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Impaired Amygdala-based Learning and Decreased Anxiety in a Murine Model of Pseudohypoparathyroidism type 1A.

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Abstract

Pseudohypoparathyroidism type 1A (PHP1A) is a genetic disorder caused by maternally inherited mutations in the gene *Gnas*. PHP1A is characterized by multiple hormone impairment, early onset obesity and cognitive impairment. Animal models of PHP1A are needed to investigate the mechanism of cognitive impairment. In the present study we used the cre-lox murine model to investigate behavior and cognition in maternally vs. paternally inherited *gnas* mutations expressed in the central nervous system. We observed a behavioral phenotype of decreased anxiety and impaired amygdala-based learning in the PHP1A mutant mouse model though there was no difference in hippocampal based learning tasks. In the elevated zero maze and open field analysis in the locomotor activity chambers, mutant mice showed diminished anxiety/increased impulsivity which could correlate with the attention deficit phenotype of children with PHP1A. The mutant mice also demonstrated poorer motor strength on the inverted screen test. These findings mirror some clinical features of PHP1A though overall the murine phenotype was milder than expected.

Keywords

Max 6; Pseudohypoparathyroidism type 1A; Behavior; Cognition

1 INTRODUCTION

Pseudohypoparathyroidism type 1A (PHP1A) is a rare genetic disorder caused by mutations in the gene *Gnas* on the maternal allele of chromosome 20. This complex genetic locus encodes for multiple transcripts, including the alpha subunit of stimulatory G-proteins ($G_s\alpha$). In certain tissues, the paternal allele is imprinted and the maternal allele

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preferentially expressed. Maternally inherited mutations therefore result in a more severe phenotype, PHP1A, that includes multi-hormone resistance, early onset obesity and cognitive impairment. In contrast, paternally inherited mutations result in a mild phenotype called pseudopseudohypoparathyroidism (PPHP) that does not include hormone resistance or cognitive impairment.

Cognitive impairment was noted in the original description of PHP1A by Fuller Albright in 1942.¹ Subsequent descriptions of the disorder include the finding of cognitive impairment and developmental delays though further characterization of the impairment or mechanistic studies have been lacking. A review of the literature showed that in 70 patients with PHP1A, 78% were described as having cognitive impairment vs. only 10% of patients with PPHP (n=30).² Patients with PHP1A have an intelligence quotient (IQ) approximately 20 points (1.4 SD) lower than their unaffected siblings.³ Cognitive impairment continues to be a consistent feature of PHP1A despite newborn screening programs which lead to early treatment of thyroid stimulating hormone resistance.³ It is likely that the difference in cognitive impairment between PHP1A and PPHP is due to tissue specific imprinting in the central nervous system (CNS).⁴ In PHP1A, imprinting in the CNS would decrease signaling through G_sα-coupled receptors such as the dopamine receptor D₁, potentially causing delayed developmental milestones and decreased cognition.

Animal models of PHP1A are needed to investigate the mechanism of cognitive impairment. Murine models of PHP1A have successfully recapitulated the multi-hormone resistance and early onset obesity phenotype.⁵ A G_sα-flox/nestin-cre mouse model was successfully used to elucidate the role of the CNS in the metabolic changes seen in PHP1A without concomitant hormone resistance.⁴ In the present study we used the cre-lox murine model to investigate behavior and cognition in maternally vs. paternally inherited *gnas* mutations expressed in the CNS.

2. MATERIAL AND METHODS

2.1 Animals.

All animal experiments complied with the National Institutes of Health guide for care and use of laboratory animals and were performed in accordance with the local Institutional Animal Care and Use Committee. Black swiss mice with loxP sites surrounding G_sα exon 1 were obtained from the Weinstein lab⁶ and backcrossed for 10 generations onto a C57/B6 background (Jackson Laboratory). Female G_sα-flox mice were mated with nestin-cre males (Jackson Laboratory) to generate maternally inherited “mutant” mice. Paternally inherited “control” mice were generated with reciprocal crosses. Testing began when mice were aged 6–8 weeks of age (see Table 1 for group details). Male and female mice were tested. Mice were tested in four separate cohorts with mice from each genotype and sex represented in each cohort.

2.2 Elevated Zero Maze (EZM).

A single 5-min. trial was conducted using a standard white elevated zero maze (width 5 cm; diameter 50 cm; wall height 15 cm, Stoelting Co. IL). Mice were video-recorded using a

ceiling-mounted camera and viewed remotely by the experimenter from an adjacent room. Position and movement of the mice were analyzed using AnyMaze (Stoelting Co., Wood Dale, IL, USA). Analysis parameters were set so that 80% of the mouse needed to be present in a zone for an entry into that zone to be registered.

2.3 Locomotor Activity.

Locomotor activity was recorded in square activity chambers (27.5 cm × 27.5 cm) contained within individual sound-attenuating chambers (MedAssociates, Fairfax, VT, USA). Mice were placed in the center of the chamber and permitted to explore for 30 min. Activity was recorded via the breaking of infra-red beams (MedAssociates ENV-510 software). Time spent in the periphery was calculated for exploration in the edge versus a central square (19.05 cm × 19.05 cm) where the peripheral area accounted for approximately 52% of the overall area of the chamber.

2.4 Inverted Screen.

Mice were placed on a square of wire mesh (131 mm × 92 mm) which was gently inverted until mice were hanging upside down approx. 30 cm above an enclosure lined with bedding. Mice were observed until they fell, or the full 180 s. trial time had elapsed.

2.5 Rotarod.

Rotarod testing was conducted using an accelerating rotarod (Ugo Basille, Italy) that increased in speed from 4 to 40 revolutions per minute over the course of 4 min. with a maximum trial time of 5 min. Three trials were conducted per day, on three consecutive days. The time taken to fall from the apparatus was recorded automatically.

2.6 Marble Burying.

A clean cage identical to the home-cage (Allentown, 194 mm × 181 mm × 398 mm) was filled to a depth of approx. 2.5 cm inch with white Diamondsoft bedding (Teklad). Twenty dark blue marbles were placed in a 5 × 4 configuration, leaving a clear space of approx. 4.0–5.0 cm to allow entrance and removal of the mouse without disturbing the marbles. Mice were left undisturbed in the cage for 30 min. after which time they were removed and an image taken of the cage from directly above. The number of marbles that were at least 50% buried was scored by two independent investigators who were blinded to animal group at the time of coding. Agreement between coders was very high (Pearson correlation coefficient $r=0.97$, $P<0.001$) and therefore final value is calculated as an average of the scores from the two coders.

2.7 2-trial Y-maze.

A custom-built Y-maze of clear plexiglass with arms 5.0 cm width and 34.5 cm length was used to measure preferential exploration of a novel area. During the first trial one arm was blocked and mice were permitted to freely explore the remaining two arms for 5 min. During the second, test trial, which followed a 5-min. inter-trial interval, all three arms were open and the mouse was permitted to explore for a further 5 min. Trials were video-recorded using a ceiling-mounted camera and viewed remotely by the experimenter from an adjacent

room. Position and movement of the mice were analyzed using AnyMaze. Entry into an arm was defined as 80% of the mouse crossing at least 1 cm. into the arm, which left a small central triangle that was not included in analyses. Cues proximal to the maze were kept constant throughout the trials, and between cohorts. Blocked arms and entry arms during both trials were counterbalanced among mice across all trials and groups.

2.8 Tail-suspension test.

Tails were secured to the force plate using tape at the proximal half of the tail. Movement data were recorded automatically using MedAssociates Inc. software, with time in which struggling was greater than a pre-defined threshold (7, arbitrary units) which represent a minimal level of movement associated with breathing. Data were recorded per minute, for 6 minutes.

2.9 Fear Conditioning.

Fear conditioning was measured using commercially available shock chambers (dimensions: $30.5 \times 24.1 \times 21$ cm, Med Associates) and analyzed using Med Associates software. On the first testing day mice were placed in the chambers for an 8-min. training trial. The trial consisted of a 2-minute no stimulus period, followed by three tone-shock pairings (tone 70 dB, 30 s; shock 0.5 mA, 0.5 s) each separated by 90 s. 24 hours later, mice were placed in the same chambers for 4 min. with no additional tones or shocks (context memory trial). One hour later, mice were placed into an identical chamber for a final 4 min trial in which the floor was lined with a white plexiglass insert, and a second white plexiglass insert was placed in the chamber to form a curved back wall, changing the shape of the chamber. A few drops of vanilla essence were also present in the chamber to provide novel olfactory cues. The previously exposed conditioned stimulus (tone) was played for the final 2 min. of the trial only (cued memory trial). The testing room had two ante-chambers. For training and context-dependent trials mice were transported into the room from ante-chamber 1 in a clean, empty home cage. For the cue-dependent trial, this was varied so that mice were transported into the test room from ante-chamber 2, providing an altered context. Following testing, a shock threshold assessment was performed on a subset of animals. Mice were observed as shocks were administered at increasing intensities (0.075, 0.1, 0.15, 0.2, 0.25, 0.3 mA) once every 30 s for 0.5 s each. An experimenter noted the shock level at which each mouse flinched or jumped, and continued administering shocks until the animal vocalized.

2.10 Forced Swim test.

Mice were placed in a clear plexiglass cylinder (15.3 cm. diameter) filled to a depth of 16.0 cm with water at room temperature. Mice were placed in the water and left to swim for 6 min. while trials were video recorded from the front. Time spent immobile was coded by a trained coder from the videos. Data provided are all from a single coder to ensure consistency. However, forty percent of trials were also coded by a second investigator and were strongly correlated (Pearson correlation coefficient $r=0.61$, $P<0.001$).

Behavioral testing was conducted in the order described above (see also, Table 2). Testing was conducted in the order presented below, with testing performed from the least invasive to the most invasive or potentially stressful, in order to avoid potentially confounding data.

2.10 Statistical analyses.

All mice are included in analyses for all tasks except in the case of one mouse which could not be tested on the tail suspension test due to a damaged tail. Data for most single measures were analyzed by Univariate analyses with independent variables of Genotype and Sex. Repeated Measures ANOVA analyses were conducted for Tail Suspension data (repeated measure: time, min (6)) and for Rotarod performance (repeated measure: day (3)). Separate paired t-tests were conducted for each sex and genotype for 2-trial Y-maze to compare time spent in novel arm versus average time spent in the familiar arms. All analyses were conducted using SPSS (IBM) version 24.

3. RESULTS

Animal weights were recorded at least once during the behavioral testing period. Weights of adult mice (age 9–12 weeks) did not vary according to gene inheritance pattern for either male or female mice ($t(21)=0.503$, $P=0.62$; $t(22)=-1.214$, $P=0.238$, respectively).

3.1 Elevated zero maze.

Exploration on the maze was indexed through distance travelled, and number of transitions between open and closed zones (figure 1A–B). Neither of these measures varied according to genotype or sex ($F_s<1.569$, $P_s>0.216$). Nevertheless, mutant animals spent less time than control animals in the less anxiogenic, closed zones of the maze ($F_{1, 57}=15.752$, $P<0.001$, figure 1C) indicating either decreased anxiety or heightened impulsivity. There were no main effects of sex or interaction ($F_s<0.990$, $P_s>0.324$).

3.2 Locomotor activity.

Mutant animals travelled significantly less distance than control animals in the activity chambers ($F_{1, 57}=5.821$, $P=0.019$, figure 1D). There were no main effects of sex or interaction ($F_s<1.585$, $P_s>0.213$). Vertical beam breaks were used to index rearing events as a possible repetitive behavior. Rearing did not differ according to genotype or sex ($F_s<0.699$, $P_s>0.407$, figure 1E). Mutant animals also spent significantly less time than control animals in the periphery of the activity chambers exhibiting wall-hugging or thigmotaxic activity ($F_{1, 57}=4.493$, $P=0.038$, figure 1F). There were no main effects of sex or interaction ($F_s<1.878$, $P_s>0.176$).

3.3 Forced swim test.

Neither sex nor genotype impact immobility time in the forced swim test ($F_s<0.888$, $P_s>0.350$, figure 1G).

3.4 Tail suspension test.

Time spent immobile increased over the first 3 min. of the session and remained high until the end of testing ($F_{5, 280}=61.237$, $P<0.001$). Immobility time did not vary according to sex or genotype inheritance and there were no interactions $F_s<2.502$, $P_s>0.068$, figure 1H–I).

3.5 Inverted Screen.

Mutant animals fell more quickly from the inverted screen than control animals ($F_{1, 57}=4.137$, $P=0.047$, figure 2A). There was no additional effect of sex on performance ($F_s<0.701$, $P_s>0.406$).

3.6 Rotarod.

Latency to fall from the rotarod increased across the 3 days of testing in all groups ($F_{2, 114}=49.814$, $P<0.001$), there were no interactions between test day and either genotype or sex ($F_s<1.578$, $P_s>0.211$, figure 2B–C). Mutant animals fell from the apparatus moderately faster than control mice, although this trend was not significant ($F_{1, 57}=2.956$, $P=0.091$). There were no additional sex effects or interactions ($F_s<0.339$, $P_s>0.563$).

3.7 Marble Burying.

All groups buried a similar number of marbles during the 30 min. trial ($F_s<0.892$, $P_s>0.349$, figure 2D).

3.8 Y-maze.

During the Y-maze test trial mice of both genotypes and sexes travelled similar distances in the maze ($F_s<0.355$, $P_s>0.554$, figure 3A). Time spent in the novel arm did not vary between genotypes indicating similar learning between groups ($F_{1, 57}=0.044$, $P=0.834$, figure 3B). Female mice showed a slightly stronger preference for the novel arm than male mice ($F_{1, 57}=5.911$, $P=0.018$), with no Sex x Genotype interaction ($F_{1, 57}=2.629$, $P=0.110$). All groups spent more time in novel compared to familiar arms ($t_s>2.545$, $P_s<0.029$).

3.9 Conditioned Fear.

Time spent freezing was similar in both genotypes and sexes during testing in the familiar context (hippocampal-dependent learning) ($F_s<0.79$, $P_s>0.38$, figure 3C). Although there were no main effects of sex or genotype on time spent freezing during the first 2-min. of the in the novel context trial, freezing during the 2-min. tone was calculated minus the time spent freezing during the initial 2 min. period to account for potential baseline differences in the new context. Mice with the maternally-inherited mutation spent less time freezing than control animals indicating poorer learning ($F_{1, 57}=4.447$, $P=0.039$, figure 3D). There were no additional effects of sex ($F_s<1.521$, $P_s>0.223$) on either measure. There were no differences according to genotype or sex on response to the shocks administered during threshold testing ($F_s<2.924$, $P_s>0.105$). All mice vocalized at or below 0.3 mA indicating similar sensitivity to the conditioned stimuli shocks.

4. DISCUSSION

Cognitive impairment and developmental delays are an important yet understudied clinical feature of PHP1A. We have previously shown that children with PHP1A have lower intelligence quotient (IQ) scores, impaired adaptive behavior, behavior problems and decreased executive function.³ In this study, maternally inherited, brain-specific $G_{\beta 3}$ knockout mice served as a model of PHP1A. To control for behavioral changes specific to

nestin-cre mice,⁷ we used paternally inherited G_{α} knockout mice, a model of PPHP, as opposed to wild type controls. A behavioral phenotype of decreased anxiety and impaired amygdala-based learning was observed in the PHP1A mutant mouse model. These findings are similar to some clinical features of PHP1A though overall the murine phenotype was milder than expected.

In the EZM and open field analysis of the locomotor activity data, mutant mice showed increased exploration of the more anxiogenic areas of test arenas (open zones of EZM, center of activity chambers), while children with PHP1A tended to have increased anxiety problems by parental report.³ The increased exploration of open spaces can also be interpreted as increased impulsivity.⁸ Consistent with this result, children with PHP1A did perform poorly on inhibition and switching tasks in a performance test of executive function.³ Children with PHP1A are also at higher risk of ADHD which is often characterized by impulsivity, though this is a more prominent feature of the hyperactive subtype while patients with PHP1A have symptoms consistent with the inattentive subtype.⁹

During 30 minutes of locomotor activity testing, the mutant mice had decreased activity, though this was not observed in the shorter (5 minute) EZM or Y-maze tests. Decreased exploration is sometimes associated with increased repetitive behaviors such as rearing, although this was not observed in these mice. The mutant mice also demonstrated poorer motor strength on the inverted screen test along with a non-significant trend toward poorer performance on rotarod testing. Children with PHP1A typically demonstrate gross and fine motor developmental delays, often severe enough to require both physical and occupational therapy.³

Children with PHP1A have a composite IQ score 21 points (-1.4 SD) lower than their unaffected siblings and 93% require special education services at school.³ In one kindred study, family members with PHP1A had composite IQ scores 30 points (-2 SD) lower than family members with PPHP.² Patients with PHP1A have a particular deficit in working memory.³ In contrast, performance did not differ according to genotype in our murine model of PHP1A/PPHP on either of the hippocampal based learning tasks (Y-maze or context trial of fear conditioning). Mice in this study were exposed to multiple behavioral tests, including several that are invasive or aversive (conditioned fear, tail suspension, forced swim). The amount of cognitive testing performed was therefore limited since excessive handling and testing could impact performance on the depressive-like and anxiety-like behavior tests, and conversely, additional stress could have impacted learning and memory. Since our data suggested that the cognitive deficits in this model are subtle, future testing should incorporate tests that can be manipulated to increase difficulty level, such as lengthening inter trial intervals, or Morris water maze testing followed by reversal learning (new platform positions) to fully investigate the cognitive abilities in these mice. In contrast, amygdala based learning may be impaired because we observed that after accounting for the baseline freezing levels in the cue trial of the fear conditioning paradigm, mutant animals froze less when they heard the tone that had previously been paired with the shock.

In this study we compared maternally vs. paternally inherited G_{α} mutations. The advantage of this strategy is minimizing the effect of confounders inherit to this genetically modified

mouse model, such as behavioral changes attributable to nestin-cre. The differences demonstrated between groups in this study were less than expected and did not fully recapitulate the human PHP1A phenotype of impaired cognition and decreased working memory. It is possible, however, that paternally inherited $G_{s\alpha}$ mutations do confer a behavioral phenotype which would minimize the differences between our two groups. Since this is a disorder that is typically diagnosed in early childhood, it would also be interesting to study these mice at an earlier time point. Further research is needed to understand behavior and cognition in humans with PPHP and to determine if this cre-lox system can serve as a model for mechanistic studies. In particular it would be informative to conduct more in-depth cognitive analyses, and to probe specifically for signs of impulsivity including the use of operant testing, along with other behaviors modeling altered anxiety and depressive like behavior (REF 8). It will also be useful to document the emergence of deficits in this model to assess the developmental trajectory of behavioral differences and how well these may track with the clinical phenotype observed in human populations.

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Figure 1. Activity, anxiety and depressive like behaviors.

(A) Distance travelled in the elevated zero maze, (B) number of transitions between open and closed zones (zone entries), and (C) percent time spent in the closed zones of the maze. (D) Distance travelled in locomotor activity chambers and (E) number of vertical beam breaks (rearing). (F) Time spent in the periphery for the entire 30-min, locomotor session. (G) Total time spent immobile (not swimming) during the 6-min. forced swim trial. (H, I) Time spent not struggling across the six 1-min. time bins during tail suspension testing for male and female mice. Paternally-inherited mutation = Control; Maternally-inherited mutation = Mutant. M=Male, F=Female. Pct. = percent. * $P < 0.05$, *** $P < 0.001$ main effect of genotype.

Figure 2. Sensorimotor function and repetitive behaviors.

(A) Average time to fall across three trials on the inverted screen test. (B, C) Average time taken to fall from the rotarod on three consecutive days with three trials per day, in male and female mice. (D) Number of marbles buried greater than 50% in the 30-min. trial.

Paternally-inherited mutation = Control; Maternally-inherited mutation = Mutant. M=Male, F=Female. * $P < 0.05$, main effect of genotype.

A - 2-trial Y-maze

B - 2-trial Y-maze

C - Conditioned fear

D - Conditioned fear

E - Conditioned fear

Figure 3. Cognitive function.

(A) Distance travelled and (B) time spent in the novel arm ('Nv') versus the average time spent in familiar arms ('Fm') during the test trial of the two-trial Y-maze paradigm. (C) Time spent freezing during the familiar context trial and (D) the novel-context, familiar cue trial (G) of the fear conditioning test. Paternally-inherited mutation = Control; Maternally-inherited mutation = Mutant. M=Male, F=Female. * $P<0.05$, *** $P<0.001$ main effect of genotype, or difference between novel and familiar arms.

Table 1.

Group and genotype information for mice included in behavioral testing.

	Sex	Mice included in behavioral analyses (N)	Mean age at start of testing (S.D.)	Total number of litters tested
	Male	14	10.64 weeks (S.D. 0.92)	6
<i>Paternally inherited</i>	Female	20	9.42 weeks (S.D. 2.04)	6
<i>“Control”</i>				
<i>Maternally inherited</i>	Male	11	8.96 weeks (S.D. 1.22)	5
<i>“Mutant”</i>	Female	16	9.78 weeks (S.D. 1.12)	5

Table 2.

Order for behavioral testing

Test order and information	
<i>Week 1</i>	Elevated Zero maze, one trial 5-min Locomotor activity, one trial 30-min Inverted screen, three trials, max. 60s/trial Rotarod, three trials per day, 3 days, max. 300 s/trial
<i>Week 2</i>	Marble Burying 2-trial Y-maze, 6-min trials Tail suspension, one 6-min trial
<i>Week 3</i>	Fear conditioning, 8-min training, two 4-min recall trials Forced swim, one 6-min trial