



## Original

# A C1976Y missense mutation in the mouse *Ip3r1* gene leads to short-term mydriasis and unfolded protein response in the iris constrictor muscles

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**Abstract:** *Ip3r1* encodes an inositol 1,4,5-trisphosphate-responsive calcium channel. Mutations in the *IP3R1* gene in humans may cause Gillespie syndrome (GS) typically presents as fixed dilated pupils in affected infants, which was referred to as iris hypoplasia. However, there is no report of mice with *Ip3r1* heterozygous mutations showing dilated pupils. Here, we report a new *Ip3r1* allele with short-term dilated pupil phenotype derived from an N-ethyl-N-nitrosourea (ENU) mutagenesis screen. This allele carries a G5927A transition mutation in *Ip3r1* gene (NM\_010585), which is predicted to result in a C1976Y amino acid change in the open reading frame of IP3R1 (NP\_034715). We named this novel *Ip3r1* allele *Ip3r1*<sup>C1976Y</sup>. Histology and pharmacological tests show that the dilated pupil phenotype is a mydriasis caused by the functional defect in the iris constrictor muscles in *Ip3r1*<sup>C1976Y</sup>. The dilated pupil phenotype in *Ip3r1*<sup>C1976Y</sup> was referred to as mydriasis and excluding iris hypoplasia. IHC analysis revealed increased expression of BIP protein, the master regulator of unfolded protein response (UPR) signaling, in *Ip3r1*<sup>C1976Y</sup> mice that did not recover. This study is the first report of an *Ip3r1* mutation being associated with the mydriasis phenotype. *Ip3r1*<sup>C1976Y</sup> mice represent a self-healing model that may be used to study the therapeutic approach for *Ip3r1*-related diseases.

**Key words:** *Ip3r1*, mouse, mydriasis, N-ethyl-N-nitrosourea (ENU), unfolded protein response (UPR)

## Introduction

Inositol 1,4,5-trisphosphate receptors (IP3Rs) are ligand-gated ion channels that release calcium ions (Ca<sup>2+</sup>) from the endosarcoplasmic reticulum (ER) to the cytoplasm in response to the binding of inositol 1,4,5-trisphosphate (IP3) [3, 4]. Three subtypes of this receptor have been identified, namely, IP3R1, IP3R2, and IP3R3 (also known as ITPR1, ITPR2, and ITPR3, respectively). These three isoforms share a common structure consist-

ing of an IP3-binding core in the N-terminal portion, a channel domain at the extreme C-terminal end and a central coupling or modulatory domain [10, 14, 38]. These subtypes are co-expressed in a variety of cells and can form either homotetramer or heterotetramer Ca<sup>2+</sup> release channels with each having distinct channel properties [18].

*Ip3r1* is predominantly expressed in the central nervous system (CNS), especially in cerebellar Purkinje cells (PCs) [11]. *IP3R1* gene mutations in humans are

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Supplementary Figures: refer to J-STAGE: <https://www.jstage.jst.go.jp/browse/expanim>



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associated with different types of autosomal dominant spinocerebellar ataxia (SCA) including late-onset spinocerebellar ataxia type 15 (SCA15) [15, 22, 26, 32–34], congenital nonprogressive spinocerebellar ataxia and mild cognitive impairment (SCA29) [2, 21, 36, 39], infantile-onset cerebellar ataxia with mild cognitive deficit [28], and childhood-onset ataxic cerebellar palsy with moderate intellectual disability [27]. Recently, mutations in the *IP3R1* gene were identified in patients with Gillespie syndrome. Gillespie syndrome typically presents as fixed dilated pupils (referred to as iris hypoplasia) in affected infants. The key extra-ocular features of Gillespie syndrome are congenital hypotonia, non-progressive cerebellar hypoplasia, ataxia and variable, usually mild, neurocognitive impairment [2, 9, 23]. However, no major abnormality in eye development has been described in mice with *Ip3r1* heterozygous null mutations [8, 9, 13, 23].

We have previously shown that an ENU-induced mutation in the *Nrg1* gene leads to the dilated pupil phenotype in *Dp1* (Dilated pupil mutation 1) mice [5]. Another short-term dilated pupil phenotype mice is also reported in this study. Here, we report that the abnormal phenotype is due to a C1976Y mutation in the *Ip3r1* gene, which causes a functional defect in the iris constrictor muscles. We named this novel *Ip3r1* allele *Ip3r1<sup>C1976Y</sup>*. Apart from the dilated pupil phenotype (mydriasis), there are no other abnormal phenotypes including ataxia that may be found.

## Materials and Methods

### Mice

The *Ip3r1<sup>C1976Y</sup>* mutant heterozygote mouse was generated via ENU mutagenesis using C57BL/6 (B6) mice. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the Yangzhou University Animal Experiments Ethics Committee with permission number SYXK (Su) IACUC 2017–0044, and carried out in accordance with the approved guidelines.

### Preparation of DNA and RNA

Genomic DNA was isolated from mouse tail tips by proteinase K digestion, phenol chloroform extraction, and ethanol precipitation. Total RNA was extracted from the brains of *Ip3r1<sup>C1976Y</sup>* and wild type mice using TRIzol reagent (Invitrogen, Carlsbad, CA, USA).

### Linkage analysis

*Ip3r1<sup>C1976Y</sup>* heterozygotes (*Ip3r1<sup>C1976Y/+</sup>*) of the B6 background were mated to DBA/2 (D2) mice to generate

F1 mice. Subsequently, F1 mice with dilated pupils were backcrossed with B6 mice to generate N2 mice. DNA samples from N2 mutant mice were screened by PCR for microsatellite markers, with PCR products were separated on 4% agarose gels by electrophoresis. After refining the mutation location to a critical region, these genes in the region were then sequenced.

### Histology and immunohistochemistry analysis

Eyes enucleated immediately after all animals were anaesthetized by intramuscular injection of Zoletil 50 (50 mg/kg) and fixed in 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.4) at 4°C overnight. The cerebellum samples were then dissected after euthanasia by cervical dislocation and fixed in 4% paraformaldehyde. Fixed samples were dehydrated through a series of ethanol washes, cleared in xylene and embedded in paraffin. Prior to staining, 6- $\mu$ m sections were deparaffinized, rehydrated using a graded series of ethanol washes and rinsed in distilled water. Sections were stained in hematoxylin and eosin using standard protocols.

For immunohistochemistry, the sections were incubated in 3% H<sub>2</sub>O<sub>2</sub> for 10 min and an antigen retrieval with citrate buffer was performed prior the antibodies incubation. The slides were then incubated overnight at 4°C with the appropriate primary antibodies, which included antibodies against IP3R1 (1:400, Abcam, Cambridge, MA, USA, Cat. ab5804), BIP (1:150, Abcam, Cat. ab108615), Calbindin (1:400, Abcam, Cat. ab108404), Caspase 3 (1:100, Cell Signaling Technology, Danvers, MA, USA, Cat. 9579). Immunocomplexes were detected by using a biotinylated secondary antibody (1:400, Vector labs, Burlingame, CA, USA, Cat. BA1000) and ABC complex (Vector labs, Cat. PK6100) and AEC substrate kit (Zsbio, Beijing, China, Cat. ZLI-9036) or DAB substrate kit (Vector labs, Cat. SK4100), followed by brief counterstaining with Mayer's hematoxylin.

### Mutational analysis

The exons sequences (protein coding genes) or all sequences (non-coding RNA gene) of genes in critical region were amplified from *Ip3r1<sup>C1976Y/+</sup>* and B6 RNA using RT-PCR and genomic DNA using PCR. cDNA was synthesized using a RevertAid First-Strand cDNA Synthesis Kit (Thermo Scientific Fermentas, St. Leon-Ro, Germany) with oligo (dT) 18 primers. PCR conditions consisted of one cycle of denaturation for 5 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 61°C, and 1 min at 72°C, and finally, one cycle of elongation for 5 min at 72°C. The refseq accession number

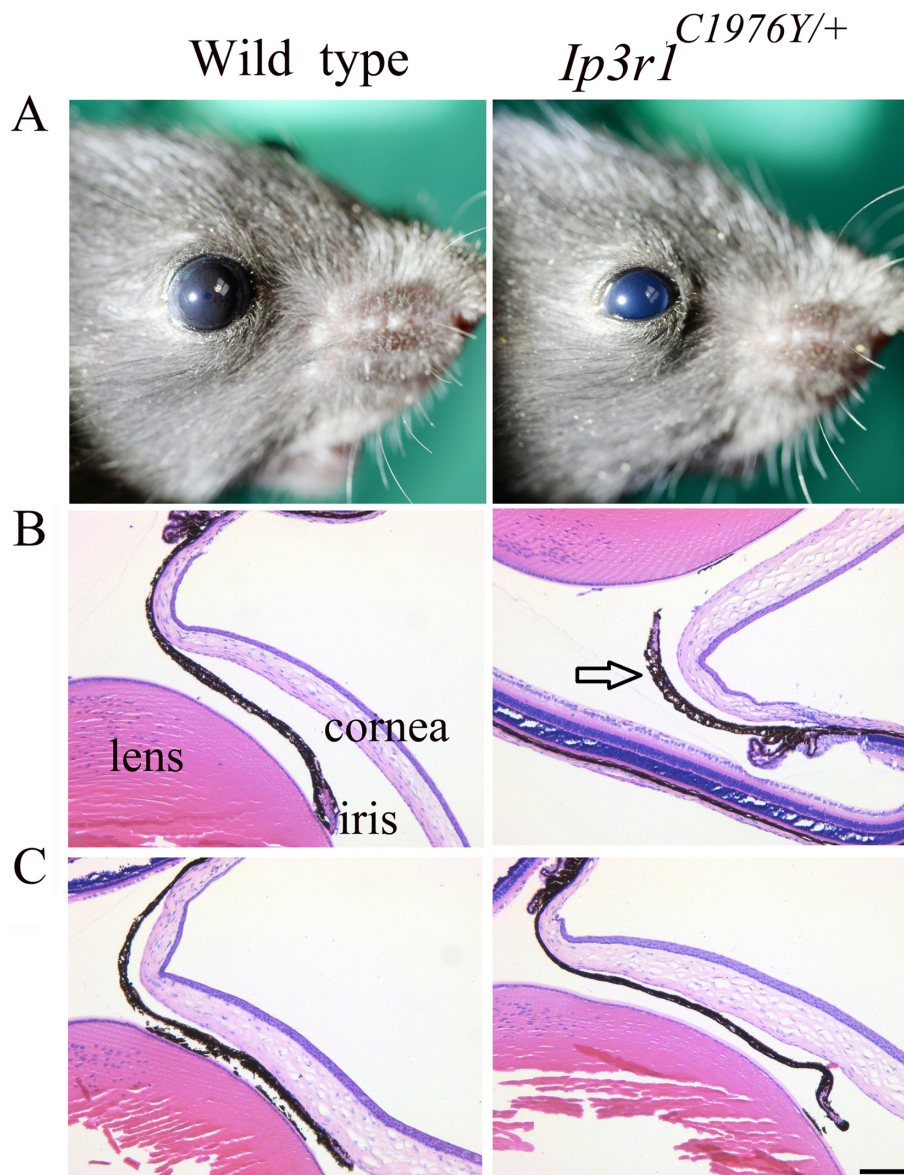
of *Ip3r1* is NM\_010585. Primer sequences are available on request. RT-PCR and PCR products were purified and sequenced by the dideoxy chain termination method using an ABI310 automated DNA sequencer (Sangon Biotech, Shanghai, China).

## Results

### Generating a mutant mouse with short-term dilated pupil phenotype using ENU mutagenesis

In a dominant ENU mutagenesis screen for abnormal

eyes, we identified a mouse mutation *Ip3r1*<sup>C1976Y</sup> with the bilaterally dilated pupil phenotype at 2-week time point (Fig. 1A). The ENU-mutagenesis parental strain was C57BL/6 (B6) male mice, and this was crossed with untreated B6 female mice for screening. After mating the *Ip3r1*<sup>C1976Y</sup> mice with wild-type B6 mice, a ratio of 359 of the 770 progeny were recorded to have the dilated pupil phenotype. Interestingly, all pupils of *Ip3r1*<sup>C1976Y</sup> recovered and were basically back to normal size after 4 weeks.



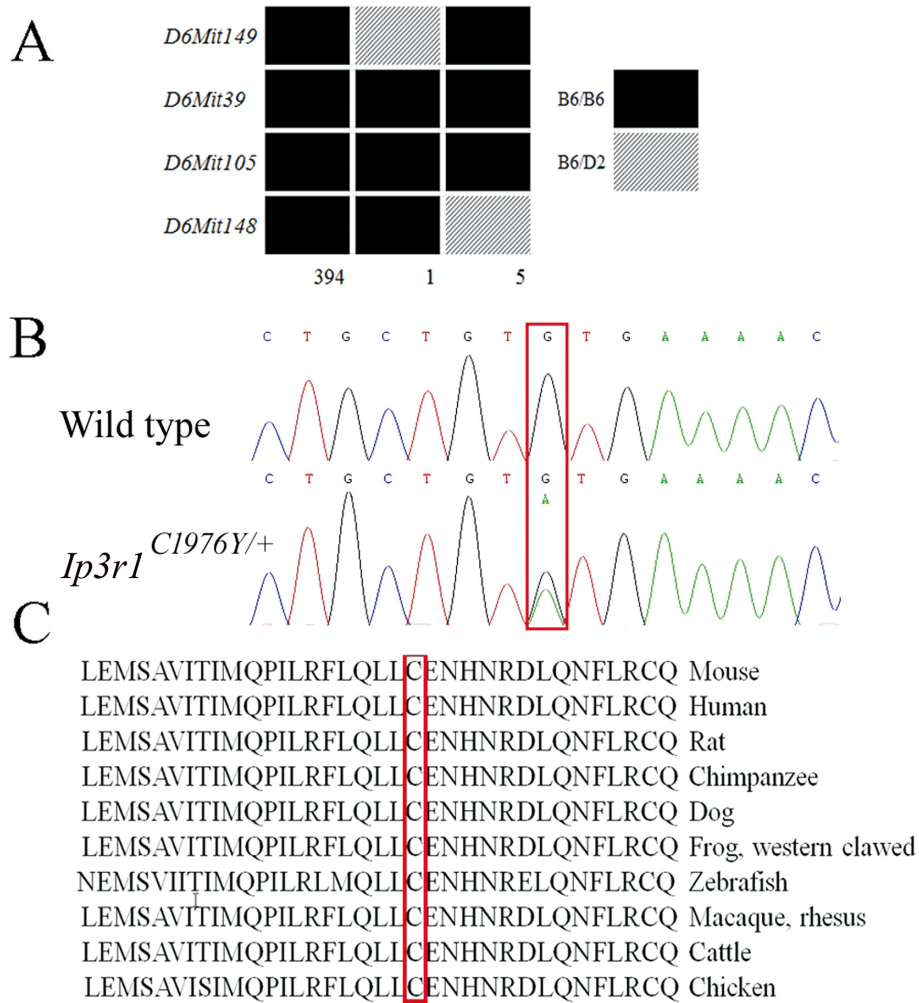
**Fig. 1.** Short-term mydriasis phenotype in *Ip3r1*<sup>C1976Y</sup> mice. (A) Wild-type mouse with normal pupil and *Ip3r1*<sup>C1976Y</sup> heterozygous (*Ip3r1*<sup>C1976Y/+</sup>) mice with a dilated pupil phenotype. (B, C) Eye pictures of hematoxylin and eosin staining from wild-type and *Ip3r1*<sup>C1976Y/+</sup> mice. *Ip3r1*<sup>C1976Y/+</sup> mice that did not recover and littermate controls (B). *Ip3r1*<sup>C1976Y/+</sup> mice that were recovered and littermate controls (C). The arrow in (B) shows the short iris in *Ip3r1*<sup>C1976Y/+</sup> mice that did not recover. At least three mice were used for each analysis, and the representative data were shown. Scale bar=100  $\mu$ m.

The dilated pupil phenotype is the result of a functional defect of the iris constrictor muscles

Hematoxylin and eosin staining showed *Ip3r1*<sup>C1976Y</sup> mutants that did not recover to have intact but short irises, compared with littermate control mice and *Ip3r1*<sup>C1976Y</sup> mice that were recovered (Figs. 1B and C). The results indicate that the defect in *Ip3r1*<sup>C1976Y</sup> mutants affects the pathway for the pupillary light response or in the muscles of the iris. The dilated pupil phenotype in *Ip3r1*<sup>C1976Y</sup> was therefore referred to as mydriasis, thereby excluding iris hypoplasia. To further analyze the defect, a 1% pilocarpine solution (a nonselective muscarinic cholinergic receptor agonist) was applied as drops in the eyes of *Ip3r1*<sup>C1976Y</sup> mice. Consequently, the mutant pupils fail to constrict indicating that the defect in *Ip3r1*<sup>C1976Y</sup> mutants is in the iris constrictor muscles.

The dilated pupil phenotype is caused by a point mutation in the *Ip3r1* gene

For initial mapping, we tested genomic DNA from 50 N2 samples with microsatellite markers across the whole genome. The mutation was mapped to chromosome 6 and had 1 exchange with marker *D6Mit230*, which was located at 45.74cM. In order to further refine the map position, genomic DNA from 400 N2 mutants was analyzed using microsatellite markers on chromosome 6. The mutation was mapped to a 4.78Mb region on chromosome 6, between microsatellite *D6Mit149* and *D6Mit148* (Fig. 2A). The region contained 10 protein coding genes (*Arl8b*, *Bhlhe40*, *Crbn*, *Edem1*, *Il5ra*, *Ip3r1*, *Lrrn1*, *Setmar*, *Sumf1* and *Trnt1*), 2 unclassified non-coding RNA genes (*0610040F04Rik* and *Gm17055*). Sequence analysis of these genes revealed no apparent nucleotide changes in *Ip3r1*<sup>C1976Y</sup> mice except for *Ip3r1*.



**Fig. 2.** Mapping and mutation analysis. (A) Genetic mapping places the mutant in the region between markers *D6Mit149* and *D6Mit148*. (B) Sequence analysis of the *Ip3r1* gene showed a G to A transition mutation, which resulted in a C1976Y amino acid change in the open reading frame in *Ip3r1*<sup>C1976Y/+</sup> mice compared to wild-type B6 mice. (C) Sequence alignment across multiple species revealed that this cysteine acid residue is highly conserved among vertebrates.

In the *Ip3r1* gene, we identified a G5927A transition mutation in exon 46 (Fig. 2B), which is predicted to result in a C1976Y amino acid change in the open reading frame. Sequence analysis of three different wild-type strains (C3H/He, 129 and DBA/2) excluded a general polymorphism at this site (data not shown). Sequence alignment across multiple species revealed that Cys-1976 is a highly evolutionarily conserved amino acid in IP3R1 suggesting conservation of function (Fig. 2C).

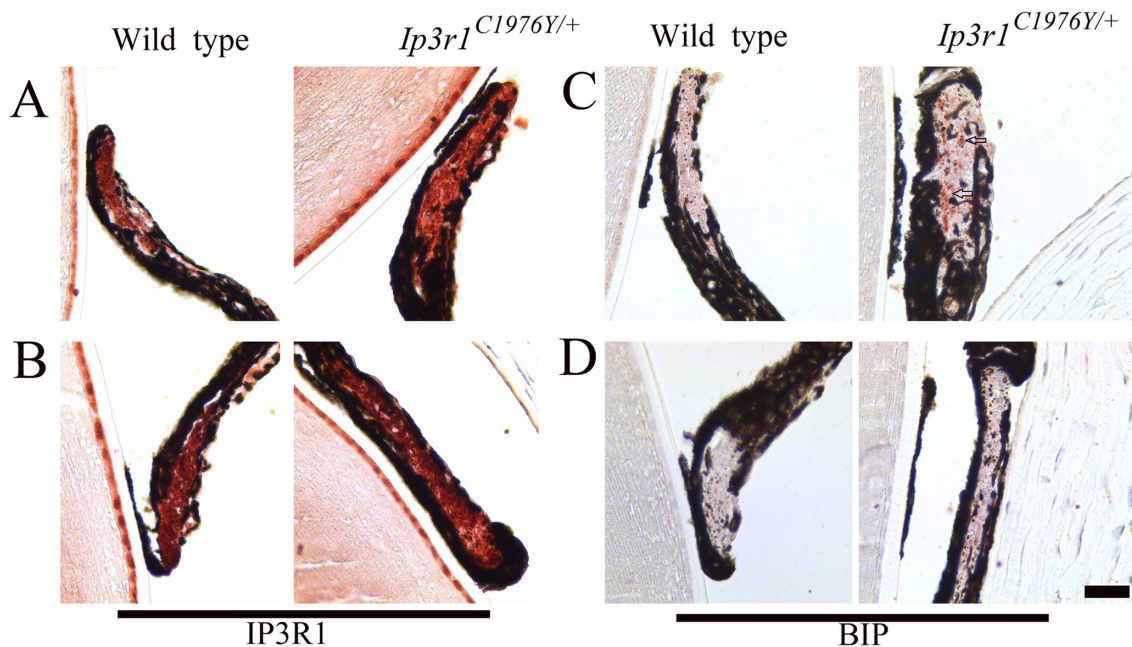
### Unfolded protein response (UPR) activation in the iris constrictor muscles of *Ip3r1*<sup>C1976Y</sup> mice that did not recover

To test whether the *Ip3r1*<sup>C1976Y</sup> mutation leads to alterations of IP3R1 protein levels in the iris constrictor muscles of *Ip3r1*<sup>C1976Y</sup> mice, we performed immunohistochemistry (IHC) tests on eye sections from *Ip3r1*<sup>C1976Y</sup> mice that were recovered and those that did not recover. The results revealed that IP3R1 protein expression was detected and similar in iris constrictor muscles of all mice (Figs. 3A and B). Mutant proteins within both the cytoplasm and the ER stimulate UPR. An ER chaperone, BIP acts as the master regulator of UPR signaling [1, 16]. IHC analysis using anti-BIP antibody showed that,

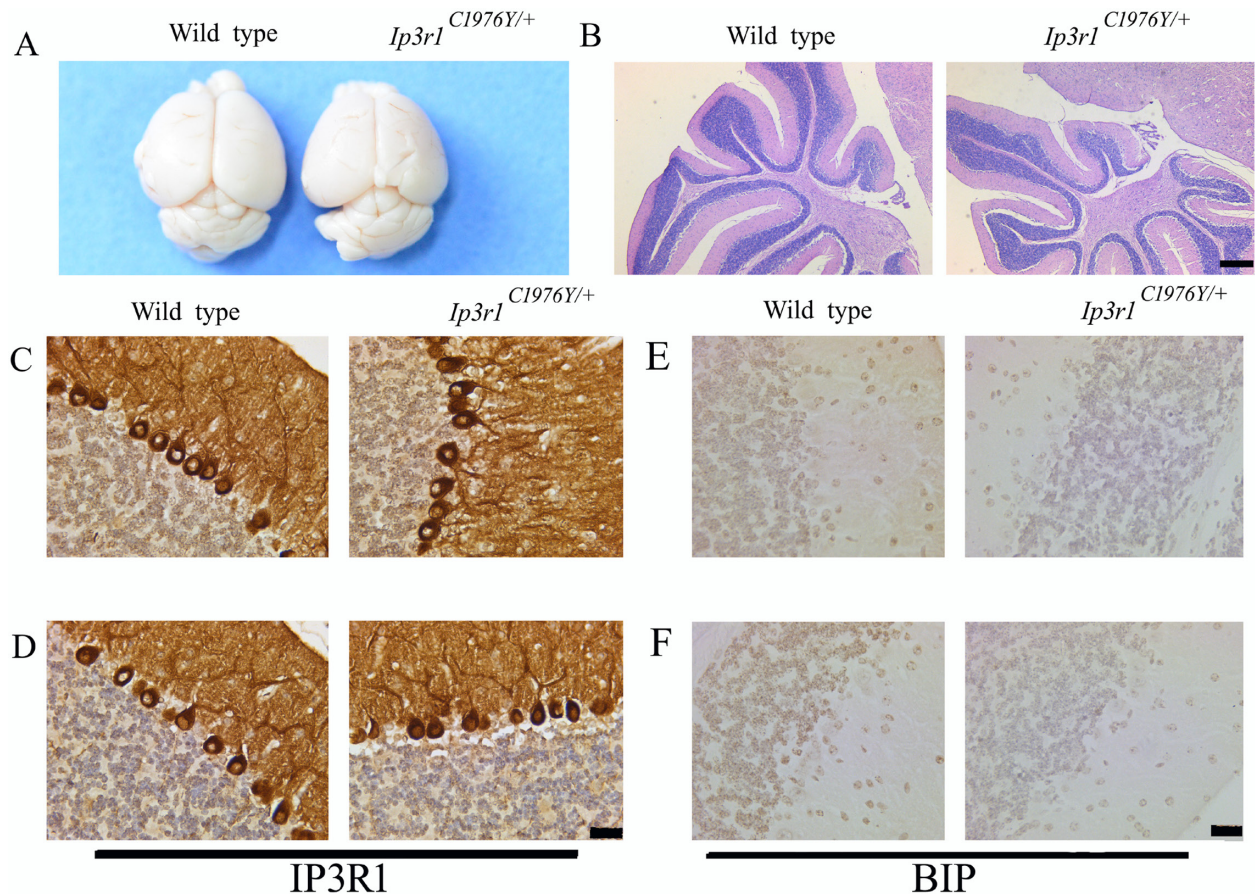
compared with wild-type littermates, BIP levels were increased in *Ip3r1*<sup>C1976Y</sup> mice that did not recover (Fig. 3C). There was no significant difference in recovered *Ip3r1*<sup>C1976Y</sup> mice and wild-type littermates (Fig. 3D). Failure of the UPR to resolve ER stress results in the stimulation of caspase-mediated apoptotic pathways [1, 20]. IHC analysis however, showed no significant apoptotic caspase 3 cells observed in the iris constrictor muscle of all mice (Supplementary Fig. 1).

### *Ip3r1*<sup>C1976Y</sup> mutants showed no obvious cerebellar ataxia symptom

Apart from the dilated pupil phenotype (mydriasis), there are no other abnormal phenotypes including ataxia that may be found. Cerebellar size of *Ip3r1*<sup>C1976Y</sup> was comparable to wild-type littermates (Figs. 4A and B). The expression level of IP3R1 in the cerebellum of *Ip3r1*<sup>C1976Y</sup> mice was equivalent to that of wild-type littermates (Figs. 4C and D). IHC for Calbindin (a marker of PC) and BIP did not reveal obvious differences between *Ip3r1*<sup>C1976Y</sup> mice and wild-type littermates (Supplementary Figs. 2A, B, Figs. 4E and F). No significant apoptotic caspase 3 cells were observed in PCs of all mice (Supplementary Figs. 2C and D).



**Fig. 3.** IHC analysis of iris constrictor muscle using anti-IP3R1 and anti-BIP antibodies. (A, B) IHC analysis of iris constrictor muscle using anti-IP3R1 antibody. *Ip3r1*<sup>C1976Y/+</sup> mice that did not recover and littermate controls (A). *Ip3r1*<sup>C1976Y/+</sup> mice that were recovered and littermate controls (B). The results showed similar IP3R1 protein level in the iris constrictor muscles between *Ip3r1*<sup>C1976Y/+</sup> mice and wild-type littermates. (C, D) IHC analysis of iris constrictor muscle using anti-BIP antibody. *Ip3r1*<sup>C1976Y/+</sup> mice that did not recover and littermate controls (C), IHC results revealed that the expression of BIP (arrow) was upregulated in iris constrictor muscles of *Ip3r1*<sup>C1976Y/+</sup> mice that did not recover, compared with wild-type littermates. The stained area fraction (%) for BIP is  $5.58 \pm 0.4$  and  $0.39 \pm 0.23$ , respectively ( $P < 0.01$ ,  $n = 4$ ). *Ip3r1*<sup>C1976Y/+</sup> mice that were recovered and littermate controls (D). The expressions of IP3R1 and BIP are shown in red. At least three mice were used for each analysis, and the representative data were shown. Scale bar = 25  $\mu\text{m}$ .



**Fig. 4.** There are no obviously abnormal phenotypes in the cerebellum of *Ip3r1*<sup>C1976Y/+</sup> mice. (A) Gross appearance of the brain from wild-type and *Ip3r1*<sup>C1976Y/+</sup> mice. (B) Hematoxylin and Eosin (HE) staining of the cerebellum in wild-type and *Ip3r1*<sup>C1976Y/+</sup> mice. Scale bar=250  $\mu$ m. (C, D) IHC analysis of cerebellum using anti-IP3R1 antibody. *Ip3r1*<sup>C1976Y/+</sup> mice that did not recover and littermate controls (C). *Ip3r1*<sup>C1976Y/+</sup> mice that were recovered and littermate controls (D). The results showed the expression level of IP3R1 in the cerebellum of *Ip3r1*<sup>C1976Y/+</sup> mice was equivalent to that of wild-type littermates. (E, F) IHC analysis of cerebellum using anti-BIP antibody. *Ip3r1*<sup>C1976Y/+</sup> mice that did not recover and littermate controls (E). *Ip3r1*<sup>C1976Y/+</sup> mice that were recovered and littermate controls (F). IHC analysis of cerebellum using anti-BIP antibody did not reveal obvious differences between *Ip3r1*<sup>C1976Y/+</sup> mice and wild-type littermates. The expressions of IP3R1 and BIP are shown in brown. Scale bar=25  $\mu$ m. At least three mice were used for each analysis, and the representative data were shown.

## Discussion

The pupil is a hole located in the center of the iris of the eye. The iris is a contractile structure, consisting two layers: the front pigmented fibrovascular layer known as a stroma and, beneath the stroma, pigmented epithelial cells. The stroma connects to an iris constrictor muscle (pupillary sphincter) and a set of iris dilator muscle (pupillary dilator). Pupil size is controlled by the iris constrictor muscle and the iris dilator muscle, which act in opposition to cause miosis (constriction) or mydriasis (dilation) of the pupil in response to different levels of light. A greater intensity of light causes the pupil to constrict, thereby allowing less light in, to prevent damage to the retina from excess light [7]. Hematoxylin and eosin staining showed an intact but short and unstretched iris, and the pilocarpine solution failed to constrict the mutant pupils in *Ip3r1*<sup>C1976Y</sup> mice that did

not recover. These results suggest that the dilated pupil phenotype is characterized by mydriasis, which is caused by a functional defect of the iris constrictor muscles.

Calcium ions influxes into the cytoplasm and their release from ER, allows actin-myosin interactions and contraction. One of the regulators of the intracellular  $Ca^{2+}$  is the IP3R1, which upon stimulation by the second messenger IP3 releases  $Ca^{2+}$  from the ER [6, 19, 25, 30, 35, 40]. It is therefore reasonable to assume that the C1976Y mutation in IP3R1 affects  $Ca^{2+}$  release from the ER, which lead to contraction defect of the iris constrictor muscles.

Mutations in the *Ip3r1* gene are associated with different types of autosomal dominant spinocerebellar ataxia (SCA) and Gillespie syndrome. The variation in the phenotype could be related to the location of the mutations in the protein; however, a clear phenotype-genotype could not be established. One can speculate

that mutant residues altered in Gillespie syndrome are located near the center of the channel, within or close to the transmembrane region [23, 34]. Mice with *Ip3r1* heterozygous null mutations showed no mydriasis [23]. These data suggest that the role of IP3R1 in iris constrictor muscle is tolerant of 50% residual channel activity. Given that IP3R1 forms a homotetramer, then only 1/16 assembled tetramers will contain four wild-type subunits. If a single variant subunit can block channel function, then 94% of tetramers will be non-functional, thus the mutation in *Ip3r1*<sup>C1976Y</sup> is likely to be acting by a dominant-negative effect.

UPR is a cellular stress response to prevent misfolded protein accumulation in ER, which strictly controls protein quality. However, prolonged induction of the UPR because of continuous expression and misfolding of mutant protein can lead to severe ER stress and induces cell apoptosis. This occurs in a variety of conditions with a genetic basis, including Alzheimer disease and cystic fibrosis. An ER chaperone, GRP78 (also known as the immunoglobulin heavy chain binding protein, BIP), acts as the master regulator of UPR signaling to improve biogenetic processes and has a cytoprotective function against ER stress. BIP can interact with IP3R1 monomers and tethers them to ensure the fidelity of subunit assembly without stochastic misassembly [5, 24, 29, 37]. GRP78 overexpression significantly enhances IP3R-mediated Ca<sup>2+</sup> release [1, 17]. Genetic studies have shown that the loss of BIP function leads to defective neural development and involuntary movement [24, 37]. Our study suggests that the unfolded protein response is activated by the *Ip3r1*<sup>C1976Y</sup> mutation, which leads to the recovery of pupil size in *Ip3r1*<sup>C1976Y</sup> mice.

Mammalian normal ocular surface development involves a transient closure and reopening of the eyelid. In mice, eyelid re-opening takes place two weeks after birth [12, 31]. So, the functional defect of the iris constrictor muscles may not be harmful to mice in the first two weeks after birth. However, the fidelity of the subunit assembly of IP3R1 is important to PCs from an earlier period. Thus we can hypothesize that in PCs an unfolded protein response may be happen at an earlier period in *Ip3r1*<sup>C1976Y</sup> mice and therefore cannot be detected after 2 weeks. If this is indeed the case, it may be a reason that no obvious cerebella ataxia in *Ip3r1*<sup>C1976Y/+</sup> mutants.

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