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ORIGINAL ARTICLE

Establishment of mouse line showing inducible priapism-like phenotypes

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Abstract

Purpose: Penile research is expected to reveal new targets for treatment and prevention of the complex mechanisms of its disorder including erectile dysfunction (ED). Thus, analyses of the molecular processes of penile ED and continuous erection as priapism are essential issues of reproductive medicine.

Methods: By performing mouse N-ethyl-N-nitrosourea mutagenesis and exome sequencing, we established a novel mouse line displaying protruded genitalia phenotype (PGP; priapism-like phenotype) and identified a novel *Pitpna* gene mutation for PGP. Extensive histological analyses on the *Pitpna* mutant and intracavernous pressure measurement (ICP) and liquid chromatography–electrospray ionization tandem mass spectrometry (LC–ESI/MS)/MS analyses were performed.

Results: We evaluated the role of phospholipids during erection for the first time and showed the mutants of inducible phenotypes of priapism. Moreover, quantitative

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analysis using LC–ESI/MS/MS revealed that the level of phosphatidylinositol (PI) was significantly lower in the mutant penile samples. These results imply that PI may contribute to penile erection by PITPα.
Conclusions: Our findings suggest that the current mutant is a mouse model for priapism and abnormalities in PI signaling pathways through PITPα may lead to priapism providing an attractive novel therapeutic target in its treatment.

KEYWORDS
corpus cavernosum, erectile dysfunction, erection, phosphatidylinositol transfer proteins alpha (*Pitpna*), priapism

1 | INTRODUCTION

Erection is triggered by complex systems with fine vasculature, nervous systems, and small cavities termed sinusoids.[1–3] We established a novel mouse line displaying a unique copulatory phenotype, the protruded genitalia phenotype (PGP; priapism-like phenotypes). Priapism is one of the serious erectile abnormalities that shows continuous erection. Delayed treatment of sustained erectile disorders often leads to erectile dysfunction (ED).[4–6] ED is a serious reproductive problem in elderly societies and its high frequency has been recently reported in advanced countries. As such, Priapism is considered a serious pathological condition and an essential issue for the analysis of erectile processes.

N-ethyl-N-nitrosourea (ENU), an alkylating agent, is one of the efficient reagents for screening phenotypes by inducing mutations in mouse germline stem cells. This allows for the discovery of gene functions in an unbiased manner.[7–11] Mice treated with ENU were mated on a genetic screen to identify recessive mutations.[12] Through a series of genetic analyses for mutagenesis screening using ENU, we identified the gene, phosphatidylinositol transfer protein alpha (*Pitpna*) as the gene responsible for PGP. To confirm the causative gene, we established a CRISPR/Cas9-mediated mouse line for *Pitpna*. Subsequent phenotypic analyses revealed identical PGP of ENU- and CRISPR/Cas-9-mediated mutants. Thus, we identified a novel *Pitpna* gene mutation that causes PGP.

PITPα transports phosphatidylinositol (PI) from the endoplasmic reticulum (ER) to the plasma membrane.[13–19] Previous reports have shown that the *Pitpna* null mutation causes spinocerebellar degeneration including gait abnormality, hypoglycemia, and liver dysfunction, leading to neonatal lethality.[20] In contrast, the current *Pitpna* mutant mice were still alive at 12 months of age showing phenotypes similar to those of *Pitpna*-deficient mice. The current experiments suggest that the *Pitpna* mutation is not only associated with gait abnormalities but also responsible for abnormalities in penile erection. The current analyses reported inducible priapism-like phenotypes promoting further analyses of erection-induced pathological events. Genetic studies on the pathology of priapism in mutant mouse models have rarely been reported. Most reports have reported mutations in nitric oxide (NO)-related signaling pathways.[21,22] We revealed the priapism-like phenotype is inducible by cavernous nerve stimulation (CNS) for the first time. Based on a series of current analyses, it is suggested that the transfer impairment of PI by PITPα may cause abnormalities in erectile functions. These results suggest the utility of the current mouse model in physiological and pathological penile studies.

2 | MATERIALS AND METHODS

2.1 | Animals

C57BL/6JJcl, DBA/2J, Jcl:ICR, and C.B.-17/Icr-*scid/scid* mice were purchased from CLEA Japan and heterozygous Crlj:CD1-*Foxn1^nu^* mice from Charles River Laboratories Japan. Mice were maintained at the RIKEN BioResource Research Center (BRC) and Wakayama Medical University. All mice in this study were housed under controlled temperature (21°C) with a 12:12 h light–dark cycle. Animal studies were reviewed and approved by the Animal Research Committee of Wakayama Medical University and the Institutional Animal Care and Use Committee of the RIKEN BRC.

2.2 | ENU mutagenesis and whole-exome sequencing

Mouse ENU mutagenesis was performed as described at http://www.brc.riken.go.jp/lab/gsc/mouse/ and in previous reports.[23,24] Mutagenized G0 males were crossed with C57BL/6J females. Males of the generated G1 founders were crossed again with the C57BL/6J females. To produce G3 progeny, in vitro fertilization (IVF) with G2 male and C57BL/6J female. Intercrossing by IVF was performed on G3 females x G2 males, resulting in the generation of G4 offsprings. The G4 mice were screened for various phenotypes (abnormal gait and PGP). Mice were weighed every 2 weeks from 3 weeks of age to 31 weeks unless indicated elsewhere. Serum samples from 11-week-old to 18-week-old mice were obtained from the inferior vena cava under anesthesia. Heterozygous mice were obtained by transplanting ovaries from phenodeviant G4 female mice into C.B-17/Icr-*scid/scid* strain mice, crossing the C57BL/6J strain with them. Homozygous mice were obtained by sibling

heterozygotes. Homozygous mice are perceived as individuals derived from ovarian transplants of phenodeviant G4 female mice. To generate F1 generation, heterozygous mice were obtained by transplanting ovaries from phenodeviant G6 female mice into C.B-17/Icr-*scid/scid* strain mice, crossing the DBA/2J strain with them. The F1 mice were interbred to produce homozygous mice on an ENU-injected mouse background. Genome-wide mapping studies using DNA samples and SNPs from 118 F2 male mice (23 with and 95 without PGP) were performed. After the detection of linkage on chromosome 11, the DNA of 118 F2 male offspring was genotyped using microsatellite markers to develop a chromosomal structure map of the region. The candidate genes localized to the critical interval between markers D11Mit219 (72.1M) and y D11Mit354 (82.4M) were identified by scanning the mouse genomic sequence available in NCBI. Genomic DNA was purified from mouse tail samples using the GenElute Mammalian Genomic DNA Miniprep kit (Sigma). Exome sequencing and bioinformatics analyses were performed at RIKEN GENESIS CO., LTD. Target capture for the exome was performed on each sample using SureSelectXT Mouse All Exon kit (Agilent Technologies) as previously described.[25] Exome-enriched DNA was then subjected to paired-end DNA library preparation. The length of the library, including the adaptors, was 294–310 bp. Whole-exome sequencing was performed using HiSeq2500. Sequencing data were generated using 101 bp paired-end reads.

2.3 | Generation of the Cas9 mediated *Pitpna* mutant mouse

Four-weeks-old C57BL/6NTac female mice were injected with 7.5 IU/mouse of pregnant male serum gonadotropin, followed 48 h later with 7.5 IU/mouse of human chorionic gonadotropin. The females were then mated to C57BL/6Ntac males, and fertilized oocytes were collected at 0.5 dpc. The final mixture was microinjected into the cytoplasm of pronuclear zygotes using piezoelectricity (Prime Tech).[26,27] On the next day, zygotes were transferred into pseudopregnant Crlj:CD1-*Foxn1*$^{nu/+}$ females. Founder male mice were mated with C57BL/6JJcl female mice to generate N1 generation. After an additional five generations of backcrossing to C57BL/6JJcl, we interbred for two generations to produce homozygous mice on the B6J background (N6F2). The mice were mated with Jcl:ICR mice for two generations and finally interbred to produce homozygote mice in a mixed background. The mouse strain is available from RIKEN BRC (RBRC09387). The guide RNA was selected using CRISPR Design (http://crispr.mit.edu/) website.[28] A DNA template for single-guide RNA (sgRNA) was produced using DR274 (Addgene #42250, a gift from Keith Joung).[29] The PCR product was used as a template for in vitro transcription using the MEGAshortscript T7 Transcription kit from Thermo Fisher. Cas9 mRNA was transcribed in vitro using the mMessage mMachine T7 ULTRA Transcription kit (Thermo Fisher) after linearizing T7-NLS hCas9-pA (RIKEN BRC #RDB13130, a gift from Tomoji Mashimo).[30] Single-stranded oligodeoxynucleotide (ssODN) template was purchased from Integrated DNA Technologies. The final injection mixture consisted of Cas9 mRNA (30 ng/μl), sgRNA (30 ng/μl), and ssODNs (100 ng/μl) to a volume of 15 μl in DNase- and RNase-free water. The sgRNA target sequence and ssODN sequences are listed in Table S1.

2.4 | Hematoxylin and Eosin stain

Penile tissues were harvested and fixed overnight at 4°C in 4% (w/v) paraformaldehyde (PFA) dissolved in phosphate-buffered saline (PBS). Tissues were subsequently dehydrated in methanol, paraffinized, and embedded in paraffin. H.E. staining were performed by standard procedures as previously described.[1,2,31,32]

2.5 | Immunofluorescence staining

Penile tissues were harvested and fixed overnight at 4°C in 4% (w/v) PFA dissolved in PBS The detailed protocols were previously described.[1,2,31,33] For primary antibody staining, the following antibodies were utilized: anti-CD34 (1/200, RAM34, eBioscience™), anti-ACTA2 (1/1000, U7033, DAKO), and anti-β-III-tubulin (1/200, ab18207, Abcam). For secondary antibody reaction, the following antibodies were utilized: Invitrogen goat antirabbit IgG Alexa Fluor 488 and Invitrogen goat antimouse IgG Alexa Fluor 488 (1/200, Thermo Fisher Scientific).

2.6 | CNS and ICP measurement

Intracavernous pressure (ICP) elicited by electrical stimulation was previously described.[34] In brief, mice are anesthetizedby 4% isoflurane for induction, and 1.5–2.0% isoflurane for maintenance using an inhalationanesthesia apparatus (Nakazawa Seisaku-sho, Funabashi, Japan). The left crus of the corpuscavernosum (CC) was cannulated using a 23-G needle for continuous ICP monitoring. The pressure transducer was connected through a transducer amplifier to a data acquisition board (PowerLab 2/26, AD Instruments Pty). Stainless steel bipolar wire electrodes (Unique Medical) and a pulse generator (Nihon Kohden) were used for cavernous nerve stimulations to electrically stimulate penile erections. The stimulation parameters were as follows: 5 V, 16 Hz, and duration time: 1 min.

2.7 | Lipid extract preparation from mouse CC

The frozen penile tissue sample was weighed in a Lysing Matrix D tube (MP Biomedical). After adding 800 μl of methanol, the tissue was homogenized using FastPrep-24TM 5G (MP Biomedical) at 6.0 m/s for four 40-s cycles. Homogenate (600 μl) was transferred into a glass tube and mixed with methanol (900 μl)/ultrapure water (750 μl)/2 M HCl (750 μl)/1 M NaCl (200 μl) solution containing 1 nmol C8:0/C8:0 PI(4,5)P_2 (as absorption inhibitor) and 20 pmol

of C17:0/C14:1 PI (as internal standard). After vigorous vortex-mixing, 3 ml $CHCl_3$ was added followed by further vortexing for 2 min. After centrifugation at 1200 rpm for 4 min at room temperature, the lower organic phase (crude lipid extract) was collected and transferred to a new glass tube. Pre-concentration and methylation reactions were performed before LC–MS/MS analysis as described previously.[35]

2.8 | LC-MS/MS analysis for lipid extracts

LC/MS/MS analysis was performed on an UltiMate 3000 LC system (Thermo Fisher Scientific) equipped with an HTC PAL autosampler (CTC Analytics) and a TSQ-Vantage (Thermo Fisher Scientific) using a multiple-reaction-monitoring experiment (MRM) as described previously.[36] MRM transitions are shown in Table S2. A total of the 17 acyl variants of PI was calculated.

3 | RESULTS

3.1 | Identification of a novel *Pitpna* mutation responsible for phenotypes like priapism

In this study, a recessive mutation causing PGP (priapism-like phenotype) and gait abnormalities was identified by a large-scale mouse screen with combined ENU mutagenesis and phenotype-driven screening.[7–11] Males of the generated G1 founders have crossed again with the C57BL/6J females. To reproduce G3 progeny, IVF with G2 male and C57BL/6J female. Intercrossing by IVF was performed with G3 females and G2 males, resulting in the breeding of G4 offspring. Some G4 females showed gait abnormalities and one G4 male showed the PGP (Figure 1A, Movie S1). To map and locate the affected genes, the G6 female mice showing gait abnormality were mated with male DAB/2J mice by ovarian transplant. The F2 line is expected to produce affected individuals homozygous for the recessive mutation and the mutation was transmitted from the G4 founder female exhibiting gait abnormalities and PGP. Genome-wide mapping studies using DNA samples and SNPs from 118 F2 male mice (23 with and 95 without PGP) showed that the causative locus was mapped to chromosome 11 (Figure 1B). Analysis with haplotype frequencies showed that the genomic region flanked by D11Mit219 (72.1M) and D11Mit354 (82.4M) of mouse chromosome 11 showed overall high LD. In the present study, whole-exome sequencing identified G to A transition in the *Pitpna* gene at nucleotide position 514 in the 8th exon. The amino acid substitution (Gly→Arg) was converted with uniquely homozygous with PGP (Figure 1C). To further confirm the correlation between *Pitpna* gene and PGP phenotype, we generated *Pitpna* mutant mice by CRISPR technology. Cas9 mRNA and sgRNA were co-injected into the mouse zygotes. Both two lines for ENU and Crispr-mediated mutant showed indistinguishable phenotypes of defective fat absorption, stunted growth, gait abnormalities, and PGP. Hence, we concluded that mutant mice created by ENU and Crispr showed identifiable phenotypes.

3.2 | The current *Pitpna* mutant mice phenocopy *Pitpna*-deficient mice

Like the *Pitpna*-deficient mice, the abdominal cavity of the current *Pitpna* mutant mice showed abnormal morphology with markedly less fat (Figure S1A). The current mutants tended to show fat malabsorption with stunted growth. Wild-type (WT) mice continue to gain weight after birth. In comparison, the current mutants showed weight gain during the first 3 days after birth without further weight increase thereafter (Figure S1B). In the case of *Pitpna*-deficient mice, hypoglycemia and abnormalities in hepatic lipid metabolism were reported.[20] To evaluate these findings, serum levels of alkaline phosphatase (ALP), total bilirubin, aspartate aminotransferase (AST), high-density cholesterol (HDL), and glucose were analyzed. The current mutants displayed increased AST, ALP, and total bilirubin levels with decreased HDL levels (Figure S2). These results suggest the current mutants may possess fatty deposits in the liver like the *Pitpna*-deficient mice. The glucose levels also displayed prominently low values (Figure S2), suggesting that the current mutants also have abnormalities related to glucose homeostasis and transport of certain luminal lipid cargoes. Hypoglycemia is likely to be a major factor in the development of cerebellar degeneration. Like the *Pitpna*-deficient mice, the current mutants also developed difficulties in gaits (Movie S1).

In addition, postnatal lethality until day 14 was detected for the *Pitpna*-deficient mice.[20] The current mutants were able to live to adulthood even though phenocopying many aspects of the *Pitpna*-deficient mice (Figure S1B).

3.3 | *Pitpna* mutants display priapism-like phenotype

The mouse penis shows a unique structure compared with the human penis.[1,2,37,38] In general, the foreskin encases the glans region, masking it from the surface. PGP was frequently observed between 4 and 8 weeks of age in the current mutants (Figure 1A). The mouse penis possesses three corporal units (CC and corpus cavernosum glandis: CCG). The erected penis of CCG and CC contains a sinusoidal structure which is a microvascular complex and essential for erection.[1,2]

Priapism is classified into two types: "veno-occlusive (ischemic)" and high-flow (non-ischemic) priapism. In the case of veno-occlusive type, outflow from the CC is impaired and it results in tissue damage and necrosis. Tissue damage and necrosis are not observed in high flow priapism patients because the circulation is not disrupted.[39–41] To examine whether PGP displays obvious morphological abnormalities such as necrosis, hematoxylin & eosin (H&E) stains was performed. Since the body of the mouse

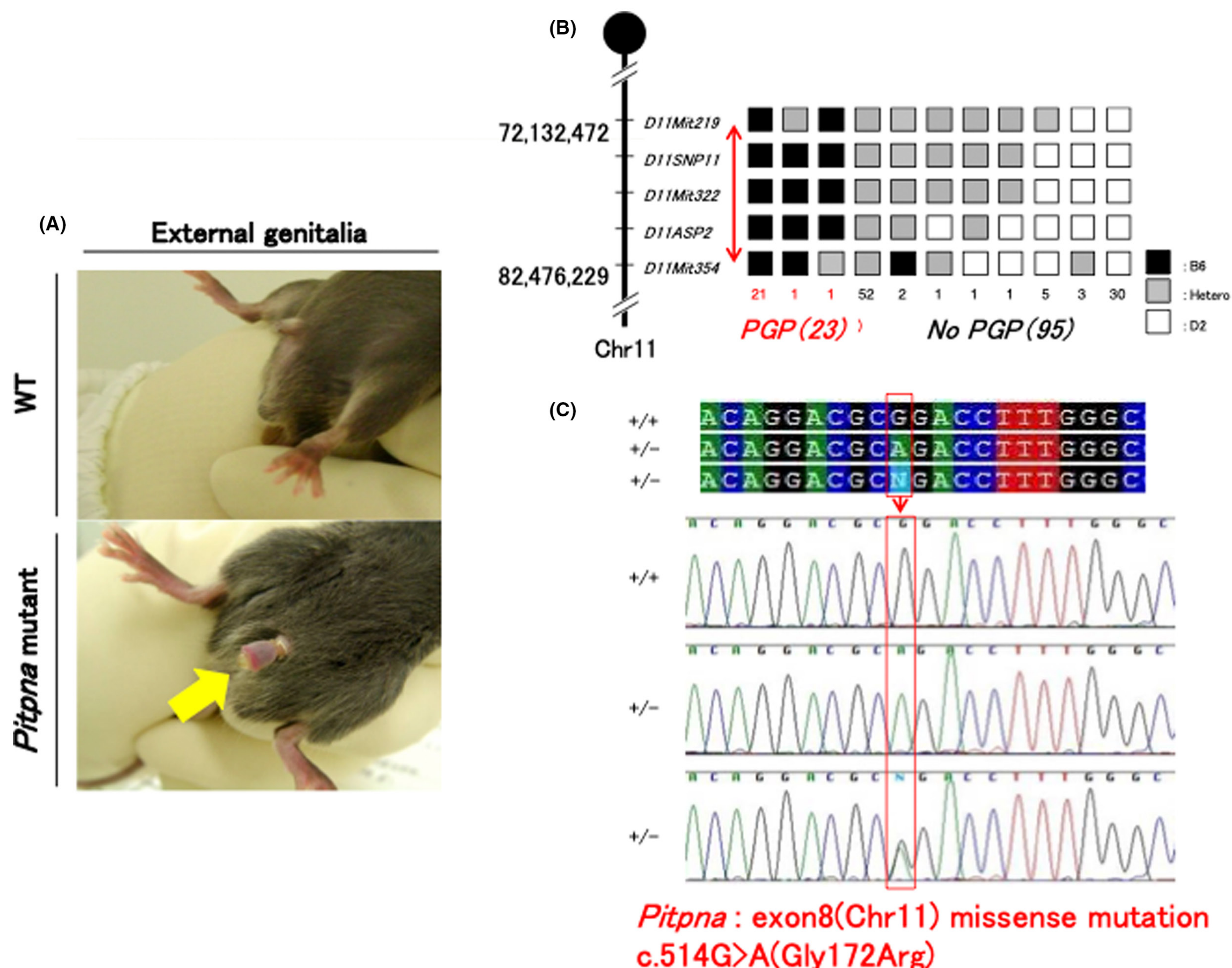

FIGURE 1 Identification of mutation responsible for protruded genitalia phenotype (PGP). (A) Representative images showing the current *Pitpna* mutant mice (*Pitpna* mutant) at 8 weeks post-partum compared to WT littermate. Protruded genitalia phenotype (PGP; priapism-like phenotype) was observed in *Pitpna* mutant (arrow, bottom). (B) Genetic map of mouse chromosome 11 showing the PGP responsible region and linkage cross-data: Gait abnormality mice were mated with DAB/6J mice and the F1 mice exhibit no abnormalities. A total of 118 inter-cross progenies from the F2 were phenotyped for PGP and genotyped for the indicated microsatellite markers. Black boxes represent haplotypes for C57BL/6J alleles and white boxes represent haplotypes for DAB/6J-derived alleles. The number of the corresponding haplotype was indicated below each column of squares. The location of marker loci was determined by minimizing the number of crossovers. (C) The nucleotide sequences around the single base substitution at position 514 (G to A) in exon8 were shown for the wild-type allele, the homozygous mutant allele, and the heterozygous mutant allele of the *Pitpna* gene. A novel mutation leads to amino acid substitution Gly172Arg in the *Pitpna* gene. WT, wild type

penis is located deeper than the body surface,[1,2] the most apparent phenotype resulting from priapism is inside of CCG. The CCG of the current mutant mice was noticeably congested with no obvious morphological abnormalities such as necrosis (Figure 2A,B). In the case of CC regions, there were no obvious changes between WT and the current mutants (Figure 2C,D). More detailed analyses by immunofluorescence of cell markers (endothelium: CD34, smooth muscle cell; ACTA2, peripheral nerve: β-III-tubulin) were performed. CD34 positive cells showed the presence of sinusoidal lumens. ACTA2 positive smooth muscle cells were observed in trabecular regions. β-III-tubulin positive cells were observed along with deep artery and sinusoids. Thus, there were no prominent alterations among WT and the current mutants (Figure S3). These results suggest that PGP may not result in ischemic effects on the penis.

3.4 | Inducible and sustained erection by electrical stimulation of the current mutants

The current mutants showed inducible and sustained erection by electrical stimulation. CNS and measurement of ICP have been utilized to evaluate erectile functions.[42] To examine whether the PGP of the current mutants was due to abnormal erectile functions, CNS

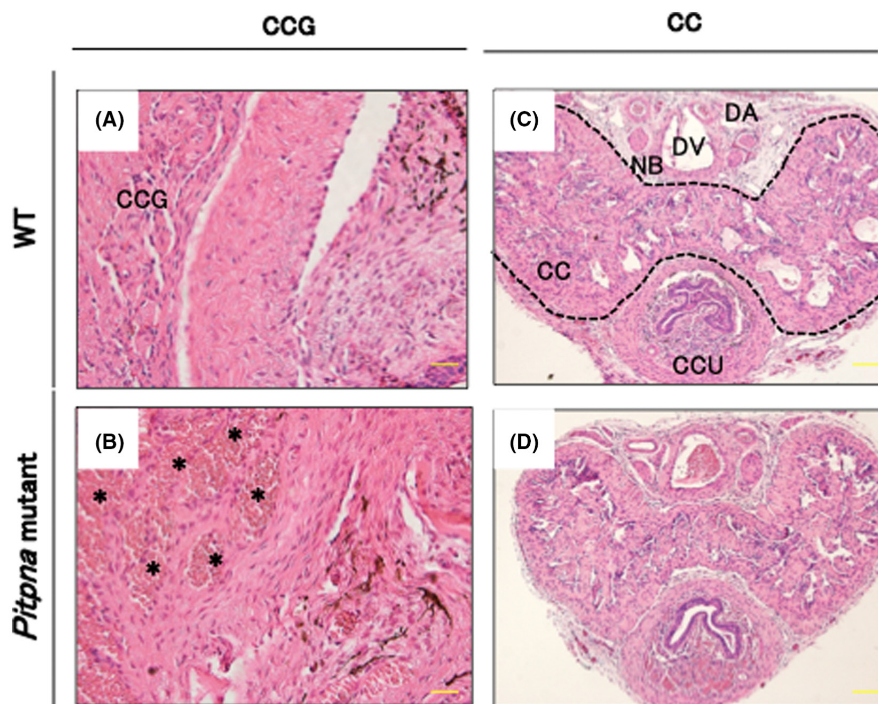

FIGURE 2 Mouse congested corpus cavernosum glandis (CCG) and corpus cavernosum (CC) of the WT and the established mutants. (A,B) The distal glans possess the penile urethra, the corpus cavernosum glandis (CCG), the corpus cavernosum urethrae, and skeletal elements. The hematoxylin-eosin staining images of WT mouse and *Pitpna* mutants CCG are shown by cross sections. (*) spaces indicate congested sinusoids in CCG. Scale bar 20 μm. (C,D) Numerous sinusoidal spaces in CC were indicated by the dotted lines. The hematoxylin-eosin staining images of WT and *Pitpna* mutants CC are shown by cross sections. Scale bar 100 μm. DV, dorsal vein; DA, dorsal artery; NB, nerve bundle; CCU, corpus cavernosum urethra; U, urethra

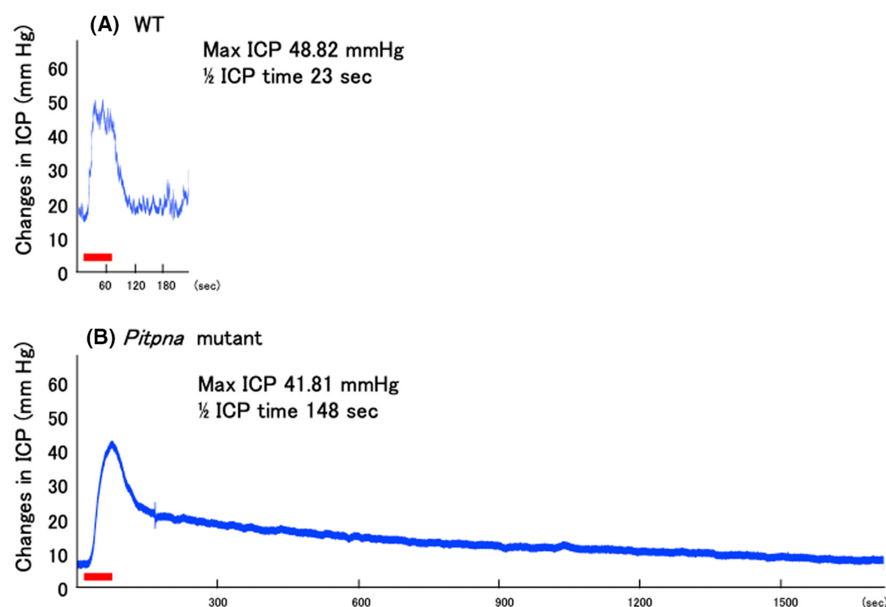

FIGURE 3 Augmented and sustained erectile responses to cavernous nerve stimulation. Representative tracings of 5-V stimulation (16 Hz) of erection for 1 min (solid red bar) in the mouse penis. The continuous irregular erectile response of the *Pitpna* mutants was clearly observed. Peak change in ICP was measured as the highest continuous pressure peak maintained for at least 150 s during the stimulus interval

and the measurement of ICP utilizing the sexually mature mutants without showing PGP were performed at 8 weeks old age. In the case of WT, ICP was dramatically increased during 5 s of stimulation and rapidly reduced approximately within 30 s after CNS (Figure 3A). The ICP of the current mutants was also increased during CNS. In contrast to WT, the current mutants kept high levels of ICP for 150 s after CNS (Figure 3B). The maximum level of ICP was similar between WT and the current mutants. However, the mutants' elevated ICP required approximately 25 min to return to their initial state levels. These results suggest that electrical stimulation could induce an erection in the current mutants and that PGP represents an abnormally persistent erection.

3.5 | Possible impaired consumption of total phospholipids in *Pitpna* mutants

PITPα binds to phospholipids such as PI and transfers them between ER and plasma membrane.[13–19] Organelle level bioassays have been only performed to measure *Pitpna* functions in vitro. However, direct in vivo measurement of PI under abnormal physiological conditions has not been performed. To investigate whether proper regulation of PI levels contributes to erectile functions, we measured PI in WT and the current mutants by liquid chromatography–electrospray ionization tandem mass spectrometry (LC–ESI/MS)/MS. The results demonstrate that PI in the current mutants was significantly reduced

after erection. Comparison between WT and the current mutants revealed that possible consumption of PI during erection was significantly reduced in the current mutants (Figure 4). The abnormal erection (priapism-like phenotype) observed in the current mutant would be related to such levels of PI consumption. This may indicate PI and its related signals contribute to erectile functions.

4 | DISCUSSION

4.1 | Establishment of a novel *Pitpna* mutant mouse line representing phenotypes like priapism

ENU mutagenesis screens have revealed previously uncharacterized phenotypes associated with mutant alleles.[7–9,43,44] We reported here the identification and characterization of the ENU-mutagenized *Pitpna* gene mice. The mutant mice displayed neurological and liver dysfunction with hypoglycemia and PGP. Genetic analyses indicate that the phenotype is recessive. Systematic genetic mapping suggests that the mutation accounted for the phenotypes is linked to chromosome 11. Analyses of the genome sequences confirmed the missense substitution, G514A(Gly172Arg) for exon 8 of the *Pitpna* gene. We also created a CRISPR/Cas9-generated mouse line with the same point mutation for exon 8 of the *Pitpna* gene. As a result, we revealed that the ENU mutant and the CRISPR mutant exhibit indistinguishable phenotypes. Hence, we have identified *Pitpna* as the gene responsible for these phenotypes. The established a novel *Pitpna* mutant mouse line displayed a reproductive phenotype as PGP.

4.2 | The current mutants were viable showing deteriorated liver function, gait abnormalities, and weight loss

PI transfer proteins (PITPs) transport PI from the site of synthesis to PI-depleted plasma membranes resulting in phosphoinositide-driven signaling.[14–16,35,45] Research on mammalian PITPs have revealed significant regulatory roles in lipid metabolism, membrane transport, and phosphoinositide signaling.[46–50] The PITP family

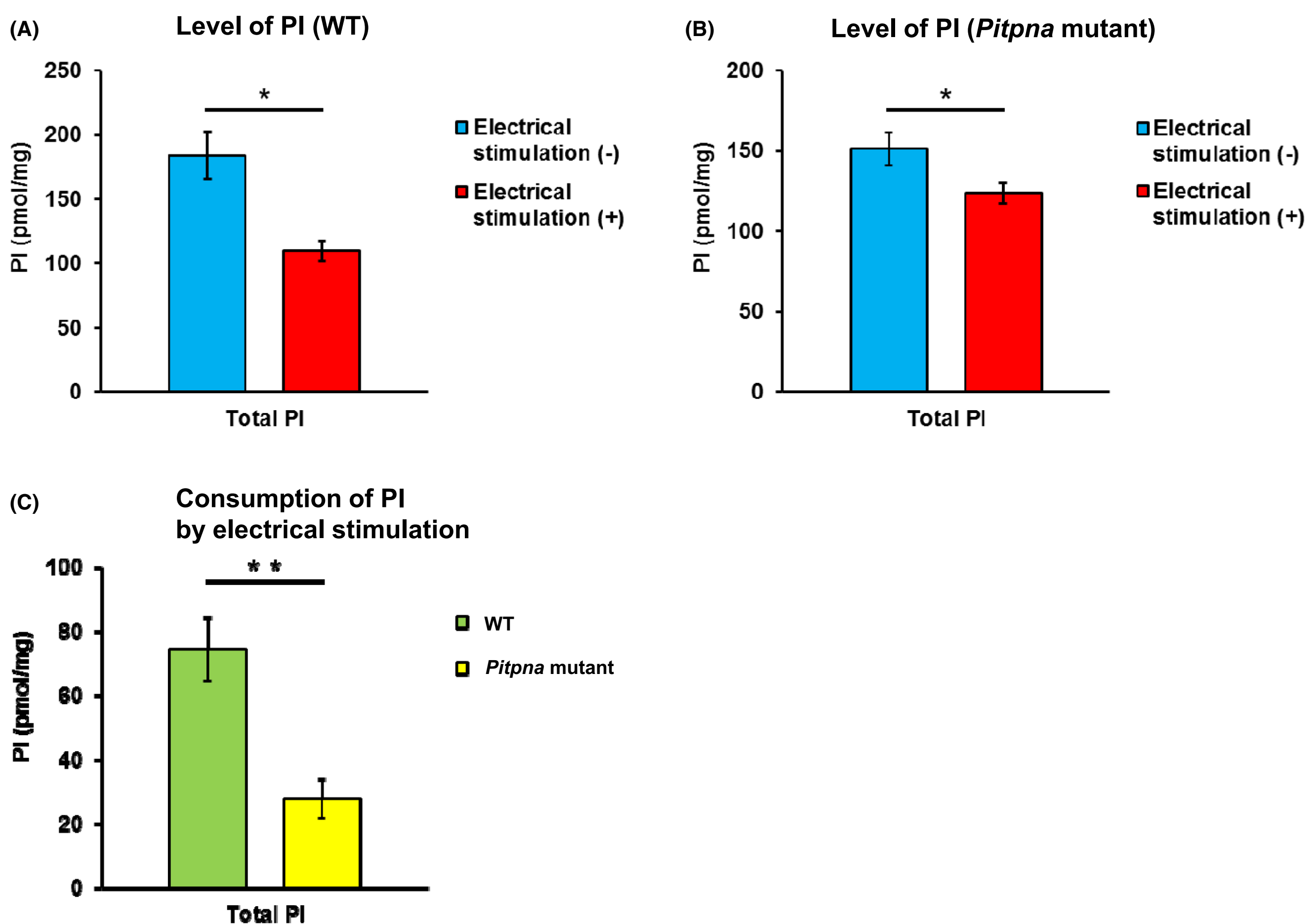

FIGURE 4 The established mutants contained a reduced level of phospholipids by quantification and analysis of penile tissue samples. (A,B) The reduced level of phosphatidylinositol (PI) in the flaccid penis (before erection) and erectile penis (after erection) were presented in the bar charts, with the *y*-axis indicating the relative intensity of phospholipid as quantified by LC–MS/MS in the WT and the *Pitpna* mutant. (C) The charts indicated the relative intensity of the WT and the *Pitpna* mutant for the levels of PI. Statistical differences between the WT and the *Pitpna* mutant's samples are assessed using a Student's *t* test for comparisons between groups. **Indicates $p < 0.01$. *Indicates $p < 0.05$

is divided into Class I and Class II according to their sequences. Class I PITPs (α and β) are relatively small proteins of about 35 kDa, including one PITP domain. PITPs (PITPα and β) bind to a single lipid and transfer it between separate membrane compartments. PITPα selectively transports and facilitates the exchange of PI between lipid bilayers. PITPα is required for cellular functions such as phospholipase C-mediated signaling.[13,19] *Pitpna*-deficient mice are not embryonic lethal but die postnatally due to multiple systemic disorders including spinocerebellar degeneration, hypoglycemia, and liver dysfunction.[20] Hepatocytes of *Pitpna*-deficient mice have been shown to accumulate neutral lipids suggesting a transport defect in the secretory pathway of lipids. The inability to effectively transport lipids from hepatocytes to the peripheral tissues has been suggested to impair fatty acid oxidation that enhances glycogenesis, resulting in hypoglycemia.[20] In addition, *Pitpna*-deficient mice develop spinocerebellar disorders with gait abnormalities. One of the essential phenotypes that triggered the identification of abnormalities in the ENU was gait abnormalities. It has been suggested that spinocerebellar disorders of *Pitpna*-deficient mice may be due to hepatic adiposity and hypoglycemia.[20] The ALP, AST, total Bilirubin, HDL cholesterol, and blood glucose levels of the current mutants were consistent with those of *Pitpna*-deficient mice, suggesting the presence of hepatic dysfunction, hypoglycemia, and dyslipidemia. As with *Pitpna*-deficient mice, the current mutants were born alive. On the other hand, they were still alive at 12 months of age, unlike *Pitpna*-deficient mice. The current mutants tended to show retarded growth postnatally with reduced abdominal fat in the abdominal cavity as with *Pitpna*-deficient mice. The heterozygotes of current mutants were similar in weight to the WT male mice, but the homozygotes of current mutants displayed marked weight loss. Although the current mutation was due to a point mutation, the current mutants represented phenotypes similar to those of *Pitpna*-deficient mice, albeit not lethal. It suggested that the current mutants were not effectively transporting lipids to peripheral tissues as in *Pitpna*-deficient mice. Some previous reports showed that the partial deletion of the C-terminus of *Pitpna* has been previously shown to impair the ability of PITPα to functions.[51,52] Nevertheless, They reported the protein retained its ability to bind lipids with a reduced transfer activity.[51,52] It is suggested that the abnormalities in the current mutants would be due to the subtle form of its mutation with reduced activity compared to *Pitpna*-deficient mice.

4.3 | The novel mutant mouse model for priapism

Priapism is the persistent, involuntary erection that occurs in the absence of sexual stimulation. The disease is critical because the severe forms of its symptom resulting in highly frequent ED. Priapism is a common complication of sickle cell disease (SCD). SCD is an inherited blood disorder that affects approximately 20–25 million people worldwide.[4,5] SCD patients tend to develop priapism, with a prevalence of about 30%–45%.[5,53] The complications associated with SCD, including priapism and ED, remain debilitating to that patients.[5]

It has been described that priapism typically involves the abnormalities of corpus cavernosum (CC).[54] Many studies reported that priapism eventually results in fibrosis of CC. Penises of the SCD model have been reported to possess severe CC abnormalities including increased collagen deposition and destruction of the endothelium which is consistent with the morphological changes of the penises of human cases with ischemic priapism.[55] However, histological characterization of many priapism models of mutant mice remains not performed. In this study, the current mutant mice displayed a similar structure of CC compared to WT mice. Detailed analyses by immunofluorescence of cell markers showed non-prominent differences in CC. Despite the absence of serious pathological changes, the current mutant mice showed phenotypes like priapism. It would be possible that the current analysis revealed the sort of prior stages of structural changes due to abnormality in the erection of mice.

On the other hand, we have evaluated for the first time CC glandis (CCG) that show phenotype-like priapism. Since the body of the mouse penis is located deeper than the body surface,[1,2] the most apparent phenotype resulting from priapism is CCG. The current mutant mice displayed sinusoidal expansion in CCG on H.E. stains compared to CC which showed no significant differences. These results suggest that CCG may be damaged earlier, and then CC may be subsequently affected. Although the mouse CCG region possesses penile bone, it could be argued that the CCG is generally equivalent to the human glans spongiosum. So far, the CCG has been ignored in many penile studies including ED and priapism because previous studies have generally focused on the CC. Analysis of CCG may contribute to further understanding of the pathophysiology of priapism.

In general, the pathogenesis of priapism has been discussed through vascular hemodynamic mechanisms.[21,22,41] The classical paradigm of venous occlusion may not explain sufficiently most of the symptoms of priapism. Traditionally accepted understandings of hematological diseases and traumatic disorders of the penis and perineum could not explain the several types of priapism.[40] Several recent studies have reported that priapism is also due to impaired regulation of penile erection.[21] Nitric oxide (NO) has been identified as a central component of the major signaling system that regulates the penile erectile response.[39] Regulatory dysfunction of NO signaling has been shown to potentially trigger priapism.[22] eNOS-deficient mice and eNOS/neuronal NOS double-deficient mice reported pronounced erectile responses.[21,22] Unlike previous mutants of *Pitpna*, the current mutants survive to maturity. We have evaluated the role of phospholipids during erection for the first time and demonstrated that current mutants showed inducible PGP. We revealed the in vivo erectile response of the current mutants and identified that the erectile response to CNS is elevated in comparison to WT mice. Thus, we think the current mutant phenotypes correspond to the priapism based on the penile congestion and abnormally persistent erection due to elevated ICP. The current study is expected to promote further understanding of the pathophysiology of priapism and

mouse model of erectile components, which will be useful for new treatments in the future.

Moreover, quantitative analysis using LC–ESI/MS/MS revealed that the level of PI was significantly lower in the penile samples from the current mutants showing continuous erection. These results imply that PI may contribute to penile erection by PITPα. Patients with spinal cord lesions or neurodegenerative diseases occasionally suffer from priapism.[56–58] *Pitpna*-deficient mice are born alive and exhibit spinocerebellar neurodegenerative disease. They die from multiple systemic disorders within a few days of birth.[20] Meanwhile, the current mutants showed neurodegenerative symptoms, but they could mature into adults and displayed characteristic priapism-like phenotypes. Therefore, reduced PI transport may relate to one of the causative dysfunctions, which may contribute to the abnormalities in the erection.

Phosphoinositides, the phosphorylated forms of PI, are the essential phospholipids as precursors of potent second messengers such as inositol triphosphate (IP_3) and diacylglycerol.[35,45,59,60] In addition, phosphoinositides possess various biological activities in vivo. Phosphoinositide 3-kinase (PI3K) generates PI tris-phosphate, which binds to and activates the protein kinase B (Akt).[45,61–64] The PI3K/Akt pathway regulates the phosphorylation of eNOS, which increases NO levels necessary for penile erection.[65–68] PITPα has been shown to regulate PI3K/Akt signaling during neuronal axon outgrowth.[69,70] Recent reports have indicated that downregulation of *Pitpna* expression is associated with upregulation of Akt signaling in Duchenne muscular dystrophy, a neuromuscular disease.[69] The current mutants revealed that reduced PI levels might influence PI3K/Akt signaling, and the dysfunction of this pathway may contribute to the pathological mechanism of priapism. Another possibility is that PI is associated with the supply of prostaglandin E1/E2 since the most abundant fatty acid in PI is arachidonic acid,[33] the precursor of prostaglandin E1/2 inducing penile erection.[71]

ENU phenotype-driven screens possess the capacity to uncover a significant number of phenotypes that show reproductive diseases. There have not been, however, reported known functions in penile disorders.[7–9,43,44] Using ENU mutagenesis, we showed phenotypes that uniquely resembling like an important vascular disease, priapism. The current mutants are not neonatally lethal and therefore potentially offer new aspects of pubertal *Pitpna* mutation. In this study, long-term alternation of tissue abnormalities in CC and ICP was not observed. Further experiments are expected to be conducted utilizing the current mutants.

Collectively, we evaluated the series of erectile responses in vivo and successfully induced erection for the first time. Our findings suggest that abnormalities in PI signaling pathways through PITPα may lead to priapism providing an attractive novel therapeutic target in its treatment.

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CONFLICT OF INTEREST

Daiki Hashimoto, Kota Fujimoto, Shin Morioka, Shinya Ayabe, Tomoya Kataoka, Ryutaro Fukumura, Yuko Ueda, Mizuki Kajimoto, Taiju Hyuga, Kentaro Suzuki, Isao Hara, Shinichi Asamura, Shigeharu Wakana, Atsushi Yoshiki, Yoichi Gondo, MasaruTamura, Takehiko Sasaki, and Gen Yamada declare that they have no conflict of interest.

HUMAN RIGHTS STATEMENTS AND INFORMED CONSENT

This work does not contain human subjects.

ANIMAL STUDIES AND APPROVAL BY ETHICS COMMITTEE

All procedures and protocols were approved by the committee on animal research at Wakayama Medical University, Wakayama, Japan (approval number: 995).

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SUPPORTING INFORMATION

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