSD comparisons where appropriate using SPSS software. Statistical outliers > 2 SD from the mean were removed from analysis. Significance was determined at $p< 0.05$. All values are reported as means +/− SEM.

3. Results

3.1 Elevated plus maze

Developmental and genotype groups—ANOVA analysis (age x genotype) showed significant differences for open arm time (OAT) ($F_{3, 155}$ =5.209, p=0.002), center area time (CT) ($F_{3, 154}$ =5.769, p=0.001), and total head dip (THD) ($F_{3, 143}$ =36.439, p=0.001). Post hoc analysis with LSD showed a significant genotype difference between adult (but not between adolescent groups) *Prkar1a*$^{+/-}$ and WT mice for OAT (Adult *Prkar1a*$^{+/-}$: 8.75± 2.4 vs. WT 22 ± 4.2 sec.; n= 51–53 per group). There were no genotype differences between adults in center zone time or head dip behavior. No gender difference was found. Developmental differences were found for *Prkar1a*$^{+/-}$ and WT in OAT (*Prkar1a*$^{+/-}$ adults vs. adolescents: 8.75± 4.2 vs. 24.2±5.5 sec., p< 0.001; WT adults vs. adolescents 45.2±5.4 vs 63±5.1 sec, p<0.04), CT (*Prkar1a*$^{+/-}$ adults vs. adolescents: 33± 5 vs. 59±7.5 sec., p< 0.004; WT adults vs. adolescents 45.2±5.4 vs 63±5 sec, p<0.03), and THD (*Prkar1a*$^{+/-}$ adults vs. adolescents: 6± 0.7 vs. 24±3 dips., p< 0.001; WT adults vs. adolescents 7±0.7 vs 23±2.8

dips, p<0.001) with increased OAT, CT, and THD for adolescents compared to adults. For risk assessment, a developmental difference was found only for *Prkar1a*$^{+/-}$ mice with higher risk assessment for adolescents versus adults (*Prkar1a*$^{+/-}$ adults vs. adolescents 0.049± 0.004 vs. 0.07±0.008 sec., p<0.03). (Figures 2–3).

Adolescent treatment groups—ANOVA (genotype x treatment group) was performed and no interaction was noted, but a treatment group difference was found within genotype. Post-hoc analysis showed a significant difference for WT between treatment groups in CT ($F_{2, 58}$ = 7.954, p=0.001); the TS group mice spent significantly more time in center zone than CNT. A treatment group effect was noted for risk assessment ($F_{2, 58}$ = 9.665, <0.001) and THD ($F_{2, 58}$ = 5.837, p<.005), with greater risk assessment in TS compared to CNT and greater total head dips (exploratory behavior) in TS and MAS groups compared to CNT. No significant treatment effects were found for OAT.

For *Prkar1a*$^{+/-}$ mice, a significant difference was found between treatment groups in THD ($F_{2, 45}$ = 6.657, p=0.003) and risk assessment ($F_{2, 45}$ = 6.936, p=0.002); the MS mice had significantly greater head dips than TS and CNT (p<0.003); MS mice also demonstrated significantly higher risk assessment than CNT. Trends were noted for OAT and CT, with higher scores for MS compared to controls (Figures 4, 5).

3.2 Spontaneous locomotor activity and reactivity to novelty (Open Field Test)

Developmental and genotype groups—A two-way ANOVA (age x genotype) showed a developmental effect for both genotypes ($F_{3, 58}$=9.787, p<0.001) but no interaction. Adult mice of both genotypes showed significantly shorter latency to approach novel object ($F_{3, 58}$ = 13.033, p=0.001), greater center time ($F_{3, 58}$ = 5.862, p=0.02), greater center time during novel object phase ($F_{3, 58}$ = 5.134, p=0.03), greater center distance ambulation in initial part of test ($F_{3, 58}$ =29.568, p<0.001) and during novel object phase ($F_{3, 58}$ =25.067, p<0.001), and less distance ambulation in peripheral area during initial part of test ($F_{3, 58}$ =43.355, p<0.001) and greater distance ambulation during novel phase of test ($F_{3, 58}$ =25.843, p<0.001) compared to adolescent mice. There was no genotype effect for adult or adolescent mice. Figures 6,7.

Adolescent treatment groups—A two-way ANOVA (genotype x treatment group) was performed and no interaction was found, but a treatment effect was noted within genotype. Treatment effects were found for WT adolescent mice for some measures during the novelty phase of the open field but only for one measure for *Prkar1a*$^{+/-}$ adolescent groups. Post-hoc analysis with Bonferroni showed that during the novel object phase, WT - TS mice spent more time in the center than controls ($F_{2, 46}$ = 3.292, p<0.05); WT -TS mice also spent less time in periphery than CNT or MAS mice ($F_{2, 46}$ = 4.507, p<0.02). (Figure 7). WT- TS mice spent more time next to novel object than controls or MS mice ($F_{2, 46}$ = 7.799, p<0.001). The only treatment effect in *Prkar1a*$^{+/-}$ adolescents was found with object time: TS mice spent more time next to a novel object than controls ($F_{2, 36}$ = 6.396, p< 0.004). A trend was noted for center time during novel object phase, *Prkar1a*$^{+/-}$ TS group mice more time in center that CNT (p<0.059). Figure 8.

3.3 Marble bury test

Developmental and genotype groups—Genotype and developmental effects were noted (F 5.537, p=0.001). Post-hoc Bonferroni analysis showed increased marble burying for adult *Prkar1a*$^{+/-}$ mice compared to adult WT littermates (p<0.006). WT adolescent mice showed increased marble burying compared to adult WT (p< 0.01); however, no developmental difference was seen for the *Prkar1a*$^{+/-}$ mice. There were no sex differences in marble bury behavior for either WT or *Prkar1a*$^{+/-}$ mice (Figure 9). A treatment effect was not found in either WT or *Prkar1a*$^{+/-}$ mice for the marble bury test.

4. Discussion

In the present study, we investigated how the loss of one allele for *Prkar1a* leads to an anxiety phenotype in adulthood, and its possible emergence in adolescence. We examined how early life experiences might influence the behavioral development of *Prkar1a*$^{+/-}$ mice. Consistent with what we previously reported, adult *Prkar1a*$^{+/-}$ mice demonstrated increased anxiety-like behaviors in the EPM (less open arm time) and marble bury test (bury more marbles) compared to WT animals. We previously reported that anxiety–like behavior in adult *Prkar1a*$^{+/-}$ mice was associated with an increase in PKA activity in both the basolateral (BLA) and central amygdala (CeA) that was not a ubiquitous effect since PKA activity was similar between heterozygotes and wild-types in other brain (orbitofrontal cortex, hippocampus, cerebellum, thalamus) and neural sensory (olfactory bulb, eyes) areas[10, 43]. This does not rule out the possibility that increased PKA activity is not acting elsewhere in the brain (i.e. paraventricular or ventromedial hypothalamus or other brain areas involved in the neural pathway of anxiety) through compensatory mechanism. However, an amygdala localized effect is consistent with extensive data on the role of the amygdala in anxiety and fear-related behaviors[10, 43].

When we examined adolescents, both WT and *Prkar1a*$^{+/-}$ mice spent more time in open and center areas of the EPM and had more THD (exploratory) behavior compared to their adult counterparts, which is consistent with prior reports of increased novelty seeking behavior in adolescent mice [44, 45]. In humans, adolescence is associated with an increase in novelty seeking behaviors that may have potential evolutionary antecedents [45–47]. Indeed, prior studies report that adolescent mice exhibit differences from adults, namely novelty seeking (open arm entries and time; head dips in EPM) behaviors in excess, a decrease in novelty-induced stress and anxiety, and an increase in impulsivity and restlessness [45, 48, 49]. For example, adolescent rodents exhibit increased exploratory drive as measured open arm exploration and head dips in the EPM and levels of locomotor activity in the OFT (49, 50). Interestingly, *Prkar1a*$^{+/-}$ adolescent mice (controls) demonstrated significantly more time in the open arms of EPM but also showed greater risk assessment behavior compared to their adult counterparts, suggesting that the anxiety-like phenotype of adult *Prkar1a*$^{+/-}$ mice is preceded by behavioral differences that are already apparent during adolescence.

In the present study, we found no genotype effect for adult mice in spontaneous locomotor activity or reactivity to novelty in the open field test. This supports that arousal level and locomotion were not factors in the difference in anxiety-like behavior noted in the EPM. Developmental differences were found for both genotypes in spontaneous locomotor activity

in the OFT; both WT and *Prkar1a*$^{+/-}$ adolescent mice demonstrated less spontaneous locomotor activity (less ambulation/locomotion in center and at periphery during main and novelty stages) and longer latency to explore novel object (indicator of less novelty-seeking) in the OFT compared to adult counterparts. No genotype effect was found for either adult or adolescent mice. General locomotor activity in a novel environment (OFT) and explorative drive were not significantly affected by the loss of one *Prkar1a* allele indicating that under low aversive conditions no phenotypic differences exist between WT and *Prkar1a*$^{+/-}$ mice.

Prior studies comparing the adolescent and adult response of rodents in the OFT have reported conflicting results. Adolescent rodents in the OFT have been reported to exhibit higher levels [50–53] or lower levels [48] of locomotor exploration compared to adults. The differences in locomotor exploration between adolescent and adults may be related to the extent that novelty drives behavior in various test situations, which may account for the apparent discrepancies across studies [45]. In our study, treatment group effect was found for WT- TS group mice in the OFT; TS-group mice spent more time in center and had less time in periphery during novel object phase, and increased object time than their respective controls. Interestingly, WT- MS groups showed differences from TS-group mice only for time in periphery during novel object phase and object time; while no differences were found for *Prkar1a*$^{+/-}$ -MS group mice compared to respective CNT or TS groups. These findings are consistent with what is known about the crucial role of developmental changes in brain structure and function that occur during adolescence with the deleterious effects of MS reported in adult rodents.

Few studies have examined the behavioral effects in adolescence of maternal separation or tactile stimulation in mice. Studies with similar protocols for maternal separation (3hrs/day x14 days starting on PN day 1) report increased motor activity in MS juveniles [54] or no difference in motor activity between controls and MS rodents (tested in adulthood) [55]. In this study, WT adolescent mice exposed to tactile stimulation had the expected response of amelioration of the anxiogenic effect of maternal separation as shown by increase in risk assessment and exploratory behavior (in the EPM) and increased time in center during the novel phase of the OFT, consistent with the stress inoculation model[56]. However, *Prkar1a*$^{+/-}$ mice exposed to TS failed to attenuate the anxiogenic effect of maternal separation, rather they demonstrated impaired stress resistance, which suggests an increased vulnerability to stress that may be a precursor to the adult anxiety phenotype noted in these heterozygote mice. The behavioral response of adolescent *Prkar1a*$^{+/-}$ mice to early life stressors, that is, a failure to discriminate between two types of stressors, is consistent with the bias to threat response of adult *Prkar1a*$^{+/-}$. We previously reported that adult *Prkar1a*$^{+/-}$ mice failed to discriminate between stress conditions (predator odor vs. novel (control)) that was associated with dysregulated PKA activity in the amygdala-prefrontal cortex circuitry, suggesting that the loss of one *Prakr1a* allele is associated with higher vulnerability to stress[43].

Interestingly, the marble bury test failed to show a developmental effect in *Prkar1a*$^{+/-}$ mice consistent with our prior report of threat bias and dysregulated PKA activity in the amygdala-prefrontal cortex in adult *Prkar1a*$^{+/-}$ mice [43]. Our finding of a lack of a genotype effect in other behavioral assays of anxiety in adolescent mice suggest that the

anxiety-like phenotype as noted in adult mice with loss of one *Prkar1a* allele (associated with increased PKA activity in the amygdala), is likely due to long-term exposure during critical developmental periods of brain development to alterations in PKA activity. PKA activity in brain areas of adolescent WT and HZ mice are similar to what has been reported for their adult counterparts (unpublished data); namely, increased PKA activity in the amygdala but no genotype differences in PKA activity in cortex, thalamus, or paraventricular hypothalamus [10, 11].

There are significant gaps in our knowledge of the ontogenetic changes in brain function during adolescence, however the incomplete maturation of neural systems that regulate emotional and inhibition behavior are thought to embody the behavioral phenotype of adolescence [45, 57–60]. Decades of research provide evidence for the impact of disruptions in maternal-neonate care in rodents and non-human primates [61–63] on behavioral and neurobiological outcomes in adulthood. Genetic predisposition to anxiety may be manipulated in interaction with the environment suggesting that maternal care may contribute to behavioral phenotype [45, 64]. Less is known about the impact of alterations in maternal care on brain and behavior in juvenile or adolescent offspring. A better understanding of the cascade of changes that occur with alterations in specific genes or neuroendocrine targets is needed. For our study, we mated WT females with *Prkar1a*$^{+/-}$ males to limit possible differences in offspring due to maternal genotype. Future studies will investigate possible differences in behavioral outcome attributed to the maternal care of *Prkar1a*$^{+/-}$ mice.

Unconditioned anxiety-related behaviors in the elevated plus maze and novel open field did not differ between genotypes in adolescent mice and reflect the evolutionarily conserved strategies in this developmental phase. The OFT elicited an immediate and consistent divergence in risk-assessment behaviors between adolescent and adult mice of both genotypes. This is consistent with the increased exploratory behavior noted in adolescent mice in the EPM and activation of the behavioral inhibition system [45, 48, 52, 65]. Although *Prkar1a*$^{+/-}$ mice demonstrated increased exploratory behavior in the EPM compared to their adult counterpart, they also showed higher risk assessment, which involves behaviors associated with the detection and analysis of threat stimuli that is associated with anxiety.

5. Conclusion

In summary, the results of the present study demonstrate that anxiety-like behaviors that were previously noted in mice with increased PKA activity are rooted in adolescence, at least in *Prkar1a*$^{+/-}$ mice. This is in line with studies of mood disorders in humans that report that signs and symptoms of many mental disorders emerge slowly [2, 58]; the development of anxiety disorders is associated with puberty [66, 67]. Anxiety disorders involve prominent symptoms of fear learning and attention to threat-related stimuli and are associated with neural structures involved in the expression or regulation of emotion-based information processing [58, 68]. The data reported here suggest that a chronic increase in PKA activity during critical periods of brain development may be essential for the development of an anxiety phenotype in adults. This is consistent with our recently reported finding of an

increased incidence of psychiatric disorders in children, adolescent and adults with *PRKAR1A* mutations (59). The most frequent psychiatric diagnosis in adults were anxiety, depression, and bipolar disorder (in that order), while for children and adolescents it was learning difficulties, attention deficit hyperactivity disorder, anxiety, and depression (in that order) [69]. Overall, our data suggest that a better understanding of the downstream targets of increased PKA activity may identify novel therapeutic targets to treat anxiety and its early development

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Highlights

- Loss of one *Prkar1a* allele is associated with an anxiety-like phenotype in adult mice.
- Developmental differences in anxiety behavior were found for WT and *Prkar1a*$^{+/-}$ mice.
- *Prkar1a*$^{+/-}$ adolescent mice demonstrated higher exploratory and novelty seeking behavior.
- Loss of one *Prkar1a* allele is associated with vulnerability to stress in adolescence.

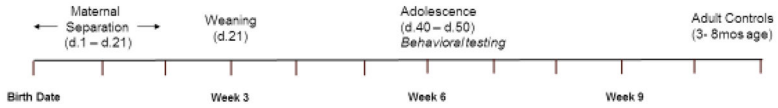

Fig. 1. Summary of experimental design

Litters were randomly assigned to group: Maternal separation, Tactile stimulation, or Facility handling. Behavioral testing of treatment groups was performed during postnatal day 40- 50 (late adolescence). Adult controls (3–8 mos age at time of testing).

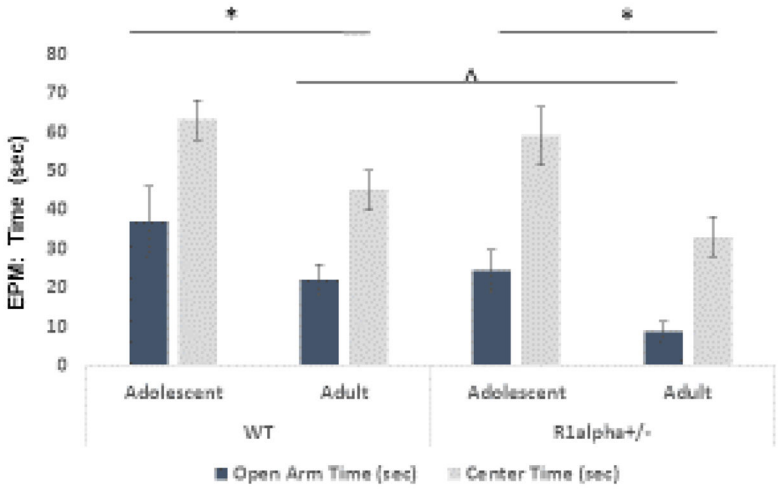

Fig. 2. Elevated plus maze (EPM),Open arm and Center area times

Comparison of wild type, *Prkar1a*$^{+/-}$ for amount of time spent (seconds) in open arm of EPM during 5 min. test period. *, p<0.05, developmental differences noted for both genotypes. ^**, p<0.05,** genotype difference found for adults. Comparison of wild type, *Prkar1a*$^{+/-}$ for amount of time spent (seconds) in center area of EPM during 5 min. test period. *, p<0.05, developmental differences noted for both genotypes.

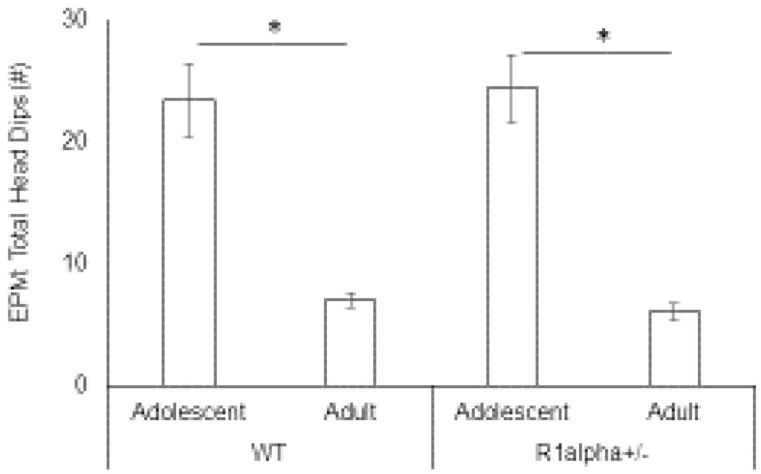

Fig. 3. Elevated plus maze (EPM), Total head dips

Comparison of wild type, *Prkar1a*$^{+/-}$ for number of head dips in EPM during 5 min. test period. *, $p<0.05$, developmental differences noted for both genotypes.

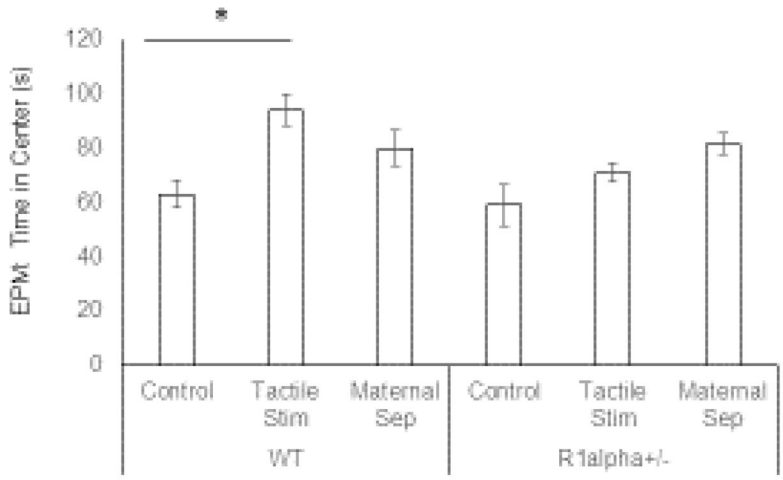

Fig. 4. Elevated plus maze (EPM), Center area time

Comparison of wild type, *Prkar1a*$^{+/-}$ adolescent treatment groups (within genotype) for amount of time spent (seconds) in center area of EPM during 5 min. test period. *, $p<0.05$, significant differences noted for wild type tactile stimulation group compared to controls.

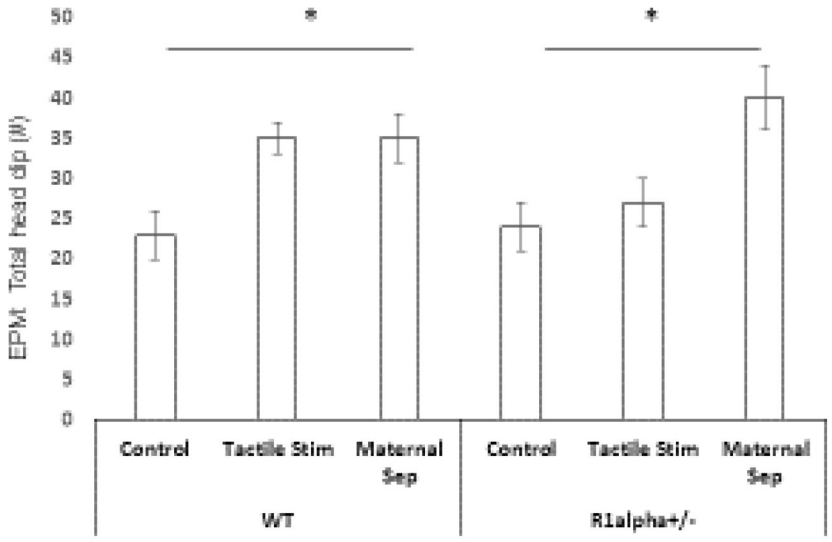

Fig. 5. Elevated plus maze (EPM), Total head dips

Comparison of wild type, *Prkar1a*$^{+/-}$ adolescent treatment groups (within genotype) for total head dips during EPM 5 min. test period. *, $p<0.05$, Wild type tactile stimulation and maternal separation groups had more head dips than controls. *, $p<0.05$, *Prkar1a*$^{+/-}$maternal separation group had more head dips than tactile stimulation or controls.

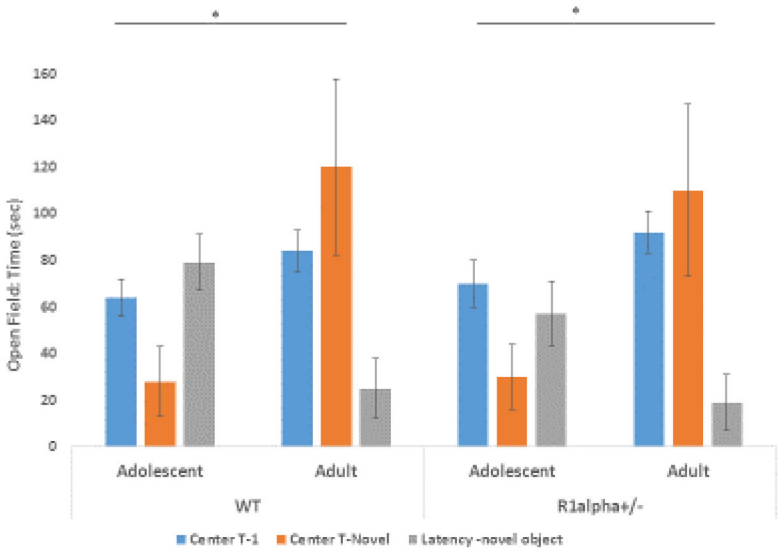

Fig. 6. Open Field Test (OFT), Latency to novel object and Center Times: T-1 test period (20min) and T-Novel object novel (5 min)

Comparison of wild type, *Prkar1a*$^{+/-}$ for amount of time to approach novel object (seconds) in center area of OFT during 5 min. test period. *, $p<0.05$, developmental differences noted for both genotypes, with shorter latency for adults compared to adolescents. Comparison of wild type, *Prkar1a*$^{+/-}$ for amount of time spent (seconds) in center area of OFT during 20 min. test period (T-1) and during 5min. novel object period (T-novel). *, $p<0.05$, developmental differences noted for both genotypes, with greater center time for adults compared to adolescents.

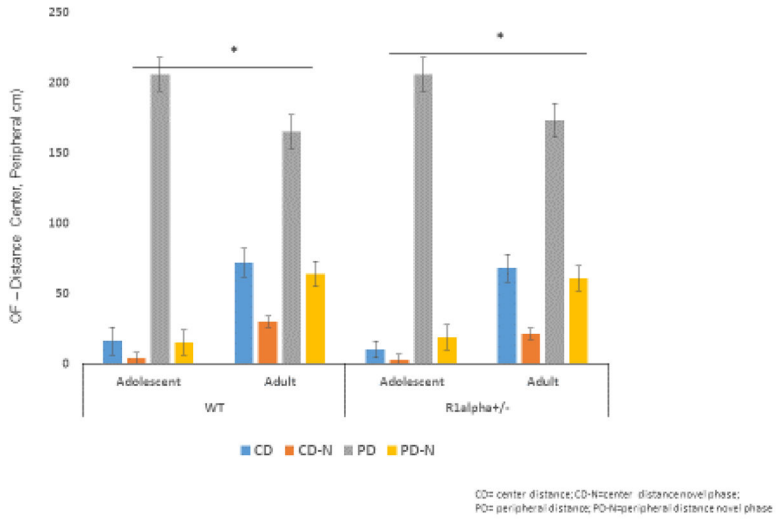

Fig.7. Open Field Test (OFT), Distance ambulated in center and peripheral areas

Comparison of wild type, *Prkar1a*$^{+/-}$ for amount of distance (cm) ambulated in center area of OFT during 20 min. test period (CD) and during 5min. novel object period (CD-novel); and amount of distance ambulated in peripheral area of OFT during 20 min. test period (PD) and during 5min. novel object period (PD-novel), *, $p<0.05$; developmental differences noted for both genotypes, with greater center distance ambulation for adults in both initial and novelty periods of test compared to adolescents, and less peripheral ambulation during initial period and greater peripheral distance ambulation during novel object period compared to adolescents.

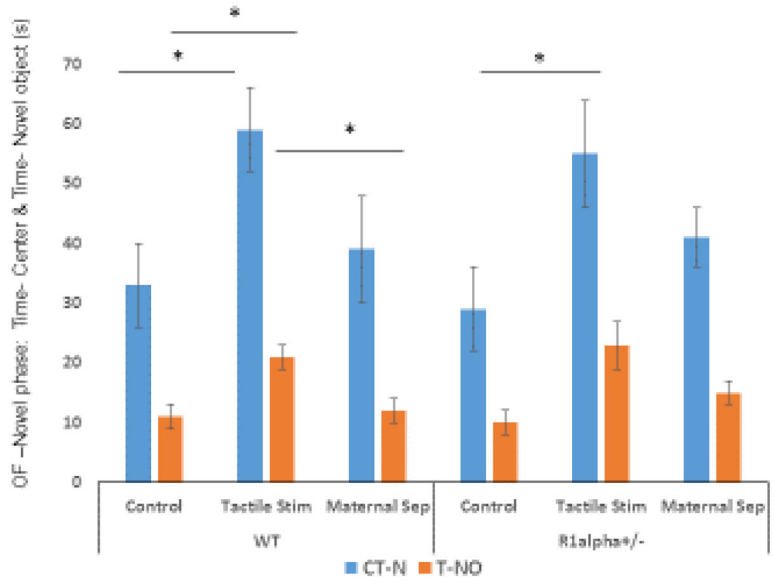

Fig.8. Open Field Test (OFT), Time in Center during novel object period and time next to novel object

Comparison of wild type, *Prkar1a*$^{+/-}$ adolescent treatment groups (within genotype) for time (seconds) in center area during 5min. novel object period, *, $p<0.05$, wild type tactile stimulation group spent more time in center than controls. Comparison of time (sec) spent next to novel object during 5min novel object period, *, $p<0.05$, wild type adolescent tactile stimulation group spent more time next to novel object than controls or maternal separation groups; *Prkar1a*$^{+/-}$ adolescent tactile stimulation group spent more time next to novel object than controls.

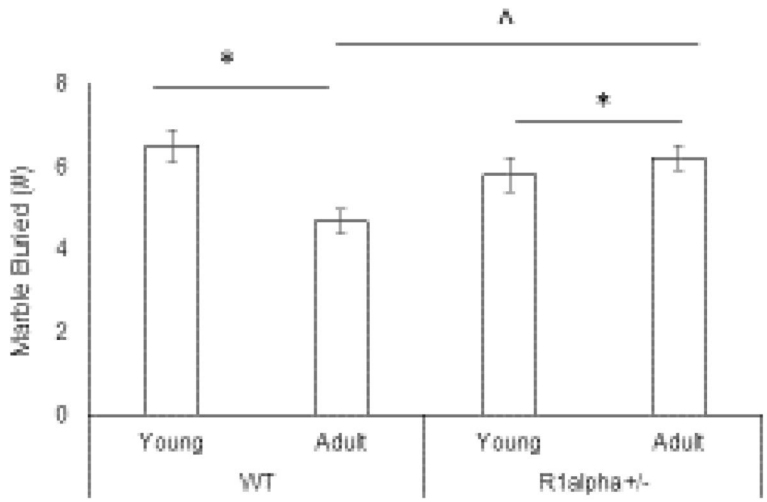

Fig 9. Marble Bury Test

Comparison of wild type, *Prkar1a*$^{+/-}$ for number of marbles buried >2/3. *, p<0.05, developmental differences noted for wild type.^**, p<0.05,** genotype difference found for adults.