DYSTONIA

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Subtle changes in Purkinje cell firing in Purkinje cell-specific *Dyt1 ΔGAG* knock-in mice

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DYT1 dystonia is an inherited early-onset generalized dystonia characterized by sustained muscle contractions causing abnormal, repetitive movements or postures. Most DYT1 patients have a heterozygous trinucleotide GAG deletion (*ΔGAG*) in *DYT1/TOR1A,* coding for torsinA. *Dyt1* heterozygous ΔGAG knock-in (KI) mice or global KI mice show motor deficits and abnormal Purkinje cell firing. However, Purkinje cell-specific heterozygous ΔGAG conditional KI mice (Pcp2-KI) show improved motor performance, reduced sensory-evoked brain activation in the striatum and midbrain, and reduced functional connectivity of the striatum with the anterior medulla. Whether Pcp2-KI mice show similar abnormal Purkinje cell firing as the global KI mice, suggesting a cell-autonomous effect causes the abnormal Purkinje cell firing in the global KI mice, is unknown. We used acute cerebellar slice recording in Pcp2-KI mice to address this issue. The Pcp2-KI mice exhibited no changes in spontaneous firing and intrinsic excitability compared to the control mice. While membrane properties were largely unchanged, the resting membrane potential was slightly hyperpolarized, which was associated with decreased baseline excitability. Our results suggest that the abnormal Purkinje cell firing in the global KI mice was not cell-autonomous and was caused by physiological changes elsewhere in the brain circuits. Our results also contribute to the ongoing research of how basal ganglia and cerebellum interact to influence motor control in normal states and movement disorders.

KEYWORDS

Purkinje cells, dystonia, torsinA, DYT1, electrophysiology

Abbreviations: BK channel, large-conductance calcium-activated potassium channel; Ch2KO mice, cholinergic neuron-specific *Dyt1* conditional knockout mice; CV, coefficient of variation; *Dyt1* KI mice, *Dyt1 ΔGAG* heterozygous knock-in mice; KI, knockin; KO, knockout; Pcp2-KI, Purkinje cell-specific heterozygous ΔGAG conditional KI mice; PCR, polymerase chain reaction; pKG, Purkinje cell-specific knockout of torsinA in global *Dyt1* KI mice; pKO, conditional knockouts of torsinA in Purkinje cells; RMP, resting membrane potential; SEM, standard error of the mean; SN, substantia nigra; SWAP, conditional knock-in locus of *Dyt1* or *Tor1a*.

Introduction

Dystonia is a movement disorder characterized by sustained or intermittent muscle contractions causing abnormal, often repetitive, movements or postures [1]. Dystonia can be caused by genetic mutations, brain injury, or the side effects of drugs. DYT1 dystonia is the most common type of early-onset generalized dystonia [2] with symptom onset from 5 to 28 years old. The majority of the individuals affected by DYT1 dystonia share a trinucleotide deletion (*ΔGAG*) located in the exon 5 of *DYT1 or TOR1A gene*, leading to a loss of a glutamate amino acid residue for torsinA (torsinA$^{\Delta E}$) [3]. It is transmitted as an autosomal dominant disorder with a 30%–40% penetrance. Affected individuals could be seriously disabled and need to use a wheelchair. There are other types of isolated mutations found in DYT1 dystonia patients with missense changes at E121K, V129L, D194V, F205I, and R288Q [4–8], and three other deletions of an 18 bp DNA fragment deletion, a frame-shift mutation caused by 4 bp deletion, and a 6 bp deletion [8–10]. The latest epidemiology data indicate between 54,366 and 80,891 *ΔGAG* mutation carriers in the United States, including 16,475-24,513 DYT1 patients due to the reduced penetrance [11].

Animal models have been used to investigate the pathophysiology of genetic diseases and contribute to developing effective treatments. Multiple animal models have been generated for DYT1 dystonia [12–18]. *Dyt1* KI mice have the corresponding in-frame trinucleotide ΔGAG deletion mutation in the endogenous *Dyt1 or Tor1a* and model DYT1 ΔGAG patients [19, 20]. The *Dyt1* KI mouse also shows dystonia-like phenotypes, including motor and sensory deficits, abnormal gait, and muscle co-contraction of the hind limbs [20–23]. A new conditional knock-in locus of *Dyt1* or *Tor1a* (referred to as SWAP) was developed, possessing loxP sites flanking exon 5 of the *Tor1a* gene, along with an additional downstream mutant exon 5 containing a ΔGAG knock-in mutation [24]. When crossed with *Pcp2-cre* mice [25], the WT exon five was deleted, and the mutant ΔGAG was expressed specifically in Purkinje cells. The resulting conditional knock-in mice, referred to as Pcp2-KI mice, show improved motor performance, reduced sensory-evoked brain activation in the striatum and midbrain, and reduced striatum functional connectivity with the anterior medulla [26].

Cerebellar circuits, especially Purkinje cells, are essential players in movement and posture control, and there are multiple lines of investigations implicating their involvement in dystonia pathogenesis [16, 27–37]. The shRNA-mediated knockdown of torsinA in wild-type mice leads to overt dystonic-like movements with cell death in the deep cerebellar nuclei [38]. *Dyt1* KI mice show altered Purkinje cell morphology [39, 40] and firing, with increased large-conductance calcium-activated potassium (BK) current and the BK channel protein levels [41]. The abnormal function of cerebellar circuits is likely involved in the pathogenesis of DYT1 and other dystonias. However, whether the Pcp2-KI mice show similar altered Purkinje cell firing is unknown. Furthermore, the relative contribution of the striatum and cerebellum in the pathogenesis of DYT1 dystonia is unclear. Here, the Purkinje cells in the Pcp2-KI mice were characterized by electrophysiological recording of acute brain slices. The spontaneous firing, intrinsic excitability, and membrane properties of Purkinje cells were examined.

Materials and methods

Animals

All experiments complied with the United States Public Health Service Guide for Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committees of the University of Florida. Pcp2-KI mice and their littermate control mice were prepared and genotyped by PCR as described previously [26]. *Pcp2-cre* heterozygous mice [25] (The Jackson Laboratory strain #: 010536) were mated with *Tor1a*^{swap} heterozygous mice [24] (The Jackson Laboratory strain #: 028099) to produce Pcp2-KI (*Pcp2-cre*+/−*Tor1a*^{swap}+/−) and control mice (*Pcp2-cre*+/−). The presence of *cre* was detected with primers creF: 5′-CAGCTAAACATGCTTCATCGTC and creR: 5′-GTTATTCGGATCATCAGCTACACC. *Tor1a*^{swap} allele was determined by primers 27427: 5′-TCCTCCCCCAAGTAC ATCAG and 27428: 5′-CATAGCTCAGCCGTCCAGTC [24]. Mice were housed under a 12-h light and 12-h dark cycle with *ad libitum* access to food and water. All experiments and initial data analysis were performed by investigators blind to the genotypes. This study followed the recommended heterogenization of study samples of various ages, and the data were analyzed with age as a covariate [42].

Brain slice electrophysiology

Electrophysiological recordings and data analysis for spontaneous firing, intrinsic excitability, and membrane properties of 128 Purkinje cells were obtained from 9 control and 7 Pcp2-KI littermate male mice (212–385 days old), as described previously [41, 43–45]. Since the onset of motor deficits is about 6.5 months old in the case of Dyt1 KI mice [20, 46], mice older than the onset age were used in the present study. Briefly, the cell-attached recordings of Purkinje cells were performed in the parasagittal 300 μm-thick cerebellar brain slices. After recording the spontaneous firing, whole-cell recordings were made by breaking through the membrane. The electrophysiological intrinsic membrane properties (resting membrane potential, capacitance, membrane resistance, and time constant) were measured in the whole-cell recording mode. The current steps were injected, and the

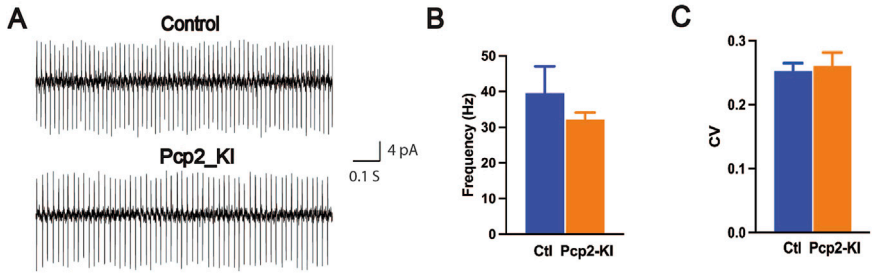

FIGURE 1
Spontaneous firing of the Purkinje cells in brain slices. **(A)** The representative traces of the Purkinje cells. Spontaneous firing frequency **(B)** and CV **(C)** were comparable between the control (Ctl) and Pcp2-KI mice. The bars represent means ± SEM.

evoked-action potentials were recorded. We used male mice to minimize the variation due to the estrous cycle and to match the sex we used in our previous study for direct comparison [41].

Statistics

Data were tested for normality first using the univariate procedure of the SAS statistical package. A generalized linear model (GENMOD) was used to compare the spontaneous firing, intrinsic excitability, and membrane properties. Age was used as a continuous variable, and data from each cell were nested within animals and treated as repeated measurements. A negative binomial distribution was used for count data, i.e., the number of action potentials in the current injection. A gamma distribution was used for data that was not normally distributed. For tonic/non-tonic cell distribution analysis, chi-square was used. Significance was assigned at $p < 0.05$. Data in the text are presented as "mean ± standard error of the mean (SEM)" unless specified otherwise.

Results

Normal spontaneous firing frequency, coefficient of variation (CV), and cell type distribution of the Purkinje cells in Pcp2-KI mice

Cerebellar Purkinje cells are the sole output of the cerebellum and play an essential role in cerebellar function. The Purkinje cells in the Pcp2-KI mice were characterized by acute brain slice recording. The spontaneous firing of the Purkinje cells was recorded by cell-attached recording mode with a voltage clamp (control, 61 cells/9 mice; Pcp2-KI, 35 cells/7 mice). The representative traces of the Purkinje cells were shown in Figure 1A. Neither the firing frequency (control, 39.6 ± 7.5 Hz; Pcp2-KI, 32.1 ± 2.0; $p = 0.30$, Figure 1B) nor CV (control, 0.254 ± 0.012; Pcp2-KI, 0.261 ± 0.021; $p = 0.77$; Figure 1C) was significantly altered in Pcp2-KI mice compared to control mice. Purkinje cells can be grouped into tonic and non-tonic types [41, 44, 47]. When analyzed separately by the cell types, neither the firing frequency nor CV was altered in both cell types (Table 1). Finally, the relative ratio of the tonic and non-tonic cells was analyzed, and there was no significant difference between the control and Pcp2-KI mice (control: tonic = 35, non-tonic = 26; Pcp2-KI: tonic = 19, non-tonic = 16, $p = 0.77$). Overall, Pcp2-KI mice had normal spontaneous firing frequency, CV, and cell type distribution of Purkinje cells.

Intrinsic excitability and membrane properties of the Purkinje cells in Pcp2-KI mice

After recording the spontaneous firing by cell-attached mode, the intrinsic membrane properties were measured in whole-cell recording mode. The resting membrane property of the Purkinje cells was determined from 9 control (73 cells) and 7 Pcp2-KI mice (55 cells). There was no significant difference in the membrane capacitance, the membrane resistance, or the time constant between the control and Pcp2-KI mice (Table 2). However, the resting membrane potential (RMP) of the Pcp2-KI mice was significantly hyperpolarized than that of the control mice, suggesting slightly decreased baseline excitability.

The intrinsic excitability of the Purkinje cells in the brain slices was measured with current step injections. The recorded neurons showed typical electrophysiological responses of the Purkinje cells (Figure 2A). The number of action potentials fired overall (control, 25.4 ± 2.3; Pcp2-KI, 26.1 ± 1.3; $p = 0.78$, Figure 2B) and at each current step (Figure 2C) were similar between control and Pcp2-KI mice. This indicates that while the neurons were less excitable at rest, their ability to respond to depolarizing stimuli was preserved, potentially reflecting compensatory mechanisms that maintained functional responsiveness despite altered baseline properties.

TABLE 1 Spontaneous firing properties of Purkinje cells by cell types.

	Tonic CV	Tonic frequency (Hz)	Non-tonic CV	Non-tonic frequency (Hz)
Control	0.123 ± 0.006	50.6 ± 7.6	0.530 ± 0.049	24.7 ± 5.5
Pcp2-KI	0.119 ± 0.012	39.1 ± 5.7	0.558 ± 0.061	23.8 ± 2.4
Z value	0.32	1.23	−0.36	0.15
p-value	0.75	0.22	0.72	0.88

CV, coefficient of variation.

TABLE 2 Intrinsic properties of Purkinje cells.

	RMP (mV)	Capacitance (pF)	MR (MΩ)	Time constant (ms)
Control	−63.3 ± 0.2	127.4 ± 5.1	23.4 ± 0.7	1.36 ± 0.02
Pcp2-KI	−63.8 ± 0.2	128.8 ± 5.1	22.4 ± 0.6	1.39 ± 0.04
Z value	2.24	−0.20	1.11	−0.70
p	0.025	0.84	0.27	0.48

RMP, resting membrane potential; MR, membrane resistance.

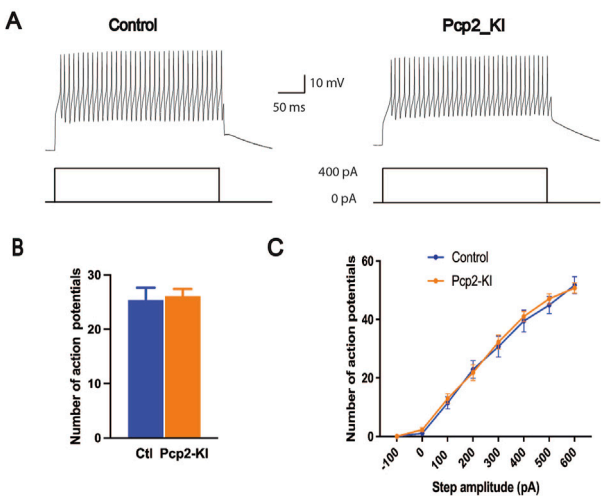

FIGURE 2
Intrinsic excitability of Purkinje cells as measured by current steps in the brain slices. Representative trace of the action potential firing in response to the current injection **(A)**. The number of action potentials of all eight steps combined **(B)** and at each current step **(C)** was not significantly altered in Pcp2-KI mice compared to control (Ctl) mice. Means ± SEM were plotted.

Discussion

The current study aimed to determine whether abnormal Purkinje cell firing in the global KI mice is cell-autonomous using Pcp2-KI mice. The Pcp2-KI mice showed no changes in spontaneous firing, intrinsic excitability, and most membrane properties. The only subtle change we found was a slightly hyperpolarized resting membrane potential. These results suggest that the abnormal Purkinje cell firing in the global KI mice is not cell-autonomous and is driven by changes elsewhere in the brain circuits. The results are reminiscent of the mouse models of DYT11 dystonia. The *Sgce* global knockout mice showed nuclear envelope deficits, while Purkinje cell- or striatum-specific Sgce KO mice have no such deficits [48, 49].

Past studies have provided strong evidence that the cerebellum is actively involved in the pathogenesis of dystonia [16, 27–37]. However, it is unknown whether the dystonia originates from the basal ganglia, cerebellum, or both [32]. The overwhelming majority of the DYT1 patients have the ΔGAG mutation in the *DYT1/TOR1A* gene. Mutant torsinA could lead to both a loss of function and a toxic gain of function [50, 51]. Conditional knockout of torsinA in the striatum, the cerebral cortex, cholinergic interneurons, and dopamine receptor 1 or 2-positive neurons leads to motor deficits or overt dystonia [52–60]. Conditional knockin of mutant torsinA in dopamine receptor 2-positive neurons (D2-KI) leads to similar motor deficits [26]. These genetic experiments in animals suggest that mutations introduced in the basal ganglia circuit alone are sufficient to induce motor deficits or overt dystonia and support a basal ganglia origin for DYT1 dystonia.

On the other hand, it is remarkable that both conditional knockouts of torsinA (pKO) and conditional knockin of mutant torsinA (Pcp2-KI) in Purkinje cells show better motor performance in mice [26, 61]. The motor phenotype in pKO and Pcp2-KI mice suggests that torsinA mutations in Purkinje cells are insufficient to produce motor deficits. The normal Purkinje cell firing in Pcp2-KI mice extends these findings. It indicates mutant torsinA in Purkinje cells alone cannot induce

abnormal Purkinje cell firing observed in the global KI mice. The behavioral and electrophysiological phenotypes in pKO and Pcp2-KI mice argue against a cerebellum origin for DYT1 dystonia. However, we can not rule out the possibility that in the global KI mice and DYT1 patients, the dystonia may still originate from Purkinje cells or other cerebellar neurons. This can be addressed with lines of *Dyt1* conditional knockin mice that express wild-type torsinA in the Purkinje cells, other cerebellar neurons, or both.

Interestingly, acute shRNA-mediated torsinA knockdown in adult mice shows overt dystonic-like movements [38]. We generated an acute torsinA cerebellar knockdown mouse model by bilateral stereotaxic injections of AAV5-CMV-Cre-GFP into the cerebellum of *Dyt1* loxP/loxP mice [57]. Expression of *cre* led to cre-loxP-mediated recombination and eliminated the expression of torsinA in AAV-infected cells. These mice showed overt dystonia similar to the shRNA-mediated torsinA knockdown mice (unpublished data). In addition, Purkinje cells in global KI mice show altered dendritic structure [39, 40] and altered spontaneous firing *in vitro* [41]. These results align with a cerebellum origin for DYT1 dystonia. Alternatively, the results could be interpreted as the cerebellum not acting as the origin but as a node downstream of the brain network abnormality that leads to the pathogenesis of DYT1 dystonia.

What might be the upstream brain network abnormality that drives Purkinje cell abnormality in DYT1 dystonia and global KI mice? The basal ganglia and the cerebellum are interconnected at the subcortical level with disynaptic pathways. The subthalamic nucleus in the basal ganglia connects to the cerebellar cortex via pontine nuclei [62]. Imaging studies in DYT1 mouse models indicate striatum alterations can influence cerebellar circuits. Forebrain torsinA knockout increases functional connectivity of the left striatum with the cerebellum [63]. Compared to controls, there is increased functional connectivity between the right dorsomedial striatum and the right cerebellar cortex in *Dyt1* Ch2KO mice, with torsinA selectively knocked out in cholinergic neurons [59]. The Purkinje cell abnormality in *Dyt1* KI mice [39–41] likely originated from the striatum. Future studies should analyze the Purkinje cell activity in the striatum-specific *Dyt1* conditional knockout [56] or knockin mice to explore such a possibility.

The cerebellum can modulate the activity of the basal ganglia, especially the striatum. The dentate nucleus in the cerebellum connects to the striatum via the thalamus [62] or directly forms monosynaptic glutamatergic connections with the dopaminergic neurons in the substantia nigra (SN), which in turn, modulate the striatal activity [64, 65]. The current study uncovered normal Purkinje cell firing in Pcp2-KI mice, except for the slightly hyperpolarized RMP. There are limitations associated with the current study. We used glutamatergic and GABAergic antagonists to block synaptic transmission in brain slice recording. Synaptic inputs to Purkinje cells were not measured. Furthermore, we did not investigate whether altered RMP leads to any physiological changes elsewhere. However, brain imaging studies of Pcp2-KI mice show reduced sensory-evoked brain activation in the striatum and midbrain and reduced striatum functional connectivity with the anterior medulla [26]. These functional changes in the basal ganglia circuit may result from the Purkinje cell-specific knockin of the mutant torsinA. Although details are unclear, these changes are likely key to understanding improved motor performance in Pcp2-KI mice and, by extension, the pKO mice. We demonstrated earlier that Purkinje cell-specific knockout of torsinA in global *Dyt1* KI mice (pKG mice) could alleviate the motor deficits associated with the *Dyt1* KI mice [61]. Future studies focusing on the electrophysiological analysis of Purkinje cells, striatal medium spiny neurons, and SN dopaminergic neurons in pKO, pKG, and Pcp2-KI will clarify the roles of cerebellum and striatum in the pathogenesis of DYT1 dystonia and how to target these connections for novel treatments. These studies will have implications in basic neuroscience research beyond the dystonia field.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was approved by University of Florida Institutional Animal Care and Use Committee. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

All authors participated in the design, interpretation of the studies, analysis of the data, and review of the manuscript; HX, PG, YnL, FY, and YqL conducted the experiments, and HX and YqL wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

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