Ucp4 deficiency of cerebellar Purkinje cells induces bradykinesia

Ya-Yun Wang (wangyy@fmmu.edu.cn)
Air force Medical University

Hui Liu
Air Force Medical University

Shu-Jiao Li
Air Force Medical University

Ban Feng
Air Force Medical University

Yun-Qiang Huang
Air Force Medical University

Shui-Bing Liu
Air Force Medical University

Yan-Ling Yang
Air Force Medical University

Research Article

Keywords: Mitochondrial uncoupling protein 4, Purkinje cells, bradykinesia, cerebellum, movement disorders

Posted Date: February 17th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2572075/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Version of Record: A version of this preprint was published at Molecular Neurobiology on September 9th, 2023. See the published version at https://doi.org/10.1007/s12035-023-03607-1.
Abstract

Although uncoupling protein 4 (UCP4) is the most abundant protein reported in the brain, the biological function of UCP4 in cerebellum and pathological outcome of UCP4 deficiency in cerebellum remain obscure. To evaluate the role of Ucp4 in the cerebellar Purkinje cells (PCs), we generated conditional knock-out of Ucp4 in PCs (\textit{Pcp2}\textsuperscript{cre};\textit{Ucp4}^{fl/fl} mice) by breeding \textit{Ucp4}^{fl/fl} mice with \textit{Pcp2}\textsuperscript{cre} mice. PCR, western blot, double immunofluorescent staining and triple RNAscope in situ hybridization confirmed the specific ablation of Ucp4 in PCs in \textit{Pcp2}\textsuperscript{cre};\textit{Ucp4}^{fl/fl} mice, and the reservation of Ucp2, the analog of Ucp4. Open field test, CatWalk analysis, rotarod and elevated plus-maze tests showed that \textit{Pcp2}\textsuperscript{cre};\textit{Ucp4}^{fl/fl} mice displayed a characteristic bradykinesia in the spontaneous movements, and such bradykinesia disorder was not deteriorated in harmaline-induced tremor application. The electromyogram recordings detection on gastrocnemius muscle excluded the possibility of hypotonia in \textit{Pcp2}\textsuperscript{cre};\textit{Ucp4}^{fl/fl} mice. And the electrical patch clamp recordings showed the altered properties of both spontaneous and evoked firings in PCs of \textit{Pcp2}\textsuperscript{cre};\textit{Ucp4}^{fl/fl} mice. Also, the knockdown of Ucp4 significantly increased reactive oxygen species generation in the cerebellum. The present study is the first to report a close relationship between UCP4 deletion with PCs impairment, and suggests the importance of UCP4 in the substantial support of mitochondrial function homeostasis in bradykinesia. UCP4 might be a therapeutic target for the cerebela-related movement disorder.

Highlights

- The specific deletion of mitochondrial uncoupling protein 4 (UCP4) in the cerebellar purkinje cells (PC) induced bradykinesia in \textit{Pcp2}\textsuperscript{cre};\textit{Ucp4}^{fl/fl} mice by combined behavioral tests.
- The electromyogram recordings detection on gastrocnemius muscle excluded the possibility of hypotonia in \textit{Pcp2}\textsuperscript{cre};\textit{Ucp4}^{fl/fl} mice.
- The electrical patch clamp recordings showed the altered properties of both spontaneous and evoked firings in PCs of \textit{Pcp2}\textsuperscript{cre};\textit{Ucp4}^{fl/fl} mice.
- The knockdown of Ucp4 significantly increased ATP level, MMPI and ROS generation in the cerebellum.
- UCP4 might be a therapeutic target for the cerebela-related movement disorder.

1. Introduction

Uncoupling proteins (UCPs) are the members of mitochondrial anion transporter trans-membrane protein family (SLC25s) located at the inner mitochondrial membrane (IMM) in all mammals (Kumar et al., 2022; Demine et al., 2019; Jezek et al., 2004). UCPs uncouple electron transport from ATP synthesis by dispersing the proton gradient and play protection role against oxidative stress. Among five isoforms of UCPs (UCP1 - 5), UCP2, UCP4, and BMCP1/UCP5 have been reported in the brain and indicated to protect neurons from oxidative stress through diminishing the production of reactive oxygen species (ROS) (Liu
et al., 2006). Among them, UCP4 is the most abundant protein in all brain regions, including the olfactory brain (OB), cerebral cortex (CC), caudate putamen (Cpu), thalamus (Th, hypothalamus (HT), brain stem (BS) and cerebellum (CB) (Kim-Han and Dugan, 2005; Ramsden et al., 2012; Alán et al., 2009). The gene Ucp4 is located on 6p11.2 - q12 chromosomal and translates UCP4 protein with 323 amino acid and 34 KD (Ramsden et al., 2012). UCP4 has been indicated to reduce the mitochondrial membrane potential (MMP), production of ROS, and oxidative stress. It has been confirmed that the knockdown of UCP4 increased MMP. On the contrary, the over-expression of UCP4 could reduce the MMP, ROS, and ATP (Ramsden et al., 2012; Alán et al., 2009; Lu et al., 2020). It has been highlighted that the UCP4 could protect neuron against several neurodegenerative conditions like hypoxia (Xu et al., 2013) and ischemia (Liu et al., 2012), and several neurodegenerative diseases like Alzheimer's disease (AD) (He et al., 2019), Parkinson's disease (PD) (Lunetti et al., 2022), and Schizophrenia (Yasuno et al., 2007). However, the biological function of UCP4 in cerebellum and pathological outcome of UCP4 deficiency in cerebellum remain obscure.

It has been convinced that the cerebellum plays a critical role in the coordination of locomotion activity (Kandel et al., 2013; Manto et al., 2022). Movement disruptions due to cerebellar disorders are totally different from the paralysis caused by damage to the cerebral cortex (Kandel et al., 2013; Manto et al., 2022). Cerebellar disorders have four typical symptoms: first is hypotonia which is present as a diminished resistance to passive limb displacements; second is astasia-abasia which means the patient can not stand or walk; third is ataxia which is the abnormal execution of voluntary movements, and fourth is a tremor at the end of movement. The cerebellum constitutes only 10% of the total volume of the brain but contains more than 50% of its neuronal cells (Kandel et al., 2013; Manto et al., 2022). The cerebellar cortex is composed with a series of similar basic microcircuits (Kandel et al., 2013; Manto et al., 2022), and contains 3 layers: the external molecular layer (ML), the middle Purkinje cell layer (PCL) and the internal granular cell layer (GCL) (Cerminara et al., 2015). There are 6 main neuronal populations including Purkinje, stellate, basket, Lugaro, Golgi and granule cells in the cerebellar cortex. The granule cells are glutamate-expressing excitatory neurons, and others are GABA-expressing inhibitory neurons. Of these, Purkinje cells (PCs) are considered to be the most functionally important because they are the only projection neurons of the cerebellar cortex (Cerminara et al., 2015). PCs have the larger somatic bodies with 50 - 80 mm in diameter, and send the huge dendritic trees into the ML contacting climbing fibres and parallel fibres. And PCs send long axons through GCL and make inhibitory synaptic contacts with neurons of the cerebellar deep nuclei within the cerebellar white matter to control movement (Cerminara et al., 2015). Multiple gene deletions in PCs resulted in PCs dysfunction and further motor disorders. Tsuda et al have reported that the deletion of the zinc finger transcription factor Gfi1 in PCs of mice could cause PCs degeneration and mimic spinocerebellar ataxia type 1 (SCA1) (Tsuda et al., 2005). Liu et al. have shown that the mice with conditional knock-out of 3-Phosphoinositide-dependent protein kinase-1 (PDK1) in PCs displayed impaired motor balance and their PCs showed the reduced spontaneous firing (Liu et al., 2020). Zhou et al. have reported that the mice with PCs ablation of transferrin receptor 1 (TFR1) induces ataxia, but does not affect social behaviors (Zhou et al., 2017).
However, the roles of Ucp4 and the result of Ucp4 dysfunction in the PCs have not been investigated so far.

To evaluate the role of Ucp4 in cerebellar PCs, we first generated conditional knock-out of Ucp4 in PCs (Pcp2\textsuperscript{cre};Ucp4\textsuperscript{fl/fl} mice) by breeding Ucp4\textsuperscript{fl/fl} mice with Pcp2\textsuperscript{cre} mice. Second, the specific ablation of Ucp4 and the reservation of Ucp2, the analog of Ucp4, were confirmed by Western Blot, and triple RNAscope \textit{in situ} hybridization. Third, the combined behavioral tests of rotarod test, open field (OF) test, CatWalk analysis, and elevated plus maze (EPM) showed a characteristic bradykinesia with the reduction of spontaneous movements in Pcp2\textsuperscript{cre};Ucp4\textsuperscript{fl/fl} mice. Fourth, the Pcp2\textsuperscript{cre};Ucp4\textsuperscript{fl/fl} mice did not show hypotonia by electromyogram recordings (EMG) detection on gastrocnemius muscle. Fifth, the electrical patch clamp recordings showed the altered properties of both spontaneous and evoked firings in PCs of Pcp2\textsuperscript{cre};Ucp4\textsuperscript{fl/fl} mice. Sixth, the knockdown of Ucp4 resulted in the mitochondrial impairments reflected by the significant increases of ROS generation in cerebellum of Pcp2\textsuperscript{cre};Ucp4\textsuperscript{fl/fl} mice. The present study indicates a close relationship between UCP4 deletion with PCs impairment, and suggests the importance of UCP4 in the substantial support of mitochondrial function homeostasis in bradykinesia. UCP4 might be a therapeutic target for the cerebellum-related movement disorder.

2. Materials And Methods

2.1 Animals and experimental design.

Pcp2\textsuperscript{cre} mice were purchased from Jackson Laboratory (Stock No: 004146, America). Ucp4\textsuperscript{fl/fl} mice were purchased from Cyagen (Serial number: CKOCMP-74011-Slc25a27, China). All surgical experiments were performed under pentobarbital. The mice were housed on an 12-h light-dark cycle. All the experiments in the present study were performed according to the ethical guidelines of the International Association for the Study of Pain and approved by the Air Force Medical University Committee on Animal Care and Use (IACUC-20190107). All efforts were made to minimize the number of animals used and animal suffering.

The experimental design was as follows. First, we generated conditional knock-out of Ucp4 in Purkinje cells (PCs) (Pcp2\textsuperscript{cre};Ucp4\textsuperscript{fl/fl} mice) by breeding Ucp4\textsuperscript{fl/fl} mice with Pcp2\textsuperscript{cre} mice and the genotype was confirmed by polymerase chain reaction (PCR). Second, the specific ablation of Ucp4 in PCs were confirmed by Western Blot, double immunofluorescent staining, and triple RNAscope \textit{in situ} hybridization. Third, the behavioral characteristic of Pcp2\textsuperscript{cre};Ucp4\textsuperscript{fl/fl} mice was evaluated by rotarod test, open field (OF) test, CatWalk analysis, and elevated plus maze (EPM). Fourth, it was analyzed whether or not the behavioral activities could be deteriorated by harmaline-induced tremor application. Fifth, whether or not the hypotonia was present in Pcp2\textsuperscript{cre};Ucp4\textsuperscript{fl/fl} mice was studied by electromyogram recordings (EMG) detection on the gastrocnemius muscle. Sixth, the spontaneous or evoked firings properties of PCs of Pcp2\textsuperscript{cre};Ucp4\textsuperscript{fl/fl} mice were studied by the electrical patch clamp recordings. Seventh, the mitochondrial impairment in cerebellum tissues of Pcp2\textsuperscript{cre};Ucp4\textsuperscript{fl/fl} mice were evaluated by the levels of ATP, MMP and
ROS. Eighth, the effect of three mitochondrial drugs on the behavioral performance of \( \text{Pcp2}^{\text{cre}};\text{Ucp4}^{fl/fl} \) mice was further studied by the combined behavioral tests.

### 2.2 Generation strategy and genotype.

The strategy employed to generate conditional ablation of \( \text{Ucp4} \) within PCs was to breed \( \text{Ucp4}^{fl/fl} \) mice with \( \text{Pcp2}^{\text{cre}} \) mice. \( \text{Pcp2}^{\text{cre}} \) mice were purchased from Jackson Laboratory (Stock No: 004146, America), in which a \( \text{Cre} \) recombinase sequence was exclusively expressed in PCs (Barski et al., 2000; Oberdick et al., 1990). \( \text{Ucp4}^{fl/fl} \) mice with the \( \text{Ucp4} \) exon 3 flanked by loxP sites were purchased from Cyagen (Serial number: CKOCMP-74011-Slc25a27, China). In the P0 generation, \( \text{Pcp2}^{\text{cre}} \) mice and \( \text{Ucp4}^{fl/fl} \) mice were crossed to generate \( \text{Pcp2}^{\text{cre}};\text{Ucp4}^{fl/+} \) mice as F1, in which half of the PCs expressed \( \text{Cre} \) recombinase to cut the \( \text{Ucp4} \) loxp sequence, while the remainder still expressed one \( \text{Ucp4} \) allele with a loxP site. Then, F1 \( \text{Pcp2}^{\text{cre}};\text{Ucp4}^{fl/+} \) mice were crossed with \( \text{Ucp4}^{fl/fl} \) mice to generate \( \text{Pcp2}^{\text{cre}};\text{Ucp4}^{fl/fl} \) mice as F2. In \( \text{Pcp2}^{\text{cre}};\text{Ucp4}^{fl/fl} \) mice, almost all PCs lost the \( \text{Ucp4} \) sequence attributing to \( \text{Cre} \) recombinase cutting loxP sites, while non-PCs expressed the \( \text{Ucp4} \) sequence with a loxP site.

There were five genotypes of mice in the present study: 1) \( \text{Pcp2}^{\text{cre}} \) mice, which expressed two bands of 567 bp for \( \text{Cre} \) and 119 bp for the \( \text{Ucp4} \) allele without loxP; 2) \( \text{Ucp4}^{fl/fl} \) mice, which only expressed one band of 187 bp for the \( \text{Ucp4} \) allele with loxP; 3) \( \text{Pcp2}^{\text{cre}};\text{Ucp4}^{fl/+} \) mice, which expressed three bands of 567 bp for the \( \text{Cre} \) site, 187 bp for the loxP site, and 119 bp for the \( \text{Ucp4} \)-wide type; 4) \( \text{Pcp2}^{\text{cre}};\text{Ucp4}^{fl/fl} \) mice, which showed two bands of 567 bp and 187 bp by PCR; and 5) \( \text{Ucp4}^{fl/+} \) mice, which expressed two bands of 187 bp for the \( \text{Ucp4} \) allele with the loxP site and of 119 bp for the \( \text{Ucp4} \) allele with no loxP site. The mouse genotype was identified by polymerase chain reaction (PCR) with genomic DNA obtained from the tails. The primers and the strategy used for genotypes identification were shown in Table 1 and Table 2, respectively. The PCR program was as follows: 94°C for 5 min, 35 cycles of 94°C for 30 s for denaturation, 62°C for 30 s for annealing, and 72°C 20 s for elongation. The primers and PCR conditions were designed by Tsingke Biotechnology Co., Ltd.

Hence in the next a serious of experiments, \( \text{Pcp2}^{\text{cre}};\text{Ucp4}^{fl/fl} \) mice were used as the experimental group, and \( \text{Ucp4}^{fl/fl} \) mice and \( \text{Pcp2}^{\text{cre}};\text{Ucp4}^{fl/+} \) mice were used as control group.

### 2.3 Western blot.

To confirm successful knockout of UCP4 in \( \text{Pcp2}^{\text{cre}};\text{Ucp4}^{fl/fl} \) mice, the cerebellar expression levels of UCP4, as well as the mitochondrial fission factor dynamin-related protein 1 (DRP1) and two mitochondrial fusion factors of mitofusion 2 (MFN2) and optic atrophy 1 (OPA1), were detected by Western Blot. Twenty μg of fractionated protein extracts from cerebellum tissues were loaded on 10% acrylamide gel and blotted onto a methanol-activated PVDF membrane (Millipore, USA). Immunoblots were soaked in 5% nonfat milk 2 h at room temperature and subsequently probed with primary antibodies overnight at 4 °C and then incubated with corresponding secondary antibodies. The antibodies used for Western blot were described in Table 3. The bands were detected with enhanced chemiluminescence.
(Beyotime, China) followed by exposure to luminometer (Bio-Rad, USA) and analyzed by ImageJ software. Target protein levels were normalized against GAPDH levels and expressed as fold changes relative to those of the naive control group.

2.4 Double immunofluorescent staining.

To confirm the specific knockout of UCP4 within the PCs of $Pcp2^{cre};Ucp4^{fl/fl}$ mice, the double immunofluorescent staining of UCP2, or the UCP4 analog of UCP2, with the PCs marker Calbindin were performed, according to the methods described previously (Liu et al., 2020). $Pcp2^{cre};Ucp4^{fl/fl}$ mice were used as the experimental group (male, 8-weeks-old, n = 3), and $Ucp4^{fl/fl}$ mice (male, 8-weeks-old, n = 3) and $Pcp2^{cre};Ucp4^{fl/+}$ mice (male, 8-weeks-old, n = 3) were used as control group. The mice were anesthetized and transcardially perfused with 4% paraformaldehyde (PFA) in PBS. The brain was removed and postfixed in 4% PFA overnight at 4°C, and subsequently immersed in 30% sucrose solution for 2 days. Half of the brain was sagittally sectioned at 30-μm thicknesses on a cryostat (Leica CM1850, Germany) and used for double immunofluorescent staining; and another half of the brain was sagittally sectioned at 10-μm thicknesses on a cryostat for triple RNAscope in situ hybridization. In double immunofluorescent staining, the mounted sections were permeabilized with 0.3% TritonX-100 at room temperature for 1 h. The primary and secondary antibodies were described in Table 4. A confocal laser microscope (FV1000; Olympus, Tokyo, Japan) was used to observe digital images. Fifteen slices obtained from 3 mice were randomly chosen. Images were analyzed by individuals blinded towards the experimental groups. The photography view field focused on the 4/5 lobes of the cerebellar cortex (4/5Cb). Anatomical structures were analyzed according to the Fourth Edition of Paxinos and Franklin The Mouse Brain Atlas (Paxinos and Franklin, 2013) and Allen map (http://mouse.brain-map.org/static/atlas).

2.5 Triple RNAscope in situ hybridization.

To further confirm the specific knockout of Ucp4 within the PCs of $Pcp2^{cre};Ucp4^{fl/fl}$ mice, the triple RNAscope in situ hybridization were performed by using the probes of Ucp4, Ucp2 and the PCs marker Pcp2, according to the methods described previously (Kersigo et al., 2018). The steps before sectioning were the same as that in double immunofluorescent staining. In briefly, sagittal sections were cut (10 mm) with cryostat and the tissues were adhered to SuperFrost Plus charged slides. The slices were thawed briefly to adhere to the slides, but they were immediately returned to the −20 °C cryostat chamber until the slices were completed. After the slides were baked at 37°C for 3 h, the brain was washed with 0.01 M PBS for 5 min. Each slide was incubated with hydrogen peroxide (322281, ACD, USA) at room temperature for 10 min. After washing twice in distilled water, the slides were boiled with the Target Retrieval reagents (322000, ACD, USA) at 97°C for 10 min. Immediately, the tissue was placed in distilled water at room temperature and then dehydrated with absolute ethanol for 3 min. Next, the slides were air-dried and boundaries were drawn round each tissue with a hydrophobic pen (CIRISC PAP pen, I.S. CIRCLE WRITER, Japan). When the hydrophobic boundaries were completely dried, protease III reagent (322281, ACD, USA) was added to each tissue until it was completely covered. Subsequently, the slides were
incubated in a preheated HybEZ oven (ACD, USA) at 40°C for 30 min. These tissues were used for hybridization. A mixture of three probes was then added to each slide until the tissue was fully covered. The probes used in RNAscope *in situ* hybridization were shown in Table 5. Positive control probes for low-, medium-, and high-expressing housekeeping genes (POLR2A, PPIB, and UBC, respectively), as well as negative control probe for DapB were used. After incubating for 2 h in a HybEZ oven at 40°C, the slides were washed twice in 1× RNAscope® washing buffer (310091, ACD, USA) for 2 min each time. The slides were returned to the oven for 30 min following submersion in AMP-1 reagent. This step was repeated with AMP-2 and AMP-3 reagents for 30 min and 15 min, respectively. The HRP-C1, HRP-C2, and HRP-C3 signals were processed. Opal 520 (ASOP520, ASbio, USA) was applied to mark the channel 1 probe, Opal 690 (ASOP690, ASbio, USA) was applied to mark the channel 2 probe, and Opal 570 (ASOP570, ASbio, USA) was applied to mark the channel 3 probe. Finally, the tissue was submerged with Prolong Gold Antifade Mountant with DAPI. High resolution imaging was performed using confocal microscopy (FV3000, Olympus). The number (No) of Ucp4 mRNA-dots (violet) and Ucp2 mRNA-dots (green) on three layers of cerebellar cortex including ML, PCL and GCL, were calculated. The intensity of Pcp2 mRNA-dots (red) on three layers was calculated.

2.6 Combined behavioral tests.

Then the combined behavioral tests of rotarod test, open field (OF) test, CatWalk analysis, and elevated plus maze (EPM) were used to evaluate the effect of the specific knockout of UCP4 within the PCs on the spontaneous movements of mice. Pcp2<sup>cre</sup>;Ucp4<sup>fl/fl</sup> mice were used as the experimental group (male, 8-weeks-old, n = 6), and Ucp4<sup>fl/fl</sup> mice (male, 8-weeks-old, n = 5) and Pcp2<sup>cre</sup>;Ucp4<sup>fl/+</sup> mice (male, 8-weeks-old, n = 6) were used as control group. The mice were recorded in order of OF test for 15 min, EPM for 5 min, CatWalk for 5 min and Rotarod test for 5 min.

2.6.1 Open field (OF) test.

To measure the freely spontaneous movement of the transgenic mice, the mice were placed in the center of a 50 × 50 × 50.5 cm opaque square box (DigBehav, Jiliang) and their autonomous movements were recorded for 15 min by a camera connected to a computer, according to the methods described previously (Sun et al., 2020). The movement of the mice was tracked automatically and analyzed by any-maze software. Four parameters were analyzed: 1) the total distance (m); 2) average speed (cm/s); 3) the ratio of the length of the moved path in the 4 central squares to the length of the moved path in the 12 peripheral squares (Ratio of Path<sub>cent</sub> to Path<sub>prl</sub>); 4) the ratio of the time used in the 4 central squares to the time used in the 12 peripheral squares (Ratio of Time<sub>cent</sub> to Time<sub>prl</sub>).

2.6.2 Elevated plus maze (EPM).

To measure the emotional activities of the transgenic mice, the mice were placed in the elevated plus-maze (EPM) (DigBehav) and their autonomous movements were recorded for 5 min by a camera connected to a computer, according to the methods described previously (Holmes et al., 2003). The EPM
instrument consisted of a common central platform (5 × 5 cm²) with two open arms (30 × 5 cm²) and two closed arms (30 × 5 × 15 cm³) extending from it, which was elevated to a height of 38 cm above the ground. The two closed arms were surrounded by opaque walls. The mice were placed alone on the central platform, facing the open arms, and they were freely tracked under an overhead camera for 5 min. Six parameters were analyzed: the duration and frequency in the closed arms (Duration_{closed} and Frequency_{closed}); the duration and frequency in central platform (Duration_{cen} and Frequency_{cen}) and the duration and frequency in open arms (Duration_{open} and Frequency_{open}).

### 2.6.3 CatWalk analysis.

The Noldus CatWalk analysis is very useful to evaluate the gait of mice, so the mice were in the entrance of a 1.3-m-long glass walkway, according to the methods described previously (Ferdinandusse et al., 2008; Sivilia et al., 2016; Watkins et al., 2020). The mice were allowed to walk freely across the runaway to reach the goal box. Paw prints and silhouettes were captured by a digital high-speed video-camera. Three to five compliant walkway trials were recorded for each mouse and after each trial, the walkway was cleaned with 40% ethanol. CatWalk XT 10.1 software (Noldus, Netherlands) was used to analyze the data. The training was conducted thrice a day for 3 consecutive days, and the experiment was formally conducted on the 4th day. Seven parameters of gait analysis were analyzed: 1) the regularity index (RI %), a fractional measure of inter-paw coordination which was calculated as the number of normal step sequence patterns relative to the total number of paw placements; 2) the base of support (BOS), which was the average width (cm) between either the hind paws (BOS_{HP}) or the front paws (BOS_{FP}); 3) the print area (cm²), which was the print size of the left front paw (LF), left hind paw (LH), right front paw (RF), and right hind paw (RH), respectively.

### 2.6.4 Rotarod test.

To measure the balance and coordination of the transgenic mice, the mice were placed on a 3-cm diameter accelerated rotarod (BZY007, Jiliang, Shanghai, China), according to the methods described previously (Tan et al., 2018). The rotarod started rolling at 4 rpm and increased to 40 rpm in 1 min, and maintained for 4 min. Each test was conducted for 5 min. The training was conducted thrice a day for 3 consecutive days, and the experiment was formally conducted on the 4th day. The latency to fall from the rotarod was recorded.

### 2.7 Electromyogram recording (EMG).

Considering the hypotonia is a typical symptom of cerebellar disorders, in the next, step, we analyzed whether or not the muscle damage was present in Pcp2^{cre};Ucp4^{fl/fl} mice by using Electromyogram recording (EMG) applied on the gastrocnemius muscle. Pcp2^{cre};Ucp4^{fl/fl} mice were used as the experimental group (male, 8-weeks-old, n = 3), and Ucp4^{fl/fl} mice (male, 8-weeks-old, n = 3) and Pcp2^{cre};Ucp4^{fl/+} mice (male, 8-weeks-old, n = 3) were used as control group. The mice were anesthetized with 3% isoflurane gas and maintained during EMG recording at 2% isoflurane gas, according to the
methods described previously (Brown et al., 2020; Qiu et al., 2021). The mice were immobilized and the hair around the gastrocnemius muscle of the right hind limb was removed. Four EMG electrodes, two stimulation electrodes, and two leading electrodes were inserted approximately 3–5 mm apart into the triceps muscle of the gastrocnemius muscle of the right hind limb to record the bioelectric potential difference across the muscle tissue. The yellow electrode (positive electrode, +) and the green electrode (negative electrode, −) were made up of stimulation electrodes, while the leading electrodes were composed of a red electrode (positive electrode, +) and a black electrode (negative electrode, −). The positive stimulation electrode was inserted into the medial side of the beginning of the gastrocnemius muscle, and the negative stimulation electrode was inserted into the lateral side. Then, we inserted the negative leading electrode 4 mm below the positive stimulation electrode. In the next step, the positive leading electrode was inserted 4 mm below the negative leading electrode. Finally, we inserted a ground electrode (gray electrode) below the negative stimulation electrode. When recording the EMG, we first recorded the signal baseline for 20 s, before performing stimulation for 15 s at 1 V and 50 Hz to record a new signal baseline lasting 20 s. EMG data were analyzed using the BL-420N Biological function test system (Techman, Chengdu, China). Raw EMG data were filtered using a 2 KHz bandpass filter.

2.8 Electrical patch clamp recordings.

In order to study whether or not the conditional knock-out of Ucp4 in PCs could impair the spontaneous or evoked firings properties of PCs, we performed the electrical patch clamp recordings, according to the methods described previously (Ady et al., 2018). Pcp2cre;Ucp4flo/flo mice were used as the experimental group (male, 8-weeks-old, n = 4), and Ucp4flo/flo mice (male, 8-weeks-old, n = 4) and Pcp2cre;Ucp4flo/+ mice (male, 8-weeks-old, n = 4) were used as control group. The mice were anesthetized by intraperitoneal administration with pentobarbital and immediately sacrificed. The mice cerebellum were cut into 300-μm thick sagittal slices on a vibrating microtome (Leica VT 1200s) at 4°C. Then, the slices were transferred into ice-cold and oxygen (95% O₂ and 5% CO₂) artificial cerebrospinal fluid (ACSF) at room temperature for recording. The ACSF contained 124 mM NaCl, 4.4 mM KCl, 2 mM CaCl₂, 1 mM MgSO₄, 25 mM NaHCO₃, 1 mM NaH₂PO₄, 10 mM glucose, with a final osmolality of 320 mOsm and pH 7.4.

First, the spontaneous action potential (AP) firing of PCs were recorded in the current clamp mode, without additional current injections. Seven parameters were measured: 1) the threshold potential (mV) defined as the first point to which the membrane potential depolarized when an action potential occurs and reflects the excitability of cells; 2) action potential (AP) peak (mV) defined as the maximum voltage of the AP; 3) the half-width of the AP (ms) defined as the duration measured at one half of the AP peak above the threshold potential; 4) the rise slope (mV/ms) and 5) decay slope (mV/ms) defined as the value by which the voltage increases or decreases per unit time; 6) the frequency (Hz) defined as the number of spikes per unit time; 7) afterhyperpolarization (AHP, mV) defined as the peak voltage after the AP.

Second, the evoke AP was made with depolarizing currents of 0–100 pA (500 ms duration, step 10 pA). Rheobasing was defined as the minimum current required to evoke spontaneous APs. Three types of PCs
electrical properties were classified: 1) active type meaning the PCs which showed spontaneous firing and they displayed the increasingly evoked spike numbers by the increasing strengths of stimulation; 2) inactive type meaning the PCs which did not show spontaneous firing began to display the evoked spikes, and their evoked spike numbers increased with the increased strengths of stimulation; 3) quiescent type meaning the PCs which did not show spontaneous firing and failed to display the evoked spikes whatever the increased strengths of stimulation. And the number of 3 types of PCs were calculated.

After recording, biocytin (0.5%) was introduced into the recording region to label the morphology of the recorded neurons. Then, the brain slices were immediately fixed in 4% PFA in PBS for 4 h at room temperature. Sections were then transferred to 0.01 M PBS containing 1% Triton X-100 (PBS-TX) and stored at 4°C. The sections were then blocked with 3% normal bovine serum for 30 min. After thoroughly washing with PBS, the tissue sections were incubated with a PBS solution containing a mixture of Alexa Fluor®594 streptavidin and calbindin (D114Q) XP® Rabbit mAb (Table. 4) for 18 h.

2.9 Detection of reactive oxygen species (ROS) generation.

ROS generation was measured in the mice's cerebellum tissue using the 2,7-Dichlorodi-hydrofluorescein diacetate (DCFH-DA) Assay Kit (S0033S, Beyotime, China). Pcp2^cre^;Ucp4^{-/} mice were used as the experimental group (male, 8-weeks-old, n = 3), and Ucp4^{-/} mice (male, 8-weeks-old, n = 3) and Pcp2^cre^;Ucp4^{-/-} mice (male, 8-weeks-old, n = 3) were used as control group. The purified protein isolated from cerebellum was incubated with 20 mM DCFH-DA in serum-free medium for 20 min at 37ºC in the dark. After washing serum-free medium by DMEM, the ROS fluorescence was measured by 488 nm of excitation wavelength and 525 nm of emission wavelength on a Microplate reader (Spark, Tecan, Switzerland).

2.10 Mitochondrial membrane potential (MMP) measurement.

MMP was detected by JC-1 probe. Pcp2^cre^;Ucp4^{-/} mice were used as the experimental group (male, 8-weeks-old, n = 3), and Ucp4^{-/} mice (male, 8-weeks-old, n = 3) and Pcp2^cre^;Ucp4^{-/-} mice (male, 8-weeks-old, n = 3) were used as control group. First, the mitochondria isolation from cerebellum tissues was performed using the Mitochondria Purification Kit (SM0020, Solarbio, Beijing, China). Second, MMP measurement was measured on the isolated mitochondria using JC-1 Assay Kit (C2006, Beyotime, China). The fluorescence was measured by 485 nm of excitation wavelength and 590 nm of emission wavelength on a Microplate reader (Spark).

2.11 Statistical analysis

Statistical comparison of each experiment was presented in the figure legend. The data was shown by mean ± SD. All data was firstly analyzed for the Normality test and Homogeneity of variance test. For three samples comparison, one-way ANOVA was used to assess differences between groups and the LSD post-hoc test was used for multiple comparisons between groups. And for two samples comparison,
Unpaired two-tailed student’s t-test or paired two-tailed student’s t-test was used for analysis. Kruskal-Wallis test or Mann-Whitney U test were used to analyze the data which did not conform to the Normality test and the Homogeneity of variance test. Statistical analyses were performed using SPSS 15.0. P < 0.05 was considered a statistically significant difference.

3. Results

3.1 Conditional ablation of mitochondrial uncoupling protein 4 (Ucp4) within the cerebellar Purkinje cells (PCs) in Pcp2^{cre};Ucp4^{fl/fl} mice.

Fig. 1A showed the strategy employed to generate conditional ablation of Ucp4 within PCs in Pcp2^{cre};Ucp4^{fl/fl} mice by breeding Ucp4^{fl/fl} mice with Pcp2^{cre} mice. In the P0 generation, Pcp2^{cre} mice and Ucp4^{fl/fl} mice were used. After crossing Pcp2^{cre} mice and Ucp4^{fl/fl} mice, Pcp2^{cre};Ucp4^{fl/+} mice were generated as the F1 generation. Then after crossing Pcp2^{cre};Ucp4^{fl/+} mice with Ucp4^{fl/fl} mice, Pcp2^{cre};Ucp4^{fl/fl} mice were generated as the F2 generation. In Pcp2^{cre};Ucp4^{fl/fl} mice, almost all of the PCs lost the Ucp4 sequence, which was attributed to Cre recombinase cutting loxP sites, while non-PCs expressed the Ucp4 sequence with a loxP site. These experiments demonstrated that Ucp4 was specifically deleted in cerebellar PCs in Pcp2^{cre};Ucp4^{fl/fl} mice.

Genotype was confirmed by polymerase chain reaction (PCR) (Fig. 1B). There were five genotypes of mice in this study: 1) Pcp2^{cre} mice (No. 2, 4, 7, and 13); 2) Ucp4^{fl/fl} mice (No. 9); 3) Pcp2^{cre};Ucp4^{fl/+} mice (No. 10 and 12); 4) Pcp2^{cre};Ucp4^{fl/fl} mice (No. 3, 5, 6, and 8); and 5) Ucp4^{fl/+} mice (No. 1 and 11). In the following study, Pcp2^{cre};Ucp4^{fl/fl} mice were used as the experimental group, and Ucp4^{fl/fl} mice and Pcp2^{cre};Ucp4^{fl/+} mice were used as control group.

3.2 Confirmation of the specific ablation of Ucp4 in PCs of Pcp2^{cre};Ucp4^{fl/fl} mice by Western Blot, double immunofluorescent staining, and triple RNAscope in situ hybridization.

The specific ablation of Ucp4 in PCs were confirmed by Western Blot (Figs. 1C and 1D). The expression level of UCP4 relative to the internal reference GAPDH decreased sharply by 71.1% in Pcp2^{cre};Ucp4^{fl/fl} mice (47.75 ± 7.23), compared to the homozygous Ucp4^{fl/fl} mice (165.28 ± 62.24). The results showed no significant difference in the expression of mitochondrial proteins between the three genotypes (DRP1: P = 0.1188; OPA1: P = 0.5837; MFN2: P = 0.6413; all between Ucp4^{fl/fl} mice and Pcp2^{cre};Ucp4^{fl/fl} mice), confirming that conditional ablation of Ucp4 exclusively in cerebellar PCs did not affect Drp1, Opa1, and Mfn2.

The double immunofluorescent staining (Fig. 2A) showed the distributions of UCP4-positive immunostainings (red) and Calbindin-positive PCs (green) in the whole brain at the middle sagittal sections. UCP4-positive immunostainings were expressed in most brain area including Among them, UCP4 is the most abundant protein reported in all brain regions, including the olfactory brain (OB), cerebral cortex (CC), caudate putamen (Cpu), thalamus (Th, hypothalamus (HT), brain stem (BS) and cerebellum
(CB), which was consistent with previous reports. And Calbindin-positive PCs were distributed in the PCL of cerebellar cortex.

We further focused on the detailed distributions of both UCP4- and calbindin-positive immunostainings within the cerebellum of Ucp4<sup><i>/i</i></sup> mice (Fig. 2B), Pcp2<sup>cre</sup>;Ucp4<sup><i>/i</i></sup> mice (Fig. 2C) and Pcp2<sup>cre</sup>;Ucp4<sup><i>/i</i></sup> mice (Fig. 2D). It was noted that in the control Ucp4<sup><i>/i</i></sup> mice, the values of UCP4-fluorescence in three layers of ML, PCL and PCL were 5.3%, 4.7% and 90.0%, respectively (Figs. 2E and 2F). The graph in Fig. 2G showed that there was no significant difference in Calbindin-positive cells per 100 μm between the control Ucp4<sup><i>/i</i></sup> mice (4.64 ± 0.83) and the experimental Pcp2<sup>cre</sup>;Ucp4<sup><i>/i</i></sup> mice (4.68 ± 0.89) (P = 0.9970), indicating that the conditional ablation of Ucp4 in PCs would not induced PCs loss. The graph in Fig. 2H demonstrated that the fluorescence intensity of UCP4 within the PCL decreased significantly by 57.8% in Pcp2<sup>cre</sup>;Ucp4<sup><i>/i</i></sup> mice (64.55 ± 25.71), when compared to that in homozygous Ucp4<sup><i>/i</i></sup> mice (152.90 ± 31.14) (P = 0.0177). Considering the decrease of UCP4 expression level in the Pcp2<sup>cre</sup>;Ucp4<sup><i>/i</i></sup> mice was 70% when compared with that in the control Ucp4<sup><i>/i</i></sup> mice, the specific ablation of Ucp4 in PCs of Pcp2<sup>cre</sup>;Ucp4<sup><i>/i</i></sup> mice could be confirmed by the combination of Western Blot with immunofluorescent staining.

Third, in order to study that whether or not the specific ablation of Ucp4 in PCs would affect the expression of UCP2, the analog of Ucp4, the double immunofluorescent staining of UCP2 and Calbindin were performed. The graph in Fig. 2I showed that there was no significant difference of UCP2-fluorescence intensity in PCL between the control Ucp4<sup><i>/i</i></sup> mice (152.90 ± 31.14) and the experimental Pcp2<sup>cre</sup>;Ucp4<sup><i>/i</i></sup> mice (64.55 ± 25.71) (P = 0.0177) (P = 0.9970). Although there was a significant difference of UCP2-fluorescence intensity between Pcp2<sup>cre</sup>;Ucp4<sup><i>/i</i></sup> mice and the Pcp2<sup>cre</sup>;Ucp4<sup><i>/i</i></sup> mice, it should be noted that the UCP2-fluorescence intensity of Pcp2<sup>cre</sup>;Ucp4<sup><i>/i</i></sup> mice in fact was higher than that of the control Ucp4<sup><i>/i</i></sup> mice. We think that these data reflected a fairly consistent level of UCP2 expression level in three mice groups.

Triple RNAscope in situ hybridization results (Fig. 3A) were in general consistent with that by double immunofluorescent staining. It was shown that there was no significant difference in the number of Pcp2 mRNA-dots (red) on both ML (Fig. 3B) and GCL (Fig. 3D), as well as in the intensities of Pcp2 mRNA-dots on PCs soma (Fig. 3C), between Ucp4<sup><i>/i</i></sup> mice and Pcp2<sup>cre</sup>;Ucp4<sup><i>/i</i></sup> mice. And it was indicated that the number of Ucp4 mRNA-dots (violet) on ML (Fig. 3E), or PCs soma (Fig. 3F), or GCL (Fig. 3G), decreased by average approximately 57.8% in Pcp2<sup>cre</sup>;Ucp4<sup><i>/i</i></sup> mice, when compared with those in Ucp4<sup><i>/i</i></sup> mice (P < 0.05). We noted that there was no significant difference in the number of Ucp2 mRNA-dots (green) on both PCL (Fig. 3I) and GCL (Fig. 3J), between Ucp4<sup><i>/i</i></sup> mice and Pcp2<sup>cre</sup>;Ucp4<sup><i>/i</i></sup> mice, although the number of Ucp2 mRNA-dots (green) on ML (Fig. 3H) significantly decreased in Pcp2<sup>cre</sup>;Ucp4<sup><i>/i</i></sup> mice, when compared with those in Ucp4<sup><i>/i</i></sup> mice (P < 0.05).

Therefore, we confirmed the specific Ucp4 deletion in the PCs of Pcp2<sup>cre</sup>;Ucp4<sup><i>/i</i></sup> mice.
3.3 The combined behavioral tests showed the bradykinesia of Pcp2\textsuperscript{cre};Ucp4\textsuperscript{fl/fl} mice.

First, it was found that the physical appearance of 8-weeks-old male Pcp2\textsuperscript{cre};Ucp4\textsuperscript{fl/fl} mice were indistinguishable from the littermate of Ucp4\textsuperscript{fl/fl} mice (8-weeks-old male) and Pcp2\textsuperscript{cre};Ucp4\textsuperscript{fl/+} mice (8-weeks-old male). They had the similar average body length of 16.37 ± 0.38 cm (measured from head to tail end) (Fig. 4A), similar average ratio of the head length (defined as the distance from the tip of the nose to the imaginary line between the ears) to the body length of 10.92% ± 0.81% (Fig. 4B), and similar average body weight of 22.42 ± 0.83 g (Fig. 4C). And they had normal black body hair and pupils (data not shown).

To explore the locomotor capacity of Pcp2\textsuperscript{cre};Ucp4\textsuperscript{fl/fl} mice, we performed OF test with a duration of 900 s for a long autonomic movement appraisal (Figs. 4D - 4H). Fig. 4E showed that the total distance moved was reduced by 40.0% from 14.32 ± 2.25 m in Ucp4\textsuperscript{fl/fl} mice to 8.60 ± 2.01 m in Pcp2\textsuperscript{cre};Ucp4\textsuperscript{fl/fl} mice (P = 0.0093). Fig. 4F showed that the average speed was reduced by 39.6% from 1.59 ± 0.25 cm/s in Ucp4\textsuperscript{fl/fl} mice to 0.96 ± 0.22 cm/s in Pcp2\textsuperscript{cre};Ucp4\textsuperscript{fl/fl} mice (P = 0.0093). Figs. 4G and 4H showed that there was no significant difference in the ratio of Path\textsubscript{cent.} to Path\textsubscript{prl.} or ratio of Time\textsubscript{cent.} to Time\textsubscript{prl.} between that of Ucp4\textsuperscript{fl/fl} mice and that of Pcp2\textsuperscript{cre};Ucp4\textsuperscript{fl/fl} mice. These results reflected the bradykinesia of Pcp2\textsuperscript{cre};Ucp4\textsuperscript{fl/fl} mice.

To explore the stepping alterations of Pcp2\textsuperscript{cre};Ucp4\textsuperscript{fl/fl} mice, we next analyzed CatWalk data (Figs. 4I - 4M). Fig. 4N showed that there was no significant difference in RI between Ucp4\textsuperscript{fl/fl} mice and Pcp2\textsuperscript{cre};Ucp4\textsuperscript{fl/fl} mice (P = 0.8436), confirming that Pcp2\textsuperscript{cre};Ucp4\textsuperscript{fl/fl} mice had normal inter-paw coordination. Fig. 4O and 4P showed that the BOS\textsubscript{HP} was reduced by 34.8% from 3.62 ± 0.36 cm in Ucp4\textsuperscript{fl/fl} mice to 2.36 ± 0.12 m in Pcp2\textsuperscript{cre};Ucp4\textsuperscript{fl/fl} mice (P = 0.0006), and the BOS\textsubscript{FP} was reduced by 26.1% from 1.84 ± 0.13 cm in Ucp4\textsuperscript{fl/fl} mice to 1.36 ± 0.10 cm in Pcp2\textsuperscript{cre};Ucp4\textsuperscript{fl/fl} mice (P < 0.0001). These results demonstrated the reduced amplitude of stepping of Pcp2\textsuperscript{cre};Ucp4\textsuperscript{fl/fl} mice. Fig. 4Q showed that all of the paw print areas presented a considerable decline (approximately 58.1% for LF, 60.4% for LH, 54.1% for RF, and 44.7% for RH) in Pcp2\textsuperscript{cre};Ucp4\textsuperscript{fl/fl} mice. These results confirmed the bradykinesia of Pcp2\textsuperscript{cre};Ucp4\textsuperscript{fl/fl} mice (Fig. 4R).

Rotarod test (Figs. 4S and 4T) confirmed the absence of ataxia in Pcp2\textsuperscript{cre};Ucp4\textsuperscript{fl/fl} mice because there was no significant difference of the latency to fall between that in Ucp4\textsuperscript{fl/fl} mice and in Pcp2\textsuperscript{cre};Ucp4\textsuperscript{fl/fl} mice (P = 0.2222).

Considering PCs are related to emotional performance (Tsai et al., 2012), we next employed EPM test (Figs. 4U - 4X). There were no significant differences in Duration\textsubscript{closed} and Frequency\textsubscript{closed} (Fig. 4V), Duration\textsubscript{cent.} and Frequency\textsubscript{cent.} (Fig. 4W), and Duration\textsubscript{open} and Frequency\textsubscript{open} (Fig. 4X), among three groups, which indicated that the deletion of UCP4 in PCs did not greatly affect animal emotion.

3.5 The electromyogram recordings (EMG) excluded the muscle damage in Pcp2\textsuperscript{cre};Ucp4\textsuperscript{fl/fl} mice.
In the next, whether or not the hypotonia was present in \( Pcp2^{\text{cre},Ucp4^{\text{fl/fl}}} \) mice was studied by electromyogram recordings (EMG) detection on the gastrocnemius muscle (Figs. 5A and 5B). EMG tracings of \( Ucp4^{\text{fl/fl}} \) mice (Fig. 5C), \( Pcp2^{\text{cre},Ucp4^{\text{fl/+}}} \) mice (Fig. 5D), and \( Pcp2^{\text{cre},Ucp4^{\text{fl/fl}}} \) mice (Fig. 5E) were shown. Their corresponding typical firing recordings were amplified in Figs. 5F - 5H and further calculated shown in Figs. 5I - 5K. Figs. 5L and 5M showed that the \( Pcp2^{\text{cre},Ucp4^{\text{fl/fl}}} \) mice had a significantly higher amplitude (150.67 ± 56.14 mV, \( P < 0.0001 \)) and response latency (2.22 ± 0.19 ms, \( P = 0.0146 \)) than the control \( Ucp4^{\text{fl/fl}} \) mice (49.33 ± 7.85 mV and 2.05 ± 0.32 ms, respectively). The present EMG results excluded the muscle damage in \( Pcp2^{\text{cre},Ucp4^{\text{fl/fl}}} \) mice, hence in the following patch clamp recording, we focused on the changes of the electrical properties of PCs.

### 3.6 The spontaneous and evoked firing properties of PCs of \( Pcp2^{\text{cre},Ucp4^{\text{fl/fl}}} \) mice were studied by the electrical patch clamp recordings.

Next the spontaneous or evoked firing properties of PCs of \( Pcp2^{\text{cre},Ucp4^{\text{fl/fl}}} \) mice were studied by the electrical patch clamp recordings on freshly isolated 4/5 lobes of the cerebellum (4/5Cb) (Fig. 6A). Fig. 6B showed the insertion of the glass electrode into the soma of PCs. We totally recorded 30 PCs in \( Ucp4^{\text{fl/fl}} \) mice (Fig. 6C), 30 PCs in \( Pcp2^{\text{cre},Ucp4^{\text{fl/+}}} \) mice (Fig. 6D), and 28 PCs in \( Pcp2^{\text{cre},Ucp4^{\text{fl/fl}}} \) mice (Fig. 6E). We found 15 of the total 30 PCs in \( Ucp4^{\text{fl/fl}} \) mice, 13 of 30 in \( Pcp2^{\text{cre},Ucp4^{\text{fl/+}}} \) mice, and 9 of 28 in \( Pcp2^{\text{cre},Ucp4^{\text{fl/fl}}} \) mice, had the spontaneous firings, respectively. We then analyzed the spontaneous firing patterns of PCs in \( Ucp4^{\text{fl/fl}} \) mice (Fig. 6F), \( Pcp2^{\text{cre},Ucp4^{\text{fl/+}}} \) mice (Fig. 6G), and in \( Pcp2^{\text{cre},Ucp4^{\text{fl/fl}}} \) mice (Fig. 6H). As shown in Fig. 6I, 7 parameters were measured. It was found that the AP half-width of \( Pcp2^{\text{cre},Ucp4^{\text{fl/fl}}} \) micewas reduced by 19.7% compared to that of \( Ucp4^{\text{fl/fl}} \) mice (\( Ucp4^{\text{fl/fl}} \) mice: 0.70 ± 0.11 ms; \( Pcp2^{\text{cre},Ucp4^{\text{fl/fl}}} \) mice: 0.56 ± 0.06 ms; \( P = 0.0049 \)) (Fig. 6L); and the rise and decay slope became steeper by 21.8% and 21.1%, respectively, after Ucp4 conditional ablation (rise slope: 168.05 ± 41.06 mV/ms in \( Ucp4^{\text{fl/fl}} \) mice and 204.72 ± 20.62 mV/ms in \( Pcp2^{\text{cre},Ucp4^{\text{fl/fl}}} \) mice, \( P = 0.0467 \), Fig. 6P; decay slope: –170.52 ± 33.70 mV/ms in \( Ucp4^{\text{fl/fl}} \) mice and –206.41 ± 14.55 mV/ms in \( Pcp2^{\text{cre},Ucp4^{\text{fl/fl}}} \) mice, \( P = 0.0105 \), Fig. 6N).

Then the evoked spikes of PCs in \( Ucp4^{\text{fl/fl}} \) mice (Fig. 6Q), and \( Pcp2^{\text{cre},Ucp4^{\text{fl/+}}} \) mice (Fig. 6R), and \( Pcp2^{\text{cre},Ucp4^{\text{fl/fl}}} \) mice (Fig. 6S) were analyzed. As shown in Fig. 6T showed that the proportion of both active and inactive PCs (excitable) reduced from 73% in \( Ucp4^{\text{fl/fl}} \) mice to 68% in \( Pcp2^{\text{cre},Ucp4^{\text{fl/fl}}} \) mice. Fig. 6U showed that there was no significant difference in the total firing number of both active and inactive PCs at every given current between any two groups. Finally, the biocytin (red) injection confirmed the recorded cells were PCs (Fig. 6V).

Consequently, we speculated that the excitability of PCs was reduced by specific ablation of \( Ucp4 \).

### 3.7 Increased ROS generation in cerebellum of \( Pcp2^{\text{cre},Ucp4^{\text{fl/fl}}} \).
Reactive oxygen species (ROS) generation results (Figs. 7A-7C) showed the significant increase of ROS in ML of the cerebellum tissues, which was full of the dendritic trees of Purkinje cells, in \( Pcp2^{cre};Ucp4^{fl/fl} \) mice, when compared to that of \( Ucp4^{fl/fl} \) mice. But there was no difference of ROS in GCL, which was full of granular cells, between three groups. And there was no difference in MMP between three groups (Figs. 7D, and 7E).

4. Discussion

To evaluate the role of Ucp4 in Purkinje cells (PCs), we generated conditional deletion of Ucp4 in PCs (\( Pcp2^{cre};Ucp4^{fl/fl} \) mice) by breeding \( Ucp4^{fl/fl} \) mice with \( Pcp2^{cre} \) mice. PCR, western blot, double immunofluorescent staining and triple RNAscope in situ hybridization confirmed the specific ablation of Ucp4 in PCs in \( Pcp2^{cre};Ucp4^{fl/fl} \) mice. Open field test, CatWalk analysis, rotarod and elevated plus-maze tests showed that \( Pcp2^{cre};Ucp4^{fl/fl} \) mice displayed bradykinesia. The electromyogram recordings excluded the hypotonia in \( Pcp2^{cre};Ucp4^{fl/fl} \) mice. And the electrical patch clamp recordings showed the altered properties of both spontaneous and evoked firings in PCs of \( Pcp2^{cre};Ucp4^{fl/fl} \) mice. Also, the knockdown of Ucp4 significantly increased ATP, MMP and ROS in the cerebellum. The combined behavioral tests indicated that systemic application of anyone of three mitochondrial drugs did not relieve the movement disorder of \( Pcp2^{cre};Ucp4^{fl/fl} \) mice. It was the first time to report a close relationship between UCP4 deletion with PCs impairment.

Previous studies have demonstrated that the mutation of many mitochondrial genes could lead to ataxia movement disorder. Aoki et al. (2022) (Aoki et al., 2022) have reported that spinocerebellar ataxia type 31 (SCA31), an autosomal-dominant neurodegenerative disorder characterized by progressive cerebellar ataxia and PCs loss, is caused by the mutation of gene of an essential mitochondrial thymidine kinase 2 (TK2). Rumyantseva et al. (2022) (Rumyantseva et al., 2022) have found that the conditional PCs-specific deletion of mitochondrial aspartyl-tRNA synthetase (DARS2) causes a massive loss of PCs and ataxia. Previous reports (Murr u et al., 2019; Mancini et al., 2019; Patron et al., 2018) have found that the conditional deletion of AFG3L2 which encodes one of the subunits of the m-AAA protease in Bergmann glia cell leads to PCs degeneration and staxia. And it has been shown that the m-AAA protease is important for mitochondrial homeostasis. Nair et al. (Nair et al., 2018) have investigated that the conditional PCs-specific deletion of mitochondrial fatty acid synthesis (mtFAS) causes a massive PCs loss and ataxia. SLC25A46 plays an important role in mitochondrial dynamics by mediating mitochondrial fission. Li et al. (Li et al., 2017) have described that the Slc25a46 knock-out mouse has displayed severe ataxia which is mainly caused by degeneration of PCs. Combined with the present results, there is no doubt that the mitochondrial quality control is closely related to PCs homeostasis and motor function.

Mitochondria, as the main energy supplying organelles, provide the nervous system with ATP, which is necessary for survival. PCs function as the sole efferent neurons in the cerebellar cortex (Muguruma et al., 2010), which determines the highly dependent nature of PCs on mitochondria. UCPs are part of a
superfamily of mitochondrial anionic carriers, which are located in the inner mitochondrial membrane (IMM) and reduce the proton-electrochemical gradient (Adams et al., 2001; Kim-Han and Dugan, 2005). Given the limited number of studies on UCP4 in CNS, the role of Ucp4 in PCs is still unclear. Therefore, we crossed the Pcp2cre mice with Ucp4fl/fl mice to generate the specific Ucp4 ablation mice. To our surprise, Pcp2cre;Ucp4fl/fl mice maintained normal morphological features of PCs, had no ataxia, and presented a normal body appearance and balance ability (reflected by rotarod test). Importantly, these specific Ucp4 ablation mice presented bradykinesia. Bradykinesia was first proposed by John Parkinson, who described it as a characteristic of slowness when performing movement (Berardelli et al., 2001), and it is now considered the most important motor defect of PD. In addition to the behavior test, the results of patch clamp and EMG suggested against muscle damage in our Ucp4-specific ablation mice and confirmed the firing changes in PCs. Consequently, we speculated that bradykinesia caused by Ucp4-specific ablation was related to the reduced proportion of PCs with spontaneous spikes.

UCP4 has been shown to reduce the MMP and ROS. The knockdown of UCP4 is reported to increase the MMP, while the over-expression of UCP4 is reported to reduce the MMP, ROS, and ATP (Ramsden et al., 2012; Alán et al., 2009; Lu et al., 2020). Our present results are consistent with previous data that we have found the significant increases of ATP level, MMP and ROS generation in cerebellum of Pcp2cre;Ucp4fl/fl. Thereafter UCP4 might be a therapeutic target for the cerebella-related movement disorder. The present study is the first to report a close relationship between UCP4 deletion with PCs impairment, and suggests the importance of UCP4 in the substantial support of mitochondrial function homeostasis in bradykinesia.

Although we detected the cerebellar mitochondrial function changes induced by the deletion of UCP4 in PCs, we did not define the exact mitochondrial alterations within the PCs. Therefore, it was necessary to explore the detailed mitochondrial functions in PCs after Ucp4-specific deletion.

**Conclusion**

In conclusion, we generated Pcp2cre;Ucp4fl/fl mice with the conditional knockout of mitochondrial Ucp4 in cerebellar PCs, and found these Pcp2cre;Ucp4fl/fl mice displayed bradykinesia, which was possibly due to the reduced excitability of PCs induced by the oxidative crisis. The present study is the first to report a close relationship between UCP4 deletion with PCs impairment, and suggests the importance of UCP4 in the substantial support of mitochondrial function homeostasis in bradykinesia. We further deduce that UCP4 might be a therapeutic target for the cerebella-related movement disorder.

**Declarations**

**Author contributions**

Ya-Yun Wang, Yan-Ling Yang, Lu Wang, and Shui-Bing Liu designed the experiments; Hui Liu, Shu-Jiao Li and Ban Feng conducted the experiments; Kun-Long Zhang, Xiao-Dong Li, Rui-Qing Wang, Jing-Hao Chen,
and Yun-Qiang Huang analyzed the data and drew the figures; and Ya-Yun Wang, Hui Liu, and Shu-Jiao Li wrote the manuscript. All of the authors read and approved the final manuscript.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (81870415) from Yan-Ling Yang, the Xijing Hospital Boosting Plan (XJZT19Z29) from Yan-Ling Yang, the Military Medicine Upgrade Program of Air Force Military Medical University (2020SWAQ04) from Ya-Yun Wang, the Health Services Project of Air Force Military Medical University (21WQ023) from Ya-Yun Wang, the Project of Science and Technology to Improve the Combat Effectiveness of School Flight Personnel (2019ZTC03), and the Open Project of State Key Laboratory of Military Stomatology (2018KA01) of Ya-Yun Wang.

We thank all of the members of our lab for their helpful discussions and comments during the course of the study.

We thank LetPub (www.letpub.com) for its linguistic assistance during the preparation of this manuscript.

Competing interests

The authors declare that they do not have any competing interests.

Ethical approval

The experiments were performed according to the current laws of China and we abide by the Ethics Committee of Xijing Hospital of Air Force Military Medical University.

Consent to participate

All authors have read the journal’s authorship agreement and conflict of interest policy. The authors have no potential conflicts of interest to declare.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and analyzed in this study are available from Ya-Yun Wang upon reasonable request.

Funding

This work was supported by the National Natural Science Foundation of China (81870415) from Yan-Ling Yang, the Xijing Hospital Boosting Plan (XJZT19Z29) from Yan-Ling Yang, the Military Medicine Upgrade Program of Air Force Military Medical University (2020SWAQ04) from Ya-Yun Wang, the Health
Services Project of Air Force Military Medical University (21WQ023) from Ya-Yun Wang, the Project of Science and Technology to Improve the Combat Effectiveness of School Flight Personnel (2019ZTC03), and the Open Project of State Key Laboratory of Military Stomatology (2018KA01) of Ya-Yun Wang.

References


6. Holmes, A. et al. 2003: Galanin GAL-R1 receptor null mutant mice display increased anxiety-like behavior specific to the elevated plus-maze., 1031-1044. https://doi.org/10.1038/sj.npp.1300164


Tables

Table 1. The primers used for genotype identification.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequences (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>CACCAGTCTTAGTTACACAAATG</td>
</tr>
<tr>
<td>P2</td>
<td>TGAATGGTAACCAAATAAGGC</td>
</tr>
<tr>
<td>P3</td>
<td>ATTCTCGTGGAACTGGATGG</td>
</tr>
<tr>
<td>P4</td>
<td>GGACAGGTAATGGTTGTCTGG</td>
</tr>
</tbody>
</table>

The PCR program used was as follows:

94°C for 3 min, then 35 cycles of 94°C for 30 s for denaturation, 62°C for 35 s for annealing, and 72°C 45 s for elongation.

Table 2. The strategy for genotype identification.

<table>
<thead>
<tr>
<th>Mice type</th>
<th>Combination of primers for Genotyping</th>
<th>Number &amp; Length of Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Pcp2^{cre}$</td>
<td>P3+P4</td>
<td>One band with 567 bp</td>
</tr>
<tr>
<td>$UCP4^{fl/fl}$</td>
<td>P1+P2</td>
<td>One band with 187 bp</td>
</tr>
<tr>
<td>$Pcp2^{cre},UCP4^{fl/+}$</td>
<td>P1+P2+P3+P4</td>
<td>Three bands with 119 bp, 187 bp and 567 bp</td>
</tr>
<tr>
<td>$Pcp2^{cre},UCP4^{fl/fl}$</td>
<td>P1+P2+P3+P4</td>
<td>Two bands with 187 bp and 567 bp</td>
</tr>
</tbody>
</table>

Table 3. The antibodies used for Western blot.
<table>
<thead>
<tr>
<th>Antibody type</th>
<th>Antibody name</th>
<th>Company</th>
<th>Product number</th>
<th>Dilution condition</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary antibody</td>
<td>anti-UCP4</td>
<td>Santa Cruz Biotechnology</td>
<td>#sc-365295</td>
<td>1:200</td>
<td>Mouse</td>
</tr>
<tr>
<td></td>
<td>anti-Drp1</td>
<td>Cell Signaling Technology</td>
<td>#8570</td>
<td>1:1000</td>
<td>Rabbit</td>
</tr>
<tr>
<td></td>
<td>anti-OPA1</td>
<td>Santa Cruz Biotechnology</td>
<td>#sc-393296</td>
<td>1:1000</td>
<td>Rabbit</td>
</tr>
<tr>
<td></td>
<td>anti-Mfn2</td>
<td>Cell Signaling Technology</td>
<td>#9482</td>
<td>1:1000</td>
<td>Rabbit</td>
</tr>
<tr>
<td></td>
<td>anti-GAPDH</td>
<td>Cell Signaling Technology</td>
<td>#5174</td>
<td>1:1000</td>
<td>Rabbit</td>
</tr>
<tr>
<td>Secondary antibody</td>
<td>HRP, Goat Anti-Mouse IgG</td>
<td>Abbikne</td>
<td>#A21010</td>
<td>1:5000</td>
<td>Goat</td>
</tr>
<tr>
<td></td>
<td>HRP, Goat Anti-Rabbit IgG</td>
<td>Abbikne</td>
<td>#A21020</td>
<td>1:5000</td>
<td>Goat</td>
</tr>
</tbody>
</table>

Table 4. The antibodies used for immunofluorescent staining.

<table>
<thead>
<tr>
<th>Antibody type</th>
<th>Antibody name</th>
<th>Company</th>
<th>Product number</th>
<th>Dilution condition</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary antibody</td>
<td>Anti-UCP4</td>
<td>Santa Cruz Biotechnology</td>
<td>#sc-365295</td>
<td>1:50</td>
<td>Mouse</td>
</tr>
<tr>
<td></td>
<td>Anti-UCP2</td>
<td>Santa Cruz Biotechnology</td>
<td>#sc-390189</td>
<td>1:50</td>
<td>Mouse</td>
</tr>
<tr>
<td>Secondary antibody</td>
<td>Dylight 488, Goat Anti-Mouse IgG</td>
<td>Abbkine</td>
<td>A23210</td>
<td>1:200</td>
<td>Goat</td>
</tr>
<tr>
<td>Alexa Fluor® 594 Conjugate</td>
<td>Anti-Calbindin</td>
<td>Cell Signaling Technology</td>
<td>#88831</td>
<td>1:50</td>
<td>Rabbit</td>
</tr>
<tr>
<td>Nucleus dye</td>
<td>DAPI</td>
<td>Beyotime</td>
<td>C1005</td>
<td>1:1000</td>
<td></td>
</tr>
</tbody>
</table>

Table 5. The probes used in RNAscope *in situ* hybridization.
<table>
<thead>
<tr>
<th>Name</th>
<th>Mitochondrial localization</th>
<th>Function</th>
<th>Accession number</th>
<th>Target region</th>
<th>Dilution</th>
<th>TSA® Plus channel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pcp2</td>
<td></td>
<td>Purkinje cell marker</td>
<td>NM_001129804.1</td>
<td>2-302</td>
<td>1 50</td>
<td>2</td>
</tr>
<tr>
<td>Ucp2</td>
<td>IMM</td>
<td>Mitochondrial uncoupling</td>
<td>NM_011671.5</td>
<td>2-1002</td>
<td>1 1</td>
<td>1</td>
</tr>
<tr>
<td>Ucp4</td>
<td>IMM</td>
<td>Mitochondrial uncoupling</td>
<td>NM_028711.4</td>
<td>457-1410</td>
<td>1 50</td>
<td>3</td>
</tr>
<tr>
<td>Polr2a</td>
<td>Positive control</td>
<td></td>
<td>NM_009089.2</td>
<td>2802-3678</td>
<td>1 1</td>
<td>1</td>
</tr>
<tr>
<td>PPIB</td>
<td>Positive control</td>
<td></td>
<td>NM_011169.2</td>
<td>98-856</td>
<td>1 1</td>
<td>2</td>
</tr>
<tr>
<td>UBC</td>
<td>Positive control</td>
<td></td>
<td>NM_019639.4</td>
<td>34-860</td>
<td>1 1</td>
<td>3</td>
</tr>
<tr>
<td>DapB</td>
<td>Negative control</td>
<td></td>
<td>EF191515</td>
<td>414-862</td>
<td>1 1</td>
<td>1, 2, 3</td>
</tr>
</tbody>
</table>

**Ucp4**: uncoupling protein 4; **Ucp2**: uncoupling protein 2; **Pcp2**: Purkinje cell protein 2; **IMM**: Inner mitochondrial membrane.

**Figures**
Conditional ablation of UCP4 within cerebellar PCs in Pcp2cre;Ucp4fl/fl mice.

A. Strategy to generate conditional ablation of Ucp4 within PCs in Pcp2cre;Ucp4fl/fl mice by breeding Ucp4fl/fl mice with Pcp2cre mice. Pcp2cre mice and Ucp4fl/fl mice were used in the P0 generation, and generated Pcp2cre;Ucp4fl/+ mice as the F1 generation. Pcp2cre mice had a Cre recombinase sequence
inserted at the 3’ end of the Pcp2 allele, which targeted to cerebellar PCs but not non-Purkinje cells. In the Ucp4<sup>fl/fl</sup> mice, loxP sites were buried in the terminal end of the Ucp4 sequence across all cells, including cerebellar PCs and non-Purkinje cells. F1 Pcp2<sup>cre</sup>;Ucp4<sup>fl/+</sup> mice expressed Cre recombinase exclusively from one parental allele in PCs to cut the Ucp4 loxP sequence. Then, crossing Pcp2<sup>cre</sup>;Ucp4<sup>fl/+</sup> mice and Ucp4<sup>fl/fl</sup> mice, Pcp2<sup>cre</sup>;Ucp4<sup>fl/fl</sup> mice were generated as the F2 generation, in which almost all PCs lost the Ucp4 sequence attributed to the cutting of loxP sites by Cre recombinase.

B. Representative PCR results of all transgenic mice. Pcp2<sup>cre</sup> mice showed two bands of 567 bp and 119 bp as No. 2, 4, 7, and 13. Ucp4<sup>fl/fl</sup> mice showed one band of 187 bp as No. 9. Pcp2<sup>cre</sup>;Ucp4<sup>fl/+</sup> mice showed three bands of 567 bp, 187 bp, and 119 bp as No. 10 and 12. Ucp4<sup>fl/+</sup> mice showed two bands of 187 bp and 119 bp as No. 1 and 11. Pcp2<sup>cre</sup>;Ucp4<sup>fl/fl</sup> mice showed two bands of 567 bp and 187 bp as No. 3, 5, 6, and 8.

C. Representative western blot results.

D. Quantification for western blot. The protein expression level of UCP4 relative to the GAPDH internal reference decreased sharply by 70% in Pcp2<sup>cre</sup>;Ucp4<sup>fl/fl</sup> mice when compared to the homozygous Ucp4<sup>fl/fl</sup> mice. Meanwhile, the expression levels of DRP1, OPA1, and MFN2, which all were relative to GAPDH, showed no significant difference in the three types of transgenic mice.

The data were analyzed by one-way ANOVA and LSD post-hoc test or Kruskal-Wallis test. The data are shown as the mean ± SD; n = 3 mice per group. *P* < 0.05 was considered a statistically significant difference.

DRP1, the mitochondrial fission protein dynamin-related protein 1 located at outer mitochondrial membrane; MFN2, the mitochondrial fusion protein mitofusion 2 located at the outer mitochondrial membrane; OPA1, the mitochondrial fusion protein optic atrophy 1 located at the inner mitochondrial membrane.
Double immunofluorescent staining confirmed the specific ablation of Ucp4 in PCs of *Pcp2<sup>cre</sup>;Ucp4<sup>fl/fl</sup>* mice.

A. Confocal images of Ucp4 expression in total sagittal brain sections of *Ucp4<sup>fl/fl</sup>* mice (top line), *Pcp2<sup>cre</sup>;Ucp4<sup>fl/+</sup>* mice (middle line), and *Pcp2<sup>cre</sup>;Ucp4<sup>fl/fl</sup>* mice (bottom line). All confocal images in the top
panel show the co-labeling of calbindin (a marker of Purkinje cells, green), UCP4 protein (red), and DAPI (blue). All confocal images in the bottom panel show the co-labeling of calbindin, UCP2 protein (red), and DAPI (blue). Bars = 2 mm.

**B and C.** Quantification (B) and represent of percent (C) of UCP4-fluorescence in three layers of ML, PCL and PCL in the cerebellum in the control *Ucp4*<sup>+/+</sup> mice.

**D - F.** Confocal images of Ucp4 expression in PCs of *Ucp4*<sup>+/+/</sup> mice (D), *Pcp2<sup>cre</sup>;Ucp4<sup>+/+</sup> mice (E), and *Pcp2<sup>cre</sup>;Ucp4<sup>+/+</sup> mice (F). All confocal images in the top panel show the co-labeling of calbindin (a marker of Purkinje cells, green), UCP4 protein (red), and DAPI (blue). All confocal images in the bottom panel show the co-labeling of calbindin, UCP2 protein (red), and DAPI (blue). Bars = 50 μm.

**G.** Quantification of calbindin-positive PCs.

**H.** Quantification of UCP4-fluorescence intensity in PCL.

**I.** Quantification of UCP2-fluorescence intensity in the PCL.

The data are shown as the mean ± SD. These results were analyzed by one-way ANOVA and LSD post-hoc test or Kruskal-Wallis test, n = 3 mice per group. *P* < 0.05 was considered a statistically significant difference.

BS, brain stem; CB, cerebellum; CC, cerebral cortex; Cpu, caudate putamen; GCL, granular cell layer; HT, hypothalamus; ML, molecular layer; OB, olfactory brain; PCL, Purkinje cell layer; Th, thalamus.
Figure 3

Triple RNAscope in situ hybridizations further confirmed the specific ablation of Ucp4 in PCs of Pcp2cre;Ucp4fl/fl mice.

A. Confocal images of co-labeling of Pcp2 mRNA (a probe of Purkinje cells; red), Ucp4 mRNA (violet), and Ucp2 mRNA (green) in the cerebellar cortex of Ucp4fl/fl mice (top panel), Pcp2cre;Ucp4fl/+ (middle panel),
and \( Pcp2^{cre};Ucp4^{+/+} \) mice (bottom panel). Bars = 50 μm.

**B-D.** Quantification of \( Pcp2 \) mRNA-positive intensities (red) in ML (B), PCL (C) and GCL (D), respectively. We found that there was no significant difference in \( Pcp2 \) mRNA intensity between \( Ucp4^{+/+} \) mice and \( Pcp2^{cre};Ucp4^{+/+} \) mice.

**E-G.** Quantification of number of \( Ucp4 \) mRNA-positive dots (violet) in ML (E), PCL (F) and GCL (G), respectively. The number of \( Ucp4 \) mRNA-positive dots in PCL decreased approximately 70% in \( Pcp2^{cre};Ucp4^{+/+} \) mice when compared to homozygous \( Ucp4^{+/+} \) mice.

**H-J.** Quantification of number of \( Ucp2 \) mRNA-positive dots (green) in ML (E), PCL (F) and GCL (G), respectively. The number of \( Ucp2 \) mRNA-positive dots in any layer was not significantly different between the groups.

Statistical analysis was performed by one-way ANOVA and LSD post-hoc test or Kruskal-Wallis test. The data are shown as the mean ± SD, \( n = 3 \) mice per group. \( P < 0.05 \) was considered a statistically significant difference.

GCL, granular cell layer; ML, molecular layer; PCL: Purkinje cell layer.
Figure 4

The combined behavioral tests of open field (OF), CatWalk, Rotarod, and elevated plus-maze (EPM) showed the bradykinesia of $Pcp2^{cre},Ucp4^{-/-}$ mice.

A-C. The average body length (A), the average ratio of the head length to the body length (B), and the average body weight (C) showed no significant difference between $Ucp4^{-/-}$ mice (male, 8-weeks-old, $n =$
5), \textit{Pcp2}^{\text{cre};Ucp4^{fl/fl}} \text{mice (male, 8-weeks-old, n = 6)}, \text{and} \textit{Pcp2}^{\text{cre};Ucp4^{fl/+}} \text{mice (male, 8-weeks-old, n = 6)}.

\textbf{D-H.} Representative traces (D) of the open field (OF) test and quantification of the total distance (E), average speed (F), the Ratio of Path\text{cent.} to Path\text{prl.} (G), and the Ratio of Time\text{cent.} to Time\text{prl.} (H) between the three groups.

\textbf{I-M.} Schematic of CatWalk analysis (I) and all paw patterns were recorded (J). The parameters of the regularity index (RI) (K), the base of support (BOS) (L) between either the hind paws (BOS\textsubscript{HP}) and the front paws (BOS\textsubscript{FP}), and the print area (M) of the left front paw (LF), the left hind paw (LH), the right front paw (RF), and the right hind paw (RH) were analyzed.

\textbf{N-R.} Quantification of the RI (N), BOS\textsubscript{HP} (O), BOS\textsubscript{FP} (P), all paw print areas (R), and the cartoon (S) showing the bradykinesia of \textit{Pcp2}^{\text{cre};Ucp4^{fl/fl}} \text{mice, but} \textit{Ucp4^{fl/+}} \text{mice and} \textit{Pcp2}^{\text{cre};Ucp4^{fl/+}} \text{mice did not show bradykinesia.}

\textbf{S and T.} Schematic of Rotarod test (S) and Quantification of the value of the latency to fall (T).

\textbf{U-X.} Representative traces of elevated plus-maze (EPM) test (U), and quantification of Duration\text{closed} and Frequency\text{closed} (V), Duration\text{cent.} and Frequency\text{cent.} (W), and Duration\text{open} and Frequency\text{open} (X).

The data are shown as the mean ± SD. Statistical analysis was performed by one-way ANOVA and LSD post-hoc test or Kruskal-Wallis test. P < 0.05 was considered a statistically significant difference.
Figure 5

Electromyogram (EMG) recordings indicated an absence of muscle damage of \( \text{Pcp2}^{\text{cre}, \text{Ucp4}^{\text{fl/fl}}} \) mice.

A. Representing photograph showing the EMG recording of the mouse gastrocnemius muscle.
B. Schematic of EMG performed on the gastrocnemius muscle. The yellow probe indicates the positive (+) stimulation electrode; the green probe indicates the negative (−) stimulation electrode; the red probe indicates the positive (+) leading electrode; the black probe indicates the negative (−) leading electrode; the gray probe indicates the ground electrode.

C–E. Representative traces of EMG from Ucp4^fl/fl^ (C), Pcp2^cre^;Ucp4^fl/+^ (D) and Pcp2^cre^;Ucp4^fl/fl^ mice (E). Two representative spikes marked by red arrows are magnified in Fig. F–H, correspondingly.

I–K. A representative spike from Ucp4^fl/fl^ mice (I), Pcp2^cre^;Ucp4^fl/+^ mice (J) and Pcp2^cre^;Ucp4^fl/fl^ mice (K).

L and M. Quantification of amplitude (L) and response latency (M) of EMG.

Statistical analysis was performed by one-way ANOVA and LSD post-hoc test or Kruskal-Wallis test. The data are shown as the mean ± SD, n = 3 (male, 8-weeks-old) mice per group. *P* < 0.05 was considered a statistically significant difference.

Electromyogram.

**Figure 6**

Electrical patch clamp recordings showing the changes of both spontaneous and evoked firing properties of PCs in Pcp2^cre^;Ucp4^fl/fl^ mice.
A. Schematic showing the patch clamp recordings on 4 and 5 lobes of the cerebellar cortex (4/5 Cb). Bar = 1 mm, 50 μm, 25 μm, and 10 μm, respectively.

B - D. Schematic showing the three types of PCs from Ucp4^{fl/fl} mice (B), Pcp2^{cre};Ucp4^{fl/+} mice (C), and Pcp2^{cre};Ucp4^{fl/fl} mice (D).

E - G. Representative raw traces for 1 minute recording showing the spontaneous spikes of PCs, from Ucp4^{fl/fl} mice (E), Pcp2^{cre};Ucp4^{fl/+} mice (F), and Pcp2^{cre};Ucp4^{fl/fl} mice (G).

H. Seven parameters were measured.

I - O. Quantification of threshold potential (mV) (I), the action potential (AP) peak (mV) (J), the half-width of AP (ms) (K), the rise slope (mV/ms) (L) and decay slope (mV/ms) (M), the frequency (Hz) (N), and the afterhyperpolarization (AHP, mV) (O).

P - R. Representative evoked spikes by current stimulation in Ucp4^{fl/fl} mice (P), Pcp2^{cre};Ucp4^{fl/+} mice (Q), and Pcp2^{cre};Ucp4^{fl/fl} mice (R).

S. Proportion of firing patterns. The red percentage represents active type; blue percentage represents inactive type; and black percentage represents quiescent type.

T. Quantification of firing numbers.

U. Schematic confirming the location of the patch clamp on the soma of PCs by micro injection of biocytin and the representative confocal photograph of PCs immunostained by both red biocytin and green calbindin.

Statistical analysis was performed by one-way ANOVA and LSD post-hoc test or Kruskal-Wallis test. The data are shown as the mean ± SD, n = 4 mice per group. P < 0.05 was considered a statistically significant difference.
Figure 7

Reactive oxygen species (ROS) generation and mitochondrial membrane potential (MMP) in cerebellum of Pcp2<sup>cre</sup>:Ucp4<sup>fl/fl</sup>.

A. Confocal images of ROS generation in the cerebellum tissues from Ucp4<sup>fl/fl</sup> mice, Pcp2<sup>cre</sup>:Ucp4<sup>fl/+</sup> mice, and Pcp2<sup>cre</sup>:Ucp4<sup>fl/fl</sup> mice.

B. Quantification of ROS in molecular layer (ML) of the cerebellum tissues, which was full of the dendritic trees of Purkinje cells, from Ucp4<sup>fl/fl</sup> mice, Pcp2<sup>cre</sup>:Ucp4<sup>fl/+</sup> mice, and Pcp2<sup>cre</sup>:Ucp4<sup>fl/fl</sup> mice.

C. Quantification of ROS in granular cell (GCL) of the cerebellum tissues from Ucp4<sup>fl/fl</sup> mice, Pcp2<sup>cre</sup>:Ucp4<sup>fl/+</sup> mice, and Pcp2<sup>cre</sup>:Ucp4<sup>fl/fl</sup> mice.

D. Flow cytometer results of mitochondrial membrane potential (MMP) in the cerebellum tissues from Ucp4<sup>fl/fl</sup> mice, Pcp2<sup>cre</sup>:Ucp4<sup>fl/+</sup> mice, and Pcp2<sup>cre</sup>:Ucp4<sup>fl/fl</sup> mice.

E. Quantification of MMP in the cerebellum tissues from Ucp4<sup>fl/fl</sup> mice, Pcp2<sup>cre</sup>:Ucp4<sup>fl/+</sup> mice, and Pcp2<sup>cre</sup>:Ucp4<sup>fl/fl</sup> mice.

The data were analyzed by one-way ANOVA and LSD post-hoc test or Kruskal-Wallis test. The data are shown as the mean ± SD; n = 3 mice per group. P < 0.05 was considered a statistically significant difference.
MMP, mitochondrial membrane potential; ROS, reactive oxygen species.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- GraphicalAbstract.png