Loss-of-function BK channel mutation causes impaired mitochondria and progressive cerebellar ataxia

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Despite a growing number of ion channel genes implicated in hereditary ataxia, it remains unclear how ion channel mutations lead to loss-of-function or death of cerebellar neurons. Mutations in the gene KCNM1, encoding the α-subunit of the BK channel have emerged as responsible for a variety of neurological phenotypes. We describe a mutation (BK\textsubscript{G354S}) in KCNM1, in a child with congenital and progressive cerebellar ataxia with cognitive impairment. The mutation in the BK channel selectivity filter dramatically reduced single-channel conductance and ion selectivity. The BK\textsubscript{G354S} channel trafficked normally to plasma, nuclear, and mitochondrial membranes, but caused reduced neurite outgrowth, cell viability, and mitochondrial content. Small interfering RNA (siRNA) knockdown of endogenous BK channels had similar effects. The BK activator, NS1619, rescued BK\textsubscript{G354S} cells but not siRNA-treated knockdown cells, by selectively blocking the mutant channels. When expressed in cerebellum via adenoassociated virus (AAV) viral transfection in mice, the mutant BK\textsubscript{G354S} channel, but not the BK\textsubscript{WT} channel, caused progressive impairment of several gait parameters consistent with cerebellar dysfunction from 40- to 80-d-old mice. Finally, treatment of the patient with chlorozoxazone, a BK/SK channel activator, partially improved motor function, but ataxia continued to progress. These studies indicate that a loss-of-function BK channel mutation causes ataxia and acts by reducing mitochondrial and subsequently cellular viability.

KCNM1 | ataxia | cerebellar degeneration

M utations in a wide array of ion channel genes underlie distinct forms of neurological disease. Prominent among these are the spino cerebellar ataxias (SCAs) that are a group of genetically diverse neurodegenerative diseases affecting the cerebellum and its connections. Approximately one-third of the nearly 30 genetically identified forms of autosomal-dominant cerebellar ataxia are due to mutations in ion channel genes, including genes for four distinct voltage-gated potassium channels (\(K_{\text{v}}\)) (1–11). Missense mutations of the KCN\textsubscript{F} (\(K_{\text{v}1.1}\)), KCNC\textsubscript{1} (\(K_{\text{v}3.1}\)), KCNC\textsubscript{3} (\(K_{\text{v}3.3}\)), and KCND\textsubscript{3} (\(K_{\text{v}4.3}\)) channel genes underlie episodic or progressive ataxia syndromes, and some of them are associated with cognitive impairment and/or epilepsy (1, 3–7). The human KCNM1 gene encodes the large-conductance, calcium- and voltage-activated \(K^{+}\) (BK) channel α-subunit. The BK pore-forming α-subunit contains seven transmembrane segments (S0 to S6) with an extracellular N terminus and an extensive intracellular C terminus containing two high-affinity Ca\textsuperscript{2+} binding sites (12). BK channels are activated by both membrane depolarization and cytosolic Ca\textsuperscript{2+}. Mutations in the BK channel lead to a variety of clinical syndromes, correlated in part to the molecular phenotype, roughly divided into gain-of-function (GOF) and loss-of-function (LOF) (13). Du et al. identified a single missense mutation (D434G) in KCNM1 that leads to a GOF change in BK channel and causes autosomal-dominant epilepsy with paroxysmal dyskinesias in a large kindred (14). Several individual patients have been reported with a KCNM1 variant predicting a point mutation (variably designated as N995S/N999S/N1053S) in the BK channel associated with epilepsy without dyskinesia. Interestingly, whereas the GOF phenotype of the D434G mutant is due to an increase in the BK Ca\textsuperscript{2+} sensitivity, in the N995S mutant the BK GOF is due to a leftward shift of the conductance–voltage (\(G-V\)) curve. LOF mutations, which reduce either channel current or protein expression, are more consistently associated with cerebellar involvement and intellectual disability (ID), with variable presence of seizures, dyskinesias, and dystonia (13, 15). Moreover, mice lacking the BK channel developed cerebellar ataxia and Purkinje cell dysfunction (15). More recently, several rare KCNM1 mutations producing an array of pathological phenotypes were reported (13, 16). However, other than the common theme of disturbed ion channel function, there is as yet little insight as to how the reported KCNM1

Significance

Genetic disruption of ion channels underlies several neurological diseases, suggesting that ionic disturbances are common neuronal stressors potentially amenable to therapies. The detailed intracellular pathways coupling ion channel mutations to neuronal damage are largely unknown. Here, we describe the finding of a single loss-of-function mutation in the BK channel in a young patient with progressive cerebellar degeneration. The mutant BK channel caused a profound dominant-negative effect on native channels, combined with reduced ion selectivity, leading to depolarization and depletion of mitochondria, and when delivered virally to mice, mimicked the disease. BK channel active drugs rescued the mutant cellular phenotype. These results point to the importance of mitochondrial ionic homeostasis in cerebellar disease and suggest therapeutic strategies.


The authors declare no competing interest.

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mutations lead to neurological diseases (17–19). Here, we describe a LOF mutation, identified by exome sequencing, in the KCNMA1 gene encoding BK channel α-subunit (BKG354S) associated with progressive cerebellar degeneration, ataxia, and cognitive impairment due to cellular toxicity and dysfunction/depletion of mitochondria.

We evaluated a 16-year-old girl who presented with cognitive impairment, multifocal dyskinesias, and progressive ataxia. Using exome sequencing, we identified a de novo mutation in the KCNMA1 gene predicting the mutation G354S in BK channels. The glycine residue 354 is in the BK selectivity filter, a highly conserved amino acid sequence among K⁺-selective ion channels. BK channels composed of the G354S mutant α-subunits (BKG354S) exhibit a dramatically reduced unitary conductance relative to BK channels bearing wild-type (WT) α-subunits (BKWT) and proportional reduction in the macromolecular K⁺ currents, suggesting no changes in protein abundance. Importantly, the mutation also reduces BK channel selectivity to K⁺ ions, increasing the relative permeability to Na⁺ ions. Expression of BKG354S also led to reductions in neurite outgrowth and cellular viability, together with depletion of mitochondria when expressed in cultured neuronal cells. Notably, application of the BK channel activator NS1619 (20–22) activates the WT channels, while inhibiting the mutant BK channels, to improve viability and mitochondrial content in cultured cells. Viral delivery to neonatal mice of the BKG354S channel, but not the BKWT channel, caused impaired gait. Clinically, treatment of the patient with chlorzoxazone, a pharmaceutical SK/BK channel activator (23), slightly improved motor coordination scores and balance, although the ataxia continued to progress. This study describes an association of progressive ataxia with a mutation in the BK channel and supports the view that BK channel function, particularly in mitochondria, is critical for neuronal viability and is a potential mechanism for Purkinje cell degeneration and cerebellar ataxia.

Results

Clinical Phenotype and Identification of the BK Channel G354S Mutation. The patient, a 16-year-old female, was noted to have ataxic gait and delayed motor milestones at the age of 18 mo. Clinical features are summarized in Table 1 and compared with those bearing previously reported KCNMA1 mutations (14, 17–19, 24). She had two febrile seizures between 3 and 5 y of age. She was developmentally delayed, estimated to read at a first-grade level, and exhibited emotional outbursts. Her language comprehension was slightly subnormal, but she had an unintelligible speech due to severe dysarthria. Her motor coordination initially improved but lagged behind that of her younger sister, until at 8 y of age when she began to have slow deterioration in her gait and upper limb coordination. She developed scoliosis at age 14, but only required conservative management. At the age of 16, her neurological examination revealed slight difficulties with comprehension and following conversations, but good orientation and memory. She also showed coarse downbeat and gaze-evoked nystagmus in all directions; perioral, truncal, and limb dyskinesias; slightly spastic tone with bilateral Babinski signs; frank anarthria, intention tremor, past pointing, and dystadiokinesia in her limbs; and severely wide-based and unsteady stance and gait. Her biological parents and younger sister are alive and doing well with no neurological complaints. Serial MRI scans from age 7 to 15 revealed severe and progressive cerebellar atrophy, most prominent in the midline, and no other abnormalities of cerebral hemispheres (Fig. 1A). The morphometric analysis estimated a 20% loss of cerebellar volume over 8 y.

Whole-exome sequencing (WES), followed by targeted di-deoxy sequencing of proband, parents, and unaffected sister, confirmed four heterozygous de novo variants in the proband (SI Appendix, Table S1). The mutation most likely to be associated with the patient’s symptoms was a 1060 G>A mutation in exon 8 in the KCNMA1 gene, which encodes the BK channel, suggesting a G354S amino acid change in this protein (Fig. 1B).

This mutation was not present in any database, including the Exome Aggregation Consortium (ExAC) and gnomAD databases of aggregated WES data, or in 765 exomes sequenced at University of Chicago, including 322 other patients with ataxia. The residue G354 is conserved in the sequence of amino acids that forms the selectivity filter of K⁺ channels, 352-TVGGYG-356. This motif is conserved in BK channels identified at least as far back as Drosophila (Fig. 2). In silico genetic prediction algorithms, such as SIFT and PolyPhen-2, that use sequence- and structure-based features and evolutionary conservation of amino acids predicted this substitution to be pathogenic (25, 26).

Of the three other de novo variants identified in WES, a variant in SLC22A5 suggests that the patient is also a possible carrier of carnitine deficiency (27) (SI Appendix, Table S2), while the other two DYFS and BRAT1 are associated with recessively inherited syndromes (28, 29) and have been seen in the normal population (https://gnomad.broadinstitute.org/).

Table 1. Summary of clinical features in proband compared with subjects with other reported KCNMA1 mutations

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<tr>
<th>Symptoms</th>
<th>LOF mutations</th>
<th>GDP mutations</th>
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<tr>
<td>Zygosity</td>
<td>Het</td>
<td>Het</td>
</tr>
<tr>
<td>Age of onset</td>
<td>18 mo</td>
<td>20 d</td>
</tr>
<tr>
<td>Ataxia</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Seizure type</td>
<td>Fabile</td>
<td>Abs/GTC</td>
</tr>
<tr>
<td>Dyskinesias/Dystonia</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Nystagmus</td>
<td>+</td>
<td>++</td>
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<tr>
<td>OHD</td>
<td>NA</td>
<td>++</td>
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<tr>
<td>Cerebellar atrophy</td>
<td>+</td>
<td>++</td>
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<tr>
<td>Developmental anomalies</td>
<td>Facial +</td>
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— indicates absent; + indicates present; and ++ indicates moderate to severe; abs, absent; DHD, developmental delay/intellectual disability; Gen, generalized; Het, heterozygous; Homo, homozygous; Inf, infancy; NA, not available.
KCNMA1 mutation on the chromosome 10. (patient, sister, and parents. Altogether, these data strongly suggest the BKWT and BKG354S, we recorded robust macroscopic K currents from BKG354S in oocyte's macropatches. Voltage clamping of macropatches of membranes expressing BKG354S also showed reduced currents compared to a comparable experiment with membranes expressing BKWT. Since the activation of BK channels is controlled by voltage as well as by intracellular concentration of Ca++, very low intracellular Ca++ concentration in cut-open oocyte experiments results in channel activation at very positive voltages (with V/2 around +170 mV), well beyond the efficient range in the cut-open oocyte preparation. Experiments with macropatches are appropriate for a wider range of voltages and have the additional advantage that Ca++ concentration can be controlled both inside and outside the membrane (Fig. 3 A and B). Channel activation in macropatches over a voltage range between −100 to +250 mV using an intracellular Ca++ concentration of about 40 nM revealed a leftward shift in the voltage-activation curves in macropatches expressing BKG354S compared with BKWT. However, at a 100 μM internal Ca++ concentration, both mutant and BKWT have similar voltage dependence and half-activation voltages (Fig. 3C). Both the BKWT and BKG354S are sensitive to Ca++, however, the smaller Ca++-induced leftward shift of the G/V in the current suggests that its apparent Ca++ sensitivity is less than that of the WT BK, further exacerbating the LOF phenotype. Noise analysis of the K+ conductance revealed a single-channel conductance of only 13.2 ± 1.5 pS (n = 5), a dramatic decrease compared to the single-channel conductance of around 180 to 200 pS from BKWT in symmetrical 110 mM K+ (Fig. 3D). Notably, the 13 pS we found for K+ conductance of BKG354S is 6.5% of that of BKWT (203 pS), which is a value very close to the 5.5% estimated with macroscopic currents from the cut-open oocytes experiments (SI Appendix, Fig. S1).

We also compared the K+ selectivity of BKG354S with BKWT by recording voltage-activated currents in symmetrical condition (140 mM K+ in both sides of the membrane) and under biionic condition (140 mM Na+ in the intracellular side and 140 mM K+ in the extracellular side of the membrane). The reversal potential was zero in symmetrical condition for both BKWT and BKG354S (Fig. 4 A and B, upper traces, and Fig. 4 C and D, K+ on the inside). Under biionic condition, the reversal potential was undetermined for BKWT (greater than +100 mV [n = 3]) and +40 ± 3 mV (n = 3) for BKG354S (Fig. 4 A and B, lower traces, and Fig. 4 C and D, K+ on the inside). These data confirm previous results showing no measurable permeation can be detected for Na+ in the case of the BKWT. From the reversal potential for the BKG354S channel in biionic condition, we calculated a PK+:PNa+ ratio of 4.8 (Fig. 4; Materials and Methods). This result indicates a considerable loss of K+ selectivity in BKG354S, and it agrees with the loss of selectivity caused by the analog mutation in the Shaker K+ channel selectivity filter (31).

BKWT: BKWT-G354S. In all three cases (BKWT, BKWT-G354S, and BKG354S), we recorded robust macroscopic K+ currents consistent with different levels of conductance (SI Appendix, Fig. S1A). Oocytes injected with 50 ng of cRNA for BKG354S showed on average (n = 10) 11% of the maximal current compared with oocytes injected with only 25 ng of cRNA for BKWT. Considering that oocytes can proportionally express channels according to the amount of cRNA injected, in the range between 25 and 50 ng, we could speculate that oocytes injected with 25 ng would express one-half of the number of channels than those injected with 50 ng. Therefore, the 11% translates to 5.5% when normalized by the amount of cRNA injected. The oocytes injected with both types of cRNA, WT and mutant, 25 ng each, served to prove the dominant-negative effect of BKG354S on heterochannels, the BKWT-G354S. If the oocytes were to express both channels equally but independently, without forming heterochannels, the level of current recorded from these oocytes would be at least the same as in those oocytes expressing BKWT only. Surprisingly, the oocytes injected with 25 ng of each cRNA for BKWT and BKG354S showed 45% of the currents from the BKWT group of oocytes (SI Appendix, Fig. S1B). We also analyzed the kinetics of the K+ current activation. The currents from BKG354S were activated up to two orders of magnitude slower than the currents from BKWT. Interestingly, the time constants from BKWT-G354S were only two to six times slower than the currents from BKWT (SI Appendix, Fig. S1C). Altogether, these data strongly suggest the BKWT and BKG354S coassemble to form heterochannels and that the BKG354S channels exert a dominant-negative effect on BKWT-G354S heteromers.
macroscopic currents is mainly due to the decrease in single-channel conductance and not to reduced channel expression. Moreover, our data from BKWT and BKGS45 channels expressed together in oocytes suggest that BKGS45 protein exerts a dominant-negative effect when coassembled with BKWT in the same hetero-channel (SI Appendix, Fig. S1).

NS1619 is a synthetic benzimidazole derivative, and it is a specific BK channel activator in the micromolar range (22). The compound produces a reversible dose-dependent leftward shift antagonized by the scorpion toxin charybdotoxin. Gesner et al. (32) proposed that NS1619 activates BK channels by interacting with the S6/RCK1 linker. Fig. 6 A–C shows that NS1619, as reported previously, robustly activates BKWT in HEK cells. Conversely, we found that the NS1619 behaves as a potent antagonist of the BKGS45 mutant channel (Fig. 6 D–F).

**The BKGS45 Mutation Reduces Neurite Outgrowth.** To investigate the effect of the BKGS45 mutation on differentiation in cultured mammalian cells, we transfected BKWT or BKGS45 into PC12 cells, which undergo differentiation into neuronal phenotype in response to nerve growth factor (NGF). Upon induction with NGF for 24 and 48 h, we observed a significantly reduced total neurite length in cells expressing BKGS45 channel compared to BKWT in the same hetero-(hetero- (SI Appendix, Fig. S1).

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in both untransfected cells and cells transfected with BK_{G354S} and BK_{WT} constructs (Fig. 8 B and C).

To characterize the subcellular distribution of the BK_{G354S} and BK_{WT} channels morphologically, we examined HEK and PC12 cells transiently transfected with BK_{WT} or BK_{G354S} channels by immunofluorescence labeling using BK channel-specific antibodies. HEK cell transiently expressing either BK channel displayed diffuse membrane fluorescence with no pattern difference between BK_{G354S} or BK_{WT} channels (Fig. 8H). Colocalization analysis using mitochondrial probe MitoTracker Red also showed that both BK_{WT} and BK_{G354S} channels are expressed in mitochondria, although, as described below, at different levels (Fig. 8H).

**The BK_{G354S} Mutation Causes Selective Toxicity to Mitochondria.** We noticed that the intensity of the 100 kDa band in the mitochondria was consistently lower in cells expressing the BK_{G354S} compared with BK_{WT} when normalized to cellular GAPDH protein (Fig. 8C). To investigate whether this reduction was due to selective loss of BK channels in mitochondria, we compared the signal for the mitochondrial voltage-dependent anion channel (VDAC), as a marker for the mitochondrial compartment. The signal for VDAC was also consistently reduced by 50% relative to BKWT when normalized to cellular GAPDH (Fig. 8B). To compare the subcellular distribution of BK_{G354S} and BK_{WT} in mitochondria, we transiently transfected HEK cells, because of their superior resolution of subcellular compartments. We stained cells expressing BK_{WT} or BK_{G354S} with Tomm20 for mitochondria (red) and immunolabeled BK channels with anti-BK channel antibody (green). Fig. 8 H and I shows that the intensity of mitochondrial labeling was decreased in BK_{G354S}-expressing cells compared with BK_{WT}-expressing cells. Colocalization of Tomm20 and BK channel antibody also revealed a significantly lower immunolabeling signal in BK_{G354S} than that in BK_{WT} (Fig. 8 H and I).

Furthermore, silencing of BK channels using siRNA KCNMA1 led to a reduction of the mitochondrial labeling intensity for Tomm20 compared with siRNA Control (SI Appendix, Fig. S2 E and F).

Finally, to test whether the reduced mitochondrial content was related to BK channel affected by the presence of BK_{G354S} subunit(s), we also treated cells transiently expressing BK_{WT} or BK_{G354S} channels with NS1619, known to improve neurite outgrowth and protect from cell death in cells expressing BK_{G354S} channels. Fig. 8D shows that pretreatment of HEK cells expressing BK_{G354S} channels with NS1619 protected the reduction of mitochondrial VDAC protein. However, treatment with NS1619 could not protect cells and prevent reduction of mitochondrial content in cells treated with KCNM1-specific siRNA (Fig. 8 D and E and SI Appendix, Fig. S2 B and C). This suggests that mutation in BK_{G354S} specifically affects channel function, and a drug acting as NS1619 might be protective in some ataxia patients.
Mitochondria Ultrastructure Is Disrupted by the BK<sub>G354S</sub> Mutation. We performed electron-microscopic (EM) image analysis of mitochondrial content and ultrastructure in stably expressing BK<sub>WT</sub> and BK<sub>G354S</sub> cells (Fig. 9 A–C). Mitochondria in BK<sub>G354S</sub> expressing cells are fewer in number (Fig. 9C) and have abnormal-appearing cristae compared with BK<sub>WT</sub> and pcDNA3-expressing cells (Fig. 9B). Quantification reveals a 58.89 ± 4.62% reduction in mitochondrial number in BK<sub>G354S</sub>-expressing cells compared with BK<sub>WT</sub> and pcDNA3-expressing cells. In addition, 44.36 ± 4.78% of mitochondria have distended and disordered cristae in BK<sub>G354S</sub> stable expressing cells (Fig. 9C).

Mitochondria Dynamics Change and Potential Decreased in Live BK<sub>G354S</sub> Mutation Cells. To observe dynamic changes of mitochondrial fusion and fission affected by the BK<sub>G354S</sub> mutation, we used MitoTracker Red, to stain live PC12 cells stably expressing BK<sub>WT</sub>, BK<sub>G354S</sub>, or pcDNA3. Live-cell imaging indicated grossly reduced content of mitochondria in cells expressing the BK<sub>G354S</sub> mutation (Movies S5 and S6) compared with cells expressing BK<sub>WT</sub> and control (Movies S1–S4). Elongated and interconnected fusion mitochondrial forms are predominant in cells expressing BK<sub>WT</sub> and control. In contrast, fragmented fission forms are more prevalent in cells expressing the BK<sub>G354S</sub> mutation.

To investigate whether the BK<sub>G354S</sub> affects mitochondrial membrane potential in addition to its effect on mitochondrial fusion and fission, we used fluorescent probes TMRM and MitoTracker green to monitor the mitochondrial membrane potential for each case. In mouse intracerebroventricularly at postnatal day 0 (P0) (33). Mice tolerated the injections well and developed normally with no visible neurological deficits initially. Analysis of the histological features of the cerebellar cortex shows no obvious difference between mice injected with BK<sub>G354S</sub> vs. BK<sub>WT</sub> channel, a finding similar to that of BK knockout mice. To investigate whether the mutant BK channel expression affects motor function, AAV9- engineered adenoassociated virus 9 (AAV9)-based expression vectors to deliver BK<sub>WT</sub> or mutant BK<sub>G354S</sub> channels to neonatal mice. We injected 2 μL at 1 × 10<sup>12</sup> vg/μL of AAV9-BK<sub>WT</sub> or AAV9-BK<sub>G354S</sub> into mouse intracerebroventricularly at postnatal day 0 (P0) (33). Mice tolerated the injections well and developed normally with no visible neurological deficits initially. Analysis of the histological features of the cerebellar cortex shows no obvious difference between mice injected with BK<sub>G354S</sub> vs. BK<sub>WT</sub> channel, a finding similar to that of BK knockout mice. To investigate whether the mutant BK channel expression affects motor function, AAV9-BK<sub>WT</sub>- or AAV9-BK<sub>G354S</sub>-treated mice were studied on a motorized treadmill equipped with a high-speed digital camera (DigiGait) (Mouse Specifics) (34) at 40 and 80 d of age to quantitatively assess gait and limb movement parameters, for both left and right sides and both forelimb and hindlimb. After post hoc correction, significant abnormalities of motor temporal and spatial parameters, either unilaterally or bilaterally, were seen at P40 in 42% and at P80 in 55% of parameters. From these parameters, an ataxia coefficient is calculated by DigiGait software. The coefficient of ataxia increased from 11.45 ± 1.4% and 8.7 ± 2.4% to 40.78 ± 2.3% and 52.36 ± 3.3% for both left and right hindlimbs, respectively, in AAV9-BK<sub>G354S</sub> mice from 40 to 80 d of age (SI Appendix, Fig. S3). SI Appendix, Fig. S3 displays representative plots gait parameters that were significantly different and impaired in the mice receiving BK<sub>G354S</sub> compared to those injected with BK<sub>WT</sub>. These findings indicate that impaired gating of the BK<sub>G354S</sub> mutation is directly responsible for depolarized or dysfunctional mitochondria.

BK<sub>G354S</sub> Causes Ataxia in Mice. Because mice with targeted deletion of the BK channel α-subunit develop ataxia, we hypothesized that the LOF BK channel mutation acts in a similar fashion. To test for a direct effect of BK<sub>G354S</sub> on cerebellar function, we engineered adenoassociated virus 9 (AAV9)-based expression vectors to deliver BK<sub>WT</sub> or mutant BK<sub>G354S</sub> channels to neonatal mice. We injected 2 μL at 1 × 10<sup>12</sup> vg/μL of AAV9-BK<sub>WT</sub> or AAV9-BK<sub>G354S</sub> into mouse intracerebroventricularly at postnatal day 0 (P0) (33). Mice tolerated the injections well and developed normally with no visible neurological deficits initially. Analysis of the histological features of the cerebellar cortex shows no obvious difference between mice injected with BK<sub>G354S</sub> vs. BK<sub>WT</sub> channel, a finding similar to that of BK knockout mice. To investigate whether the mutant BK channel expression affects motor function, AAV9-BK<sub>WT</sub>- or AAV9-BK<sub>G354S</sub>-treated mice were studied on a motorized treadmill equipped with a high-speed digital camera (DigiGait) (Mouse Specifics) (34) at 40 and 80 d of age to quantitatively assess gait and limb movement parameters, for both left and right sides and both forelimb and hindlimb. After post hoc correction, significant abnormalities of motor temporal and spatial parameters, either unilaterally or bilaterally, were seen at P40 in 42% and at P80 in 55% of parameters. From these parameters, an ataxia coefficient is calculated by DigiGait software. The coefficient of ataxia increased from 11.45 ± 1.4% and 8.7 ± 2.4% to 40.78 ± 2.3% and 52.36 ± 3.3% for both left and right hindlimbs, respectively, in AAV9-BK<sub>G354S</sub> mice from 40 to 80 d of age (SI Appendix, Fig. S3). SI Appendix, Fig. S3 displays representative plots gait parameters that were significantly different and impaired in the mice receiving BK<sub>G354S</sub> compared to those injected with BK<sub>WT</sub>. These findings
Representative nonstationary noise analysis of G354S mutant macroscopic currents. Currents were evoked using a prepulse of −80 mV from a prepulse of −80 in 10-mV steps returning to −80 mV. The holding potential was 0 mV. (B) BK_WT macroscopic currents elicited by voltage pulses from 0 to 330 mV in 10-mV steps, returning to 60 mV. The stimulation protocol consisted of a prepulse of −80 mV, followed by voltage pulses in the range of 0 to 330 mV with increments every 10 mV, returning to 60 mV. (C) Normalized tail currents (I_{tail}/I_{tail,max}) vs. voltage data. The solid lines are the best fit to the data using Eq. 2. (SI Appendix, SI Materials and Methods). For the BK_WT channels, parameters were as follows (mean ± SEM; n = 3 [red]): V_{1/2} = 185 ± 16 mV, z = 0.88 ± 0.02; BK_WT G354S, n = 7. For the BK_G354S, parameters were as follows (mean ± SEM; n = 7 [blue]): V_{1/2} = 134 ± 16 mV, z = 0.84 ± 0.03. All data were obtained in the nominal absence of Ca^{2+}. (D) Representative nonstationary noise analysis of BK_WT macroscopic currents. The patch was held at −80 mV and pulsed 200 times at 200 mV taken in the tail. The stimulation protocol consisted of a prepulse of −80 mV, followed by 200 pulses of 200 mV, returning to −20 mV. Noise analysis was done using the tail currents. Holding potential was 0 mV. The patch had 6,110 active channels with an average single-channel conductance of 172 pS. The average single-channel conductance was 170 ± 3 pS (n = 7). (E) Representative nonstationary noise analysis of G354S mutant macroscopic currents. Currents were evoked using a prepulse of −80 mV, followed by 200 pulses of 130 mV, returning to 60 mV. Noise analysis performed using the K^+ currents elicited by the 130-mV pulses. The patch had 2,180 active channels with an average single-channel conductance of 9 pS. The average single-channel conductance was 10 ± 2 pS (n = 5).

Fig. 5. Characterization of BK_WT and BK_G354S ionic currents in HEK cells. (A) Macroscopic K^+ currents in an inside-out membrane patch expressing BK_WT channels. BK_G354S currents were evoked by voltage steps from −120 to 350 mV from a prepulse of −80 in 10-mV steps returning to −80 mV. The holding potential was 0 mV. (B) BK_WT macroscopic currents elicited by voltage pulses from 0 to 330 mV in 10-mV steps, returning to 60 mV. The stimulation protocol consisted of a prepulse of −80 mV, followed by voltage pulses in the range of 0 to 330 mV with increments every 10 mV, returning to 60 mV. (C) Normalized tail currents (I_{tail}/I_{tail,max}) vs. voltage data. The solid lines are the best fit to the data using Eq. 2. (SI Appendix, SI Materials and Methods). For the BK_WT channels, parameters were as follows (mean ± SEM; n = 3 [red]): V_{1/2} = 185 ± 16 mV, z = 0.88 ± 0.02; BK_WT G354S, n = 7. For the BK_G354S, parameters were as follows (mean ± SEM; n = 7 [blue]): V_{1/2} = 134 ± 16 mV, z = 0.84 ± 0.03. All data were obtained in the nominal absence of Ca^{2+}. (D) Representative nonstationary noise analysis of BK_WT macroscopic currents. The patch was held at −80 mV and pulsed 200 times at 200 mV taken in the tail. The stimulation protocol consisted of a prepulse of −80 mV, followed by 200 pulses of 200 mV, returning to −20 mV. Noise analysis was done using the tail currents. Holding potential was 0 mV. The patch had 6,110 active channels with an average single-channel conductance of 172 pS. The average single-channel conductance was 170 ± 3 pS (n = 7). (E) Representative nonstationary noise analysis of G354S mutant macroscopic currents. Currents were evoked using a prepulse of −80 mV, followed by 200 pulses of 130 mV, returning to 60 mV. Noise analysis performed using the K^+ currents elicited by the 130-mV pulses. The patch had 2,180 active channels with an average single-channel conductance of 9 pS. The average single-channel conductance was 10 ± 2 pS (n = 5).

Directly implicate the role of this mutant channel in the clinical manifestations of ataxia in these mice.

**SK/BK Channel Activator Improves Coordination in the Patient.** Potassium channel-activating drugs have recently shown some benefit in treating ataxia (35, 36). Presently, there are not pure SK/BK channel activators approved for use in patients by the Food and Drug Administration. Chlorzoxazone is a mixed SK/BK channel activator marketed under the name Parafon Forte. We assessed the effects of this drug on motor performance in this patient. The patient relies only on proprioceptive and vestibular cues. Unfortunately, follow-up after 6 mo on the medication revealed continued disease progression. Our results suggest that activation of endogenous SK and BK channels in these BK mutant patients may provide symptomatic improvement. Continued disease progression in this case may indicate that chlorzoxazone has no effect on blocking the mutant BK_G354S channel (SI Appendix, Fig. S4).

**Discussion**

The diversity of genetic and mutational defects identified for SCAs suggests that multiple pathophysiological mechanisms lead to a common clinical and pathological phenotype. The numerous ion channel mutations implicated in the ataxias indicate that disturbances of intracellular ion milieu might be a frequent pathophysiological mechanism affecting Purkinje cells (1–4, 6, 38–41). However, the pathogenic mechanisms coupling disturbed membrane ionic flux to pathological changes at the cellular and subcellular level remain to be elucidated. Previous studies have shown that expression of mutant KCND3-encoded channels (K_V4.3) from SCA19 ataxia patients in cultured Purkinje cells reduces viability and dendritic outgrowth (42). In the present study, we demonstrate that a spontaneously occurring mutation in KCNMA1-encoded channels (BK), predicting an amino acid change in the selectivity filter of the channel, leads to the LOF of the BK channel phenotype with severely reduced channel conductance with loss of K^+ selectivity. In addition, the patient affected by this mutation presents a syndrome of progressive cerebellar ataxia, cognitive impairment, and dyskinesia. At least seven other reported KCNMA1 LOF mutations are associated
with cerebellar impairment, and two others with cognitive impairment (13). Moreover, AAV-mediated delivery of the BKG354S channel, but not the BKWT channel, produced ataxic gait in mice. Genetic ablation of the KCNMA1 also led to ataxia in mice. Together, these observations indicate a strong dependency of cerebellar function on normal BK channel activity.

BK channels are similar to other voltage-gated potassium channels in that they contain a highly conserved 352-TVGYG-356 sequence in the pore-forming domain known as the selectivity filter (Fig. 3), precisely the moiety where the G354S mutation is predicted in the present patient. It is instructive that other spontaneous mutations in the BK channel lead to enhanced rather than reduced BK channel activity and have a distinct clinical picture. D434G (BK_D434G), within the RCK domain, is a GOF mutation yielding a channel that is more sensitive to Ca2+ and that opens faster and at more negative voltages (14). N995S (BK_N995S) is a GOF mutation reported in three individuals causing a leftward shift of the conductance–voltage (G–V) curve. The GOF mutations are not associated with ataxia or cerebellar atrophy, but instead of macroscopic K+ currents recorded from these cells showed evidence of heterotetramerization. First, BKWT expression, as measured by the amplitude of the macroscopic K+ currents, was affected by the NS1619 (light red; n = 3). (D) BKG354S macroscopic currents. The voltage protocol consisted of a prepulse of −80 mV, followed by voltage pulses in the range of −80 to 230 mV with increments every 10 mV, returning to 60 mV. The holding potential was 0 mV. BKWT DNA transfected 250 ng/μL. BKG354S DNA transfected 450 ng/μL.}

Fig. 6. Effect of NS1619 on BKWT and BKG354S macroscopic currents. (A) BKWT macroscopic currents. The voltage protocol consisted of a prepulse of −80 mV, followed by voltage pulses in the range of −80 to 250 mV with increments every 10 mV, returning to −80 mV. The holding potential was 0 mV. (B) BKWT macroscopic currents in the presence of NS1619 added to a final concentration of 10 μM. The stimulation protocol consisted of a prepulse of −80 mV, followed by voltage pulses in the range of −80 to 250 mV with increments every 10 mV, returning to −80 mV. The holding potential was 0 mV. BKWT DNA transfected 250 ng/μL. (C) BKWT currents vs. voltage (I/V) curve (red) and BKWT currents vs. voltage (I/V) curve in the presence of 10 μM NS1619 (light red; n = 3). (D) BKG354S macroscopic currents. The voltage protocol consisted of a prepulse of −80 mV, followed by voltage pulses in the range of −80 to 230 mV with increments every 10 mV, returning to 60 mV. The holding potential was 0 mV. (E) BKG354S macroscopic currents in the presence of 10 μM NS1619 added to a final concentration of 10 μM NS1619. (F) BKG354S current vs. voltage (blue) and BKG354S current vs. voltage curve in the presence of 10 μM NS1619 (light blue). The voltage protocol consisted of a prepulse of −80 mV, followed by voltage pulses in the range of −80 to 230 mV with increments every 10 mV, returning to 60 mV. BKG354S DNA transfected 450 ng/μL.

A full assessment of the probable heterotetramerization of BKWT and BKG354S subunits in the same channel is beyond the scope of this study. In our preliminary results, unitary currents studied by both single-channel patch clamp and by noise analysis of macroscopic currents in membranes from oocytes injected previously with cRNA for both types of proteins, never showed intermediary conductance. On the other hand, macroscopic K+ currents, was affected by the likely coexpression, in the same oocyte, of BKG354S, suggesting dominant-negative effect and in agreement with the mitochondrial studies. Second, the time constants of the putative BKWT-G354S activation kinetics sit in between the BKWT and BKG354S values. Third, the voltage dependence of the activation kinetics resembles that of pure BKG354S channels: They are virtually voltage independent (SI Appendix, Fig. S1).

An important aspect of this BKG354S channel phenotype is that it conducts Na+ with the permeability of ~20% of the K+ permeability. This BKG354S channel Na+ permeability may play an important role in the clinicopathological phenotype since during neuronal activity BK channels normally act by damping excitatory processes mediated by an increase in internal Ca2+. In other words, the increased Na+ permeability may be as significant a deleterious property of this mutant channel as the dramatic depression of the K+ conductance. The mutant channel therefore should conduct Na+ inwardly when activated, producing longer periods of depolarization. Under physiological intracellular and extracellular concentrations of Na+ and K+, the current carried by these mutant channels has a reversal potential near −40 mV, and therefore the BKG354S effect on the membrane potential is toward keeping it near −40 mV, most likely above the threshold voltage for action potential generation by neurons. Thus, we hypothesize that when the plasma membrane
PKG354S channel is activated it should act by increasing neurotransmitter release and should not be able to modulate action potential duration adequately.

The unexpected blocking effect of NS1619 on the PKG354S conductance (to K$^+$ and more importantly to Na$^+$ as stated above) can explain the benefit of this BK activator compound (Fig. 6) on the reduced PC12 neurite outgrowth and cell viability (Fig. 7) and on the mitochondrial content, also in PC12 cells (Fig. 8).

On the other hand, the presence of PKG354S mutant channels both in the plasma membrane and the mitochondrial membrane...
would lead to the following: first, an increase in cytoplasmic \( \text{Na}^+ \) concentration during neuronal activity similar to that produced during ischemia (43); and second, as a consequence of the reduction or loss of mitoBK, the cytoprotective effect of the activation of mitoBK is lost (44). Therefore, NS1619 has the double beneficial role of inhibiting the mutant channels reducing the deleterious effect of the mutant channels present in the plasma and mitochondrial membrane and activating the remaining BKWT channels partially recovering the cytoprotective effect of mitoBK channel.

The transient beneficial effect of chlorzoxazone, a mixed SK/BK channel activator, may be attributed to the activation of residual SK channels as well, which may have even been up-regulated in the disease context.

Although the association of other LOF \( K^+ \) channel mutations in the ataxia (1, 3, 6) suggests that a persistent depolarized neuronal state is a common pathological pathway, determining the subcellular sites of action of the toxic effect of these mutations will provide significant insight into the pathogenesis of SCAs. BK channels are widely expressed throughout the central nervous system and are abundant in cerebellar Purkinje cells. Together with SK channels, they play a key role in quickly repolarizing the cell membrane when the intracellular Ca\(^{2+} \) with SK channels, they play a key role in quickly repolarizing the cell membrane when the intracellular Ca\(^{2+} \) stores are depleted (45, 46). Both BK and SK channels are also present in membranes of organelles, for example in the inner mitochondrial membrane (47) and in the inner nuclear membrane (48, 49), where they have similar biophysical and pharmacological properties. In mitochondria, they play a role in calcium retention capacity coupled to the permeability transition pore.

Thus, in the mitochondrial context, the effects of BK\(_{G354S}\) are different. In the present study, BK\(_{G354S}\) confers a toxic effect in cultured cell lines and impairs neurite outgrowth in neuronal precursor cells. We also find that BK\(_{G354S}\) is associated with dysfunctional, disrupted, and depleted mitochondria. Knockdown of the BK channel gene using siRNA replicates these cellular abnormalities. The channel mitoBK, one splicing variant of BK\(_{WT}\), is thought to protect the mitochondrial matrix from Ca\(^{2+}\) overload by depolarizing the inner membrane and therefore by decreasing its polarization voltage, the main driving force for Ca\(^{2+}\) entry (44, 50). The driving force for K\(^+\) is the voltage across the inner membrane (approximately \(-160 \text{ mV} \) negative in the matrix), since K\(^+\) concentration in the cytoplasm and in the mitochondrial matrix are similar. In the case of BK\(_{G354S}\) being present in the inner membrane of mitochondria, coassembled or not with mitoBK, its decreased unitary conductance and its partial Na\(^+\) permeability must be considered. The decreased unitary conductance would disrupt the protective effect of the channel against intracellular Ca\(^{2+}\) increases and mitochondrial Ca\(^{2+}\) buffering, a hypothesis corroborated by our findings in this present work and also by effects of experimentally blockade with paxillin, a specific BK blocker (51). As explained above, the fact that a known BK channel activator such as NS1619 blocks the BK\(_{G354S}\) mutant channel suggests that the improved survival of cells expressing the mutant may result from both actions of the drug, particularly on mitoBK, i.e., blocking the deleterious Na\(^+\) conductance of the mutant channel as well as increasing the activity of BK\(_{WT}\) channels only (Fig. 6) (44).

At present, there are no selective BK activators approved for human use. However, based on these findings, we performed an
empiric therapeutic challenge to explore the effect of a non-specific BK/SK activator, chlorzoxazone, in the proband. The patient was evaluated by semiquantitative clinical and performance measures by blinded investigators, before and after the initiation of a standard therapeutic dose of this muscle relaxant. A statistically significant improvement was noted in the patient, although disease progression continued despite therapy. One explanation for these observations is that the improvement resulted from the effect on the plasma membrane SK and BK channels, while the disease progressed due to a lack of effect of the drug on the altered ion selectivity and on mitochondrial BK channels.

Mice with targeted deletion of BK channel α-subunit (BK−/−) have predominantly a cerebellar phenotype with intention tremor, gait incoordination, and impaired eye-blink conditioning (a classic form of cerebellar motor learning). Surprisingly, these animals show no cerebellar degeneration. We found a similar picture in a mouse model induced by AAV delivery of the BK<sub>G354S</sub> mutation. This is in contrast to two siblings with the more widespread motor system disorder and marked cerebellar atrophy, attributed to homozygous LOF mutations in the BK channel. We speculate that the difference relates to age differences, or the role of compensatory pathways. The present patient has a milder phenotype in which progressive ataxia began at age 8 y, attributed to a BK channel LOF mutation.

Studies of a mouse model for another form of ataxia, SCA1, provided a possible explanation that incorporates an integrated model. In SCA1 mice, induced by genetic overexpression of the ataxin-1 protein bearing an expanded polyglutamine repeat, secondary loss of BK channel expression is accompanied by reduced spontaneous discharges. The discharges reappear during Purkinje cell shrinkage. The authors were able to rescue the cells from shrinkage by pharmacologic activation or overexpression of normal BK channels. They suggest that the shrinkage is a homeostatic adaptation to restore Purkinje cell firing in the absence of BK channels (52). However, again the viability of the mitochondria has not been evaluated in any of these mouse ataxia models.

In summary, our studies identify another genetic cause of progressive ataxia. This LOF mutation in the selectivity filter of the BK channel supports the view that delayed neuronal repolarization due to impaired potassium channel function in the membrane in addition to reduced BK channel function in the mitochondria are potential mechanisms for Purkinje cell degeneration leading to cerebellar ataxia as well as developmental delay.

Fig. 9. Abnormal mitochondrial ultrastructure and decreased mitochondrial potential in HEK cells overexpressing BK<sub>G354S</sub>, which can be rescued by NS1619. (A) Representative EM images show increased abnormal mitochondria in BK<sub>G354S</sub> overexpression cell compared with a population of normal mitochondria in pcDNA3 and BK<sub>WT</sub> overexpression cell. (B) Representative EM images show a diverse pattern of abnormal mitochondrial cristae in BK<sub>G354S</sub> mutation. (C) Quantitation of number of mitochondria per cell and percentage of abnormal cristae (n ≥ 6; *P < 0.05; **P < 0.01; ***P < 0.001). (D and E) HEK cells overexpressing BK<sub>WT</sub> or BK<sub>G354S</sub> are stained with MitoTracker green in green and TMRM in red. Representative images of TMRM decreased in BK<sub>G354S</sub> overexpression cell compared with that in pcDNA3 and BK<sub>WT</sub> overexpression cell (D); however, NS1619 treatment was able to recover TMRM expression in BK<sub>G354S</sub> overexpression cell (E). (F) Quantitation of TMRM and MitoTracker integrated intensity in D and E (n ≥ 6; **P < 0.01).
Materials and Methods

The materials and methods are described at length in SI Appendix, SI Materials and Methods. This includes human DNA samples, WES, cell culture, transient transfection, immunofluorescence staining, mitochondrial staining, cell death analysis, preparation of cell membranes and mitochondrial fractionation, antibody, retroviral transduction, live-cell imaging, expression in Xenopus oocytes with electrophysiology, injection of AAV into the ventricle of neonatal WT mice, moving platform posturography, and statistical analysis. Studies using human subjects were carried out with informed consent in accordance with the Institutional Review Board at the University of Chicago.

Data Availability. All DNA plasmids and bacterial strains used in this study are available at Dryad (https://doi.org/10.5061/dryad.1nsimbq). All data generated and analyzed over the course of the current study are included within the manuscript or SI Appendix.

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