



Electrophysiological Studies Support Utility of Positive Modulators of SK Channels for the Treatment of Spinocerebellar Ataxia Type 2

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Accepted: 18 November 2021 / Published online: 3 January 2022

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Abstract

Spinocerebellar ataxia type 2 (SCA2) is an incurable hereditary disorder accompanied by cerebellar degeneration following ataxic symptoms. The causative gene for SCA2 is *ATXN2*. The ataxin-2 protein is involved in RNA metabolism; the polyQ expansion may interrupt ataxin-2 interaction with its molecular targets, thus representing a loss-of-function mutation. However, mutant ataxin-2 protein also displays the features of gain-of-function mutation since it forms the aggregates in SCA2 cells and also enhances the IP3-induced calcium release in affected neurons. The cerebellar Purkinje cells (PCs) are primarily affected in SCA2. Their tonic pacemaker activity is crucial for the proper cerebellar functioning. Disturbances in PC pacemaking are observed in many ataxic disorders. The abnormal intrinsic pacemaking was reported in mouse models of episodic ataxia type 2 (EA2), SCA1, SCA2, SCA3, SCA6, Huntington's disease (HD), and in some other murine models of the disorders associated with the cerebellar degeneration. In our studies using SCA2-58Q transgenic mice via cerebellar slice recording and in vivo recording from urethane-anesthetized mice and awake head-fixed mice, we have demonstrated the impaired firing frequency and irregularity of PCs in these mice. PC pacemaker activity is regulated by SK channels. The pharmacological activation of SK channels has demonstrated some promising results in the electrophysiological experiments on EA2, SCA1, SCA2, SCA3, SCA6, HD mice, and also on mutant *CACNA1A* mice. In our studies, we have reported that the SK activators CyPPA and NS309 converted bursting activity into tonic, while oral treatment with CyPPA and NS13001 significantly improved motor performance and PC morphology in SCA2 mice. The i.p. injections of chlorzoxazone (CHZ) during in vivo recording sessions converted bursting cells into tonic in anesthetized SCA2 mice. And, finally, long-term injections of CHZ recovered the precision of PC pacemaking activity in awake SCA2 mice and alleviated their motor decline. Thus, the SK activation can be used as a potential way to treat SCA2 and other diseases accompanied by cerebellar degeneration.

Keywords SK channels · Purkinje cells · Spinocerebellar ataxia · Transgenic mice · Chlorzoxazone

Introduction

Spinocerebellar ataxia type 2 (SCA2) represents an inherited neurodegenerative disorder accompanied by cerebellar atrophy and progressive ataxic and cognitive symptoms [1–6]. Most of SCA2 patients are suffering from cerebellar ataxia, slow saccades, polyneuropathy, and, in some cases,

parkinsonism [6]. Past studies from patients with SCA2 have also revealed the disturbances in cognitive functions which included a decline in verbal memory, executive functions, and attention [7–10]. In addition, SCA2 patients showed higher levels of depression, anxiety, and apathy [8, 11]. So far there is still no disease-modifying treatment available for SCA2 patients [6].

The disease causative gene is *ATXN2*, and mutant ataxin-2 protein is involved in SCA2 pathology. This mutation seems to exert both gain-of-function and loss-of-function properties [6]. Thus, the interruptions in mRNA, ribosomal, and lipid metabolism were reported in studies of the lack of ataxin-2 [6]. At the same time, mutant ataxin-2 also tends to accumulate as aggregates in the cerebellar tissue from SCA2 patients, thus demonstrating its new cytotoxic functions [6]. In our previous experiments, we also

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observed gain-of-function properties of mutant ataxin-2. We reported that the mutant ataxin-2, but not the wild-type (WT) ataxin-2, is able to drastically enhance the IP₃-induced calcium release [12]. The calcium-stabilizing agents had some therapeutic effect in SCA2 model systems [12–14].

The cerebellar Purkinje cells (PCs) are primarily affected in ataxia. The precise PC pacemaking is required for the proper cerebellar functioning. The abnormal PC electrophysiology has been reported in many mouse models of ataxia [15–20]. In our studies on SCA2-58Q transgenic mice, we have also reported the loss of the PC firing precision in acute cerebellar slices from aging SCA2-58Q mice [13, 21], in urethane-anaesthetized mutant mice [22], and in awake head-fixed transgenic mice [23]. The small-conductance calcium-activated potassium channels (SK channels) are directly involved in the control of the PC pacemaking [24]. It has been also considered that these channels are associated with the abnormal calcium signaling in ataxia [25]. Thus, it has been further hypothesized that the pharmacological modulation of SK channels activity may have some therapeutic effects in SCAs. Indeed, some encouraging data were reported in murine [16, 26–28] and cellular models [14] of ataxia and even in human trials [29]. In our studies, we demonstrated that the SK activators rescue the PC firing precision in acute cerebellar slices from aging SCA2-58Q mice [21], in urethane-anaesthetized mutant mice [22], and in awake head-fixed transgenic mice [23]. The motor decline of our transgenic mice was also significantly improved [21, 23]. Based on our data and on the results of the other research ataxia groups, we assume that the abnormal PC firing most likely causes the motor symptoms in ataxias and that the SK channel activation can be used as a therapeutic target for ataxia treatment.

Pathogenesis of SCA2

The molecular pathogenesis of SCA2 has been studied for decades now, since the disease causative gene *ATXN2* was localized for the first time in 1993 [30]. The mutant protein ataxin-2 has more than 33 polyglutamine (polyQ) residues [31] and gains cytotoxic functions and/or suspends its normal functions at the same time. According to ataxin-2 domain structure, this protein is involved in the mRNA metabolism and also in the stress granule formation [6]. Of course, the polyQ expansion may change the normal conformation of ataxin-2 protein, and, thus, it may interrupt the molecular interaction between the ataxin-2 protein and its cellular targets leading to the disturbed RNA metabolism, thus displaying itself as a loss-of-function mutation. Indeed, experimental evidences support that assumption [6]. At the same time, many studies have demonstrated that polyQ-expanded ataxin-2 also exhibits features of gain-of function

mutation as it forms aggregates in the cerebellar tissues from SCA2 patients [32] and mice [18] and also disturbs calcium homeostasis in the affected neurons [12].

Our previous experiments on planar lipid bilayer system have demonstrated that polyQ-expanded mutant ataxin-2 protein, but not wild-type (WT) ataxin-2, tends to associate with IP₃ receptor and to increase its sensitivity to the IP₃ molecules, thus leading to the enhanced IP₃-induced calcium release (IICR) [12] (Fig. 1). The deranged calcium signaling is observed in many types of ataxic disorders [6, 25, 33–36]. Some attempts have been made by our research group to normalize calcium status of the affected cerebellar neurons, and some promising results have been obtained in the murine model of SCA2. Thus, the blockage of ryanodine receptors (RyRs), another intracellular calcium channels, with ryanodine and dantrolene (Fig. 1), significantly reduced the IICR in the affected cerebellar neurons, decreased the glutamate-induced apoptosis in these cells, improved the motor decline observed in ataxic mice, and also prevented neuronal loss in these mice [12]. The viral-mediated PC-specific overexpression of IP₃-phosphatase (5PP) enzyme that converts IP₃ into the inactive compound IP₂ (Fig. 1) recovered the abnormal neuronal firing, improved motor skills in SCA2-58Q mice, and ameliorated the PC morphology in these mice [13].

The small-conductance calcium-activated potassium channels (SK channels) are also directly or indirectly involved into the ionic homeostasis of the affected cerebellar neurons (Fig. 1). When SK channels are activated they pump potassium ions out of the cell thus leading to the hyperpolarization of the cell membrane and blocking the voltage-dependent calcium channels (VDCCs), hereby less calcium ions are coming into the cell through these channels (Fig. 1).

Cerebellar Purkinje cells (PCs) are primarily affected in SCA2. PCs represent the main dynamic element of the cerebellar cortex since their axons form the unique output coming from the cerebellar cortex to the deep cerebellar nuclei and to further deep brain structures. Cerebellar PC generates two types of electrophysiological signals — simple spikes and complex spikes. PC generates simple spikes when activated by parallel fibers, the branched axons of cerebellar granule cells. PC fires complex spike when activated by climbing fibers that originate in inferior olive. The precise PC firing is crucial for the proper cerebellar functioning. The disturbances in PC firing activity have been reported in many models of ataxia and other related disorders (Table 1). The loss of precision in PC firing was firstly reported by two independent research groups in *tottering* mice, a mouse model of episodic ataxia type 2 (EA2) [15, 37]. Thus, extracellular recordings of PCs in awake *tottering* mice demonstrated that the recorded simple spikes were generated much less regularly in tg mice compared to their WT littermates [37]. Similar observations were obtained via cerebellar slice

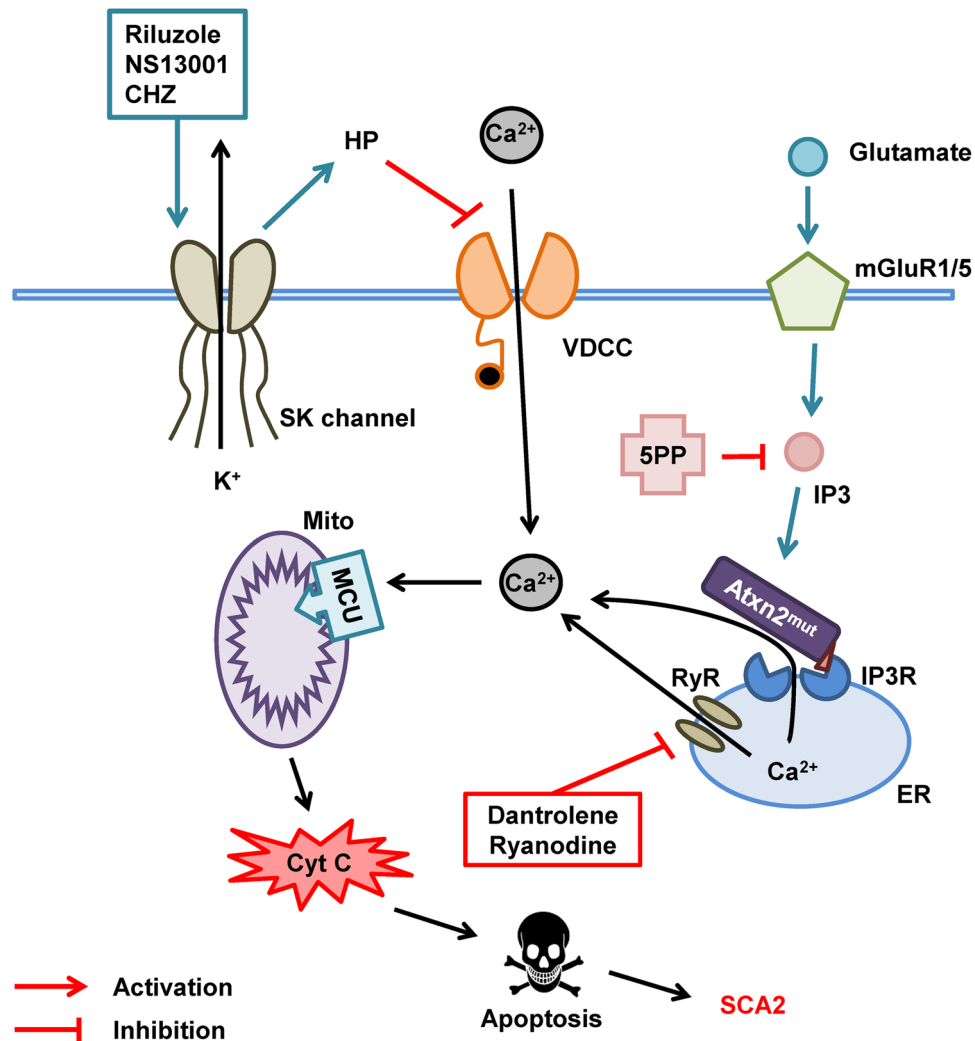


Fig. 1 Molecular pathogenesis of SCA2. Extracellular molecules of glutamate activate metabotropic glutamate receptor (mGluR), and the inositol 1,4,5-triphosphate (IP3) releases into the cytoplasm causing the further activation of the IP3 receptor (IP3R) on the endoplasmic reticulum (ER) membrane leading to the calcium entry from ER to the cytoplasm. This process is called IP3-induced calcium release (IICR). The polyQ-expanded mutant ataxin-2 protein (*Atxn2^{mut}*), but not wild-type *Atxn2*, associates with IP3R and enhances its sensitivity to IP3. Hyperactivation of IP3R results in the increased IICR in cerebellar Purkinje cells. Excessive calcium (Ca^{2+}) ions are pumped into the mitochondria (Mito) through the mitochondrial calcium uniporter (MCU), and this provokes the mitochondrial swelling, further followed by the rupture of the outer membrane and the following

release of pro-apoptotic factors like cytochrome C (Cyt C) into the cytoplasm, thus initiating apoptosis. Significantly increased IICR can be suppressed by the adeno-associated virus-mediated expression of the IP3-5-phosphatase enzyme (5PP) which converts IP3 into non-active form IP2. Another way to reduce calcium release from ER may be the inhibition of ryanodine receptors (RyRs) with dantrolene or ryanodine. Small-conductance calcium-activated potassium channels (SK channels) are involved in the control of the PC firing. SK channel activation with riluzole, NS13001, and chlorzoxazone (CHZ) enhances the hyperpolarization (HP) of the PC membrane, thus suppressing the voltage-dependent calcium channels (VDCC) leading to the decline of calcium entry from the outer space

recordings [15]. Five years later, the alterations in intrinsic PC firing were also reported for SCA3 mice [16]. After that, many other research groups started to notice the pathological changes in the cerebellar PC firing in their ataxic mice. Thus, the irregularity of PC firing was reported in ataxic mice with a mutation in P/Q voltage-dependent calcium channel [17]. Next, the progressive decline in PC firing rate was observed in SCA2-127Q transgenic mice [18]. In SCA1-82Q mice, the

abnormal membrane depolarization was reported [19]. The reduced firing frequency and higher variability of interspike intervals (ISI) were observed in SCA6 mice [20]. Interestingly, it has been reported that in various mouse models of Huntington's disease (HD), the disturbance of PC firing was also observed [38–40]. Thus, the reduction of PC firing rate was reported in presymptomatic R6/2 HD mice [38] and in 50-week-old HdhQ200 HD mice [39]. In our recent studies,

we also demonstrated the reduction of simple spike generation and the decline in PC firing precision in aging YAC128 HD mice [40]. The regularity of PC pacemaking activity was also disturbed in awake mice with a single mutation in the sodium–potassium adenosine triphosphatase (ATP1A3 mutant mice); this mutation is involved in two closely related movement disorders in humans [41]. The abnormal PC activity was also reported in awake mdx mice which represent a mouse model for Duchenne muscular dystrophy and also exert ataxic symptoms [42].

In our studies via acute cerebellar slice recordings, we firstly demonstrated that in aging SCA2-58Q transgenic mice, the number of tonic cells with highly regular activity is declining with age [13]. We also reported that the remaining tonic activity is significantly disturbed in our aging SCA2-58Q mice. Thus, the decline in firing frequency and PC firing precision was observed [13]. Next, via the single-unit extracellular recording from the urethane-anaesthetized SCA2-58Q mice, we have demonstrated the significant reduction in a portion of tonic PC cells in the aging transgenic mice and also significant decrease in the regularity of PC pacemaking in these mice [22]. We further wondered if the cerebellar pathways are affected in the SCA2 pathology in SCA2-58Q mice. To check the integrity of the olivocerebellar tract in our mice, we set out the series of experiments with harmaline intraperitoneal (IP) injections during the in vivo recording sessions of PC firing activity. Harmaline is an alkaloid that activates neurons in the olivary nucleus where the olivocerebellar fibers start. Therefore, harmaline results in an enhancement of olivary neuron activation, climbing fiber excitation, and finally PC complex spikes' (CSs) generation. In these experiments, we observed that SCA2 PCs generate much less CSs compared to WT PCs of the same age, indicating that the synaptic transmission between the terminals of climbing fibers and PC dendrites is interrupted in SCA2-58Q mice [46]. In our most recent experiments, we analyzed the spontaneous activity of cerebellar PCs in awake head-fixed WT and SCA2-58Q transgenic mice. For this purpose, we used a floating platform (Mobile HomeCage) made by Neurotar, Finland [47]. Using this method, we demonstrated that the generation of both simple and complex spikes is significantly reduced in aging SCA2-58Q mice, and the firing variability is much higher in these mice compared to their WT littermates [23].

SK Channels as a Potential Therapeutic Target for SCA2 Treatment

Recent studies have developed strategies for SCA2 therapeutic treatment. A promising approach, antisense oligonucleotide therapy (ASO therapy) where oligonucleotide sequence complementary to a target mRNA reduces protein expression [6],

improved the motor, electrophysiological, and biochemical functions in SCA2-127Q and BAC-72Q transgenic SCA2 mice [48]. Unfortunately, no clinical trials of ASO therapy have been conducted yet on human patients. Another potential therapeutic approach for SCA2 treatment is the usage of the mesenchymal stem cells (MSCs) which exhibit some immune-modulatory functions as a release of the neurotrophic factors [6]. Thus, the MSC therapy significantly improved the motor decline and PC loss observed in C57BL/6 J SCA2 transgenic mice [49]. However, the subsequent MSCs trial on SCA3 human patients did not reveal any statistically significant improvement of their SARA scores, while it surely proved the tolerability and safety of this treatment [50]. Next potential therapeutic approach for SCA2 treatment includes aggregation as a therapeutic target. Prevention of aggregate formation and accumulation of the misfolded polyQ proteins have been studied to develop the disease-modifying treatment for polyQ disorders [6]. Molecular chaperones, such as heat shock protein (HSP) 70 kDa (Hsp70), are known as suppressors of aggregation formation. Indeed, the overexpression of Hsp70 improved the morphology and the motor coordination in SCA1-82Q transgenic mice [51]. Active forms of the heat shock transcription factor (HSF1) exerted even more dramatic suppression of the aggregation formation in HD mice [52]. However, the Hsp70 overexpression had no effect on the mutant ataxin-7-induced toxicity in SCA7 mice [53]. Possibly due to these contradictory results, no human-based clinical trials have been conducted yet in this field.

Our research is mostly focused on the therapeutic strategy for SCA2 treatment that involves calcium-stabilizing agents and agents that normalize the irregular firing leading to the recovery of the abnormal PC morphology and motor decline in SCA2 transgenic mice. As previously mentioned, in our previous studies on SCA2-58Q transgenic mice, we have demonstrated that the pharmacological negative regulation of the ER calcium channels, ryanodine receptors, with such drugs as ryanodine or dantrolene, has some therapeutic effect in these mice. In particular, the recovery of calcium status, motor coordination, and PC morphology was observed after treatment [12]. In our further experiments, we have also shown that the chronic suppression of the IP3-induced calcium release via the AAV-mediated expression of the 5PP reduced the IP3 level in the SCA2 PCs and alleviated the age-dependent motor decline and PC death in our SCA2-58Q mice [13].

Almost 20 years ago now, it was demonstrated that the PC pacemaker activity is regulated by the small-conductance calcium-activated potassium channels (SK channels) [24]. Whole-cell patch clamp recordings from mice with a PC-specific knockout of SK2 channel revealed the decrease in the spontaneous PC firing rates [54], while similar reduction in PC pacemaking was also observed in some ataxic mice [13, 17, 18, 20, 23, 38–40, 42, 44, 45]. Constantly enhanced intracellular calcium levels restrain the PC pacemaker activity through the SK

Table 1 The effects of SK channel activators on the PC abnormal physiology in the murine models of the disorders associated with cerebellar degeneration

Mouse model	Modeling disease	Method of electrophysiological recording	Abnormality in PC firing	SK channel activator used	The effect of SK activation on PC firing	Reference
<i>tottering</i>	EA2	Whole-cell current-clamp Whole-cell voltage-clamp Extracellular cerebellar slice recordings	Highly irregular intrinsic pacemaking	EBIO 4-AP CHZ	Recovery of the precision of intrinsic pacemaking	[15] [27] [28]
ATXN1[82Q]	SCA1	Cell-attached and whole-cell patch clamp	Absence of repetitive spiking and dendritic hyperexcitability	CHZ SKA-31 1-EBIO + baclofen	Recovery of repetitive spiking; increase of AHP and repolarization of membrane potential	[19, 26]
Afxn1 ^{154Q/2Q}			Irregular intrinsic pacemaking and neuronal hyperexcitability	CHZ + baclofen	Recovery of the precision of intrinsic pacemaking	[43]
SCA2-58Q	SCA2	Loose patch	Irregular intrinsic pacemaking; high fraction of bursting PCs	NS309 CyPPA CHZ	Conversion of bursting PCs into tonic firing mode	[13, 21] [22]
		Extracellular single-unit recordings in vivo in anesthetized mice				
		Extracellular single-unit recordings in vivo in awake mice	Reduced SS and CS frequency; increased post-CS pause; irregular pacemaking		Recovery of the CS frequency, post-CS pause, and the precision of pacemaking while not altering the SS firing rate	[23]
ATXN2[127Q]		Cell-attached voltage-clamp Whole-cell current-clamp	Decrease in PC firing rate Absence of repetitive spiking, reduced firing frequency	ND	ND	[18] [44]
SCA3-84Q	SCA3	Whole-cell current-clamp	Hyperexcitability resulting in depolarization block and the loss of repetitive firing	SKA-31	Recovery of the depolarization block of repetitive firing	[16]
CT-10ng ^{Q27} expressing mice	SCA6	Extracellular single-unit recordings in vivo in anesthetized mice	Reduced SS frequency; irregular pacemaking	ND	ND	[20]
SCA6-84Q		Extracellular cerebellar slice recordings; extracellular single-unit recordings in vivo in anesthetized mice	Irregular pacemaking; decrease in PC firing rate	4-AP	Recovery of the precision of intrinsic pacemaking both in vitro and in vivo while not altering the firing rate in vitro	[45]
Caen1a ^{S218L}	Ataxia, migraine, epilepsy	Cell-attached and whole-cell patch clamp; extracellular single-unit recordings in vivo in awake mice	Irregular pacemaking; decrease in PC firing rate; increased post-CS pause	CHZ 1-EBIO	Recovery of the precision of intrinsic pacemaking and post-CS pause	[17]
R6/2	HD	Loose patch	Decreased PC firing rate	ND	ND	[38]
HdhQ200						[39]
YAC128		Extracellular single-unit recordings in vivo in anesthetized mice	Reduced SS frequency; increased post-CS pause; irregular pacemaking	CHZ	Recovery of the SS frequency, post-CS pause, and the precision of pacemaking	[40]
$\alpha_5^{+/D801Y}$	RDP; AHC	Extracellular single-unit recordings in vivo in awake mice	Irregular pacemaking	ND	ND	[41]
<i>mdx</i>	DMD		Decrease in PC firing rate	ND	ND	[42]

Abbreviations: EA2, episodic ataxia type 2; SCA1, 2, 3, and 6, spinocerebellar ataxia type 1, 2, 3, and 6; HD, Huntington's disease; RDP, rapid-onset dystonia parkinsonism; AHC, alternating hemiplegia of childhood; DMD, Duchenne muscular dystrophy; CHZ, chlorzoxazone; SS, simple spikes; CS, complex spikes

channels [25]. The pharmacological stimulation of SK channels activity was hereby considered to be a potential way to treat ataxia. Indeed, some promising results were obtained on mouse (Table 1) and cell models of SCA, and a clinical trial on SCA patients was conducted. Thus, the usage of SK channel activator chlorzoxazone (CHZ) and GABA_B agonist baclofen improved

the electrophysiology of PCs and also recovered the motor functions in SCA1-82Q mice [26]. SK activator CHZ and 4-AP exerted therapeutic effects in a mouse model of episodic ataxia type 2, as well [27, 28]. Similar results were reported in studies on SCA3 mice and SK channel activator SKA-31 [16]. Some attempts have been recently made to normalize the homeostasis

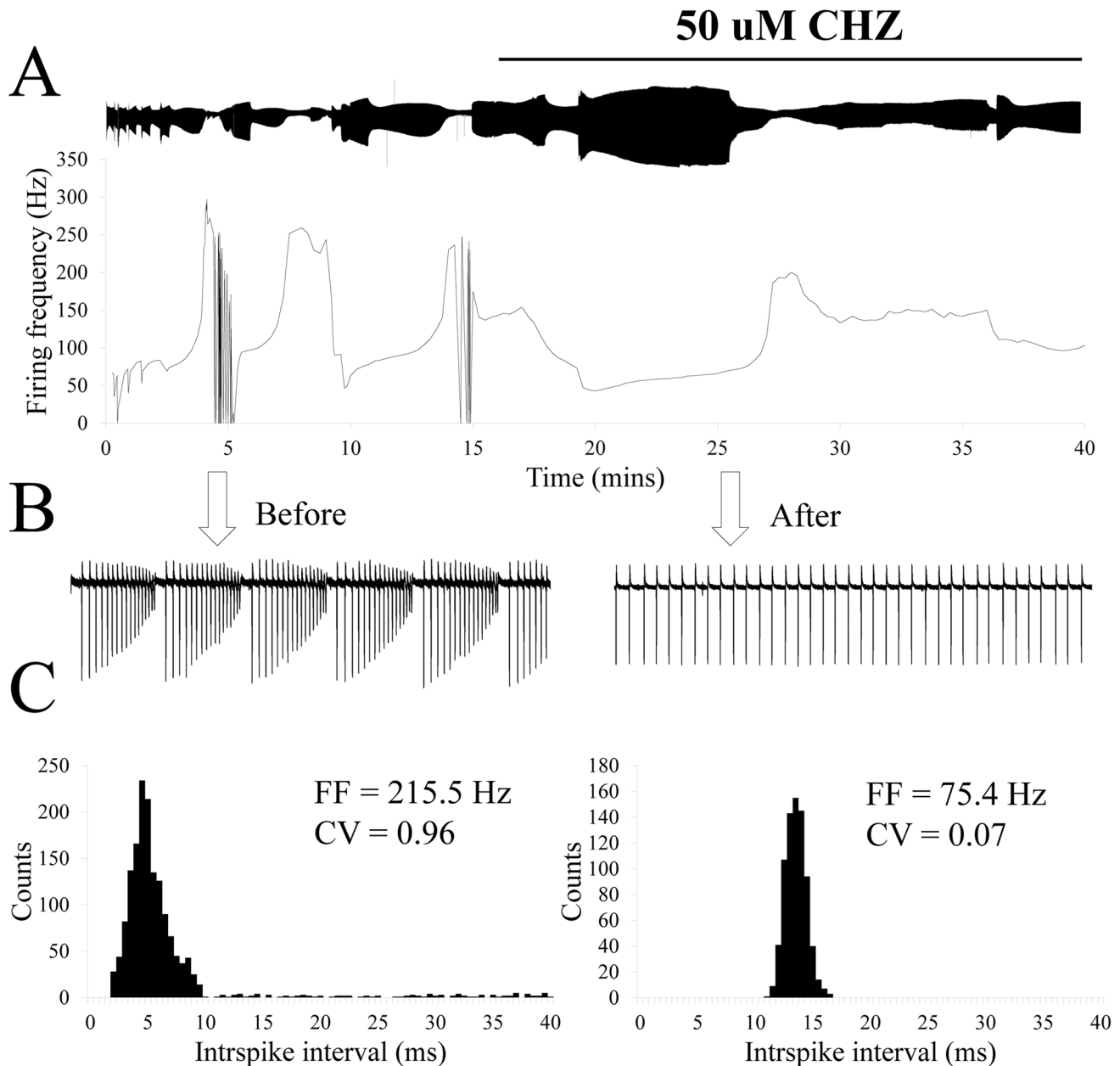


Fig. 2 SK channel positive modulator chlorzoxazone (CHZ) converts bursting patterns into tonic activity of PC in acute cerebellar slices from 8-mo-old SCA2-58Q mouse. **A** Continuous 40-min recording of PC activity. The time of 50 μ M CHZ application is indicated by a horizontal bar above the recording. A plot of the running average of firing frequency is shown below the recording. **B** 500-ms fragments of PC activity recordings before the exposure to CHZ and 10 min after the exposure are shown on the expanded timescale. **C** The distri-

butions of interspike intervals (ISI) before (left) and after (right) the exposure to CHZ were calculated from 10-s fragments of the recording shown in **A**. The average firing frequency (FF) for the analyzed fragment before CHZ application inside the bursts was 215.5 Hz, and the CV of ISI in the analyzed fragment was 0.96. The average FF for the analyzed fragment after CHZ exposure was 75.4 Hz, and the CV of ISI was 0.07

of the neurons derived from the induced pluripotent stem cells (iPSCs) donated by SCA2 human donors. Thus, the recent experiments on SCA2-iPSC-derived neurons have shown that riluzole reduced intracellular calcium levels, decreased cell death, and improved mitochondrial function in these neurons [14]. A randomized, double-blind, placebo-controlled trial on SCA patients revealed that treatment with 100 mg/day riluzole improved the SARA scores in subjects and did not have any significant side effects [29].

Using acute cerebellar slice recordings, we demonstrated that the SK channel modulators NS309 and CyPPA are able to convert highly irregular bursting activity into regular tonic mode of PCs from transgenic SCA2-58Q mice [21]. We have also observed that feeding SCA2-58Q mice with SK activators CyPPA and NS13001 significantly improved their motor performance and PC morphology [21]. We further expanded our studies of SCA2-58Q PC electrophysiological functions from *ex vivo* to *in vivo* research. Thus, in urethane-anaesthetized SCA2-58Q mice via single-unit extracellular recordings *in vivo*, we also observed that IP injections with SK activator CHZ convert bursting PCs into tonic [22]. And, finally, via the same recording method in head-fixed awake SCA2-58Q mice, we have demonstrated that the long-term IP injections of CHZ recover the depression of simple spikes (SSs) after the complex spike (CS) generation, restore CS frequency, and also significantly alleviate the regularity in SCA2-58Q PCs while not altering the SS firing rate [23]. In the same CHZ trial, we reported that CHZ treatment improves performance of SCA2-58Q transgenic mice in the beam-walk test [23]. Interestingly, similar results were obtained in the study of the effect of SK activator 4-AP in SCA6 mice *in vitro*. Thus, in these experiments, the recovery of the precision of intrinsic pacemaking was also reported while not altering the firing rate [45]. We think that the main readout of the impaired physiology of cerebellar Purkinje cells is the precision of PC firing. PCs exhibit a broad variety of their firing frequencies, while the CV ISI has more narrow means.

We propose that the therapeutic effect of SK activation in SCA pathology may be explained by the ability of SK positive modulators to convert highly irregular PC activity into a regular tonic mode (Fig. 2). The bursting activity (Fig. 2B, left) is an extreme case of the electrophysiological irregularity in PC pacemaking that occurs more frequently in aging mice. Generally, in mutant mice, tonic PCs generate activity less regularly than in their WT littermates [13, 22, 23]. In our experiments on the cerebellar slice recordings [21], urethane-anaesthetized mice [22], and head-fixed awake mice [23], we have demonstrated that the application of SK activators significantly improves the regularity of PC firing in SCA2 pathology (Fig. 2C). The normalization of electrophysiological activity pattern may be responsible for the improvement in the motor skills and PC morphology observed in our SCA2-58Q mice during the SK activator trials [21, 23].

Conclusion

The disturbed regularity of the cerebellar Purkinje cell activity most likely causes the motor symptoms in ataxias. The SK channel activators (NS309, CHZ) normalize the Purkinje cell activity in SCA2-58Q transgenic mice both *in vitro* and *in vivo*. Long-term injections of CHZ improve the electrophysiological features of cerebellar Purkinje cells and also recover the impaired motor functions in SCA2-58Q mice. Thus, the SK channel activators can be used as a potential way to treat SCA2 and other diseases accompanied by the cerebellar degeneration.

Acknowledgements IB is a holder of the Carl J. and Hortense M. Thomsen Chair in Alzheimer's Disease Research.

Funding This research was done by Peter the Great St. Petersburg Polytechnic University and supported under the strategic academic leadership program "Priority 2030" of the Russian Federation (Agreement 75-15-2021-1333 30.09.2021 to SPbPU) and by the National Institutes of Health grant R01NS056224 (IB).

Declarations

Conflict of Interest The authors declare no competing interests.

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