

Spinocerebellar ataxia type 6 knockin mice develop a progressive neuronal dysfunction with age-dependent accumulation of mutant Ca_v2.1 channels

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Spinocerebellar ataxia type 6 (SCA6) is a neurodegenerative disorder caused by CAG repeat expansions within the voltage-gated calcium (Ca_v) 2.1 channel gene. It remains controversial whether the mutation exerts neurotoxicity by changing the function of Ca_v2.1 channel or through a gain-of-function mechanism associated with accumulation of the expanded polyglutamine protein. We generated three strains of knockin (KI) mice carrying normal, expanded, or hyperexpanded CAG repeat tracts in the *Cacna1a* locus. The mice expressing hyperexpanded polyglutamine (*Sca6*^{84Q}) developed progressive motor impairment and aggregation of mutant Ca_v2.1 channels. Electrophysiological analysis of cerebellar Purkinje cells revealed similar Ca²⁺ channel current density among the three KI models. Neither voltage sensitivity of activation nor inactivation was altered in the *Sca6*^{84Q} neurons, suggesting that expanded CAG repeat *per se* does not affect the intrinsic electrophysiological properties of the channels. The pathogenesis of SCA6 is apparently linked to an age-dependent process accompanied by accumulation of mutant Ca_v2.1 channels.

cerebellum | polyglutamine | P/Q-type voltage-dependent calcium channel

Spinocerebellar ataxia type 6 (SCA6) is one of nine known dominantly inherited neurodegenerative disorders caused by the expansion of a CAG repeat. In SCA6, the CAG repeat encodes a polyglutamine tract in the Ca_v2.1 voltage-gated calcium channel subunit (*CACNA1A*) (1). The *CACNA1A* gene undergoes alternative splicing such that one splice variant translates the polyglutamine tract from a CAG repeat tract residing in the last coding exon (exon 47). Although Ca_v2.1 is expressed widely in brain, the mutation causes degeneration of cerebellar Purkinje cells (PCs) and inferior olivary neurons (2, 3).

The pathogenesis of polyglutamine diseases is quite complex in that the different proteins have diverse functions, but the diseases are all caused by an apparent gain-of-function pathogenic mechanism (4). Some features unique to SCA6 set it apart from other polyglutamine diseases. First, the disease arises from a relatively small expansion, with as few as 19 repeats (1, 5) compared with other polyglutamine diseases in which 35–300 repeats cause disease. Second, the CAG tract is present in an alternatively spliced exon, whereas in other disorders the repeat is translated in all isoforms.

The Ca_v2.1 subunit encodes P/Q-type voltage-sensitive Ca²⁺ channels, which play a critical role in neurotransmitter release (6) and generation of precise intrinsic pace making in PCs (7). Thus, it is quite logical to anticipate that the CAG repeat expansions would affect this particular function of the channel. Surprisingly, however, the data available so far do not provide conclusive evidence as to whether the small CAG repeat expansions cause disease by altering the function or expression of Ca_v2.1 channel currents (8–11). A major limitation to data interpretation is that all previous studies

have relied on overexpression models in a heterologous system. Thus, it is critical to study the consequences of glutamine-expanded Ca_v2.1 channels when they are expressed in their endogenous neuronal environment at physiologically relevant levels.

To model SCA6 in mice, we used gene targeting to generate three lines (*Sca6*^{14Q}, *Sca6*^{30Q}, and *Sca6*^{84Q}) of knockin (KI) mouse models, carrying either 14 (normal human allele), 30 (expanded SCA6 allele), or 84 (hyperexpanded allele) CAG repeats in the mouse *Cacna1a* locus. Investigating the function of the Ca_v2.1 channel in all three SCA6 KI mice allowed us to gain insight about how posttranscriptional regulation might impact channel function and the likely mechanisms mediating SCA6 pathogenesis.

Results

Generation of *Sca6* KI Mice. Mouse *Cacna1a* is highly homologous to human *CACNA1A*; however, mouse exon 47 lacks both the CAG repeat tract and its flanking sequence [supporting information (SI) Fig. S1C]. At the boundary of mouse intron 46/exon 47, we confirmed the presence of the sequence **AGGGCAGTAG**, in which each of the three AG sequences can serve as a splice acceptor site to generate distinct isoforms (12) (Fig. S1A and B). Splicing to the first AG results in an isoform with a long C-terminal tail (termed the MPI isoform), whereas splicing to the second site produces a short C terminus (MPc) caused by the introduction of a stop codon. Splicing to the third AG site gives rise to a rare isoform (MPII) in mouse PCs. We introduced the CAG repeat and its immediate flanking sequence derived from *CACNA1A* into the *Cacna1a* locus by using homologous recombination in embryonic stem (ES) cells derived from 129/SvEv strain (Fig. 1A; see *Methods* for details). Germ-line transmission of the targeted allele in the offspring was confirmed by Southern blot analysis (Fig. 1B and data not shown). To verify expression of the mutant transcripts, we performed RT-PCR analysis with primers designed to amplify the CAG repeat tract and its flanking human sequence. As shown in Fig. S2A, the bands of expected size were present in amplified cDNA from KI alleles.

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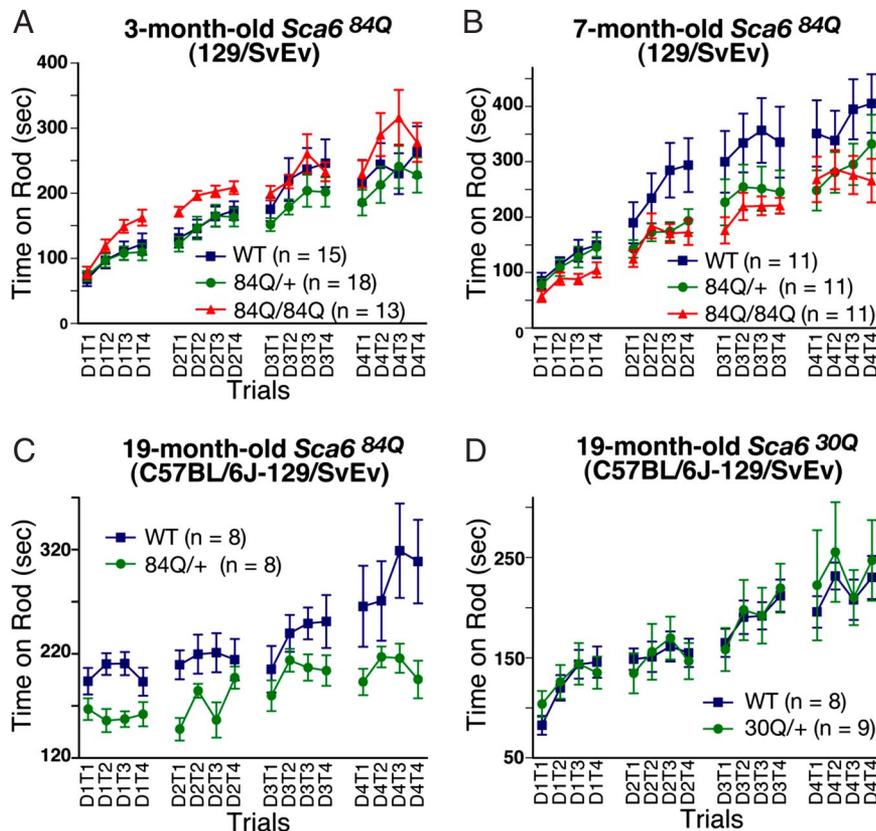


Fig. 2. Analysis of the *Sca6* KI mice on the accelerating Rotarod apparatus. (A and B) Performance of 3- (A) and 7-month-old (B) *Sca6*^{84Q/84Q} mice of 129/SvEv background. *Sca6*^{84Q/84Q} showed impaired motor performance ($P = 0.035$) at 7 months of age. (C) Performance of 19-month-old heterozygous *Sca6*^{84Q/+} mice of C57BL/6J-129/SvEv background. (D) Performance of 19-month-old heterozygous *Sca6*^{30Q/+} mice of C57BL/6J-129/SvEv background. Mice were trained in four trials per day (T1–T4) for four days (D1–D4). Error bars indicate SEM.

Sca6^{84Q/84Q} mice of 129/SvEv pure background performed similarly to WT littermates at 3 months of age (Fig. 2A). By 7 months, however, the mutant mice performed worse than WT, staying on the Rotarod significantly less time (Fig. 2B; $P < 0.05$). At this same age, heterozygous *Sca6*^{84Q/+} mice performed similarly to WT littermates ($P = 0.151$), consistent with our previous results (see above). In contrast to the *Sca6*^{84Q/84Q} mice, neither the *Sca6*^{30Q/30Q} nor *Sca6*^{14Q/14Q} mice displayed impaired performance on the Rotarod by 7 months of age (Fig. S3). Taken together, our results demonstrate that only mice bearing the *Sca6*^{84Q} allele develop motor impairment and that both the onset and severity of symptoms are gene dosage-dependent.

Properties of Ca²⁺ Channel Currents in Acutely Dissociated Cerebellar SCA6 PCs. To test the properties of SCA6 mutant channels *in vivo*, we recorded whole-cell Ca²⁺ channel currents in acutely dissociated PCs. Fig. 3 summarizes the results obtained recording WT and *Sca6* currents from PCs isolated from P12–P14 mice, using 2 mM Ba²⁺ as the charge carrier. Consistent with previous reports (14), the majority of inward current was blocked by 500 nM Aga-IVA, a specific peptide blocker of P/Q-type calcium channels; this was true for both WT and *Sca6*^{84Q/84Q} neurons (Fig. 3A and B). Although both groups of neurons contained similar Aga-IVA-insensitive components, the P/Q component was greatly decreased for the *Sca6*^{84Q/84Q} neurons. Because the Aga-IVA-insensitive component is a minor contributor to whole-cell barium current density, subsequent experiments were conducted in the absence of blocker.

We next examined the *I*–*V* relationship of barium currents in WT and *Sca6*^{84Q/84Q} neurons. In both groups, currents first became detectable with depolarization to -50 mV, peaked at -20 mV, and reversed at $+30$ mV (Fig. 3C and D). Over a wide range of voltages, current density was significantly decreased in *Sca6*^{84Q/84Q} (Fig. 3C), suggesting a voltage-independent effect.

To test whether the reduced current density seen in *Sca6*^{84Q/84Q} neurons depends on CAG repeat length, we measured whole-cell

current density in *Sca6*^{14Q/14Q} and *Sca6*^{30Q/30Q} PCs. Fig. 3E shows that all three polyglutamine-containing channels, irrespective of repeat length, exhibited a similar reduction in peak whole-cell current density relative to WT, suggesting that the decreased current does not depend on pathological expansion of the polyglutamine tract. Moreover, the reduced current density was not the result of increased cell size because whole-cell capacitance (a measure of plasma membrane surface area, and thus cell size) did not differ significantly among WT and the three groups of *Sca6* neurons (Fig. 3F).

Finally, we studied the effect of the long CAG repeat expansion on the voltage dependence of activation and inactivation, by using the protocols shown in Fig. 4A and B, respectively. Neither activation, as measured by the *G*–*V* relationship, nor steady-state inactivation was significantly different between WT and *Sca6*^{84Q/84Q} neurons (Fig. 4C).

Decreased Cav2.1 Immunoreactivity in *Sca6*^{84Q/84Q} Purkinje Neurons.

The decreased whole-cell current density observed in *Sca6*^{84Q/84Q} PCs could be explained by either impaired channel function at the plasma membrane, as reported for a mutation associated with familial hemiplegic migraine type 1 (15), or a decrease in expression or protein stability. To test these possibilities, we used immunocytochemistry to measure total expression of Cav2.1 in acutely dissociated PCs. Fig. S4A shows typical examples of a WT and an *Sca6*^{84Q/84Q} PC, identified by morphology and selective staining for type III β -tubulin using the Tuj1 Ab. The Cav2.1 protein was immunostained with an Ab raised against the intracellular linker between domains II and III (the II–III loop). Consistent with our whole-cell capacitance measurements (Fig. 3F), cell cross-sectional area (measured with the β -tubulin immunofluorescence) was not significantly different between WT and *Sca6*^{84Q/84Q}. In contrast, Cav2.1 immunofluorescence was significantly decreased for *Sca6*^{84Q/84Q} PCs (Fig. S4B). The degree of reduction in Cav2.1 immunofluorescence nearly paralleled the degree of reduction in

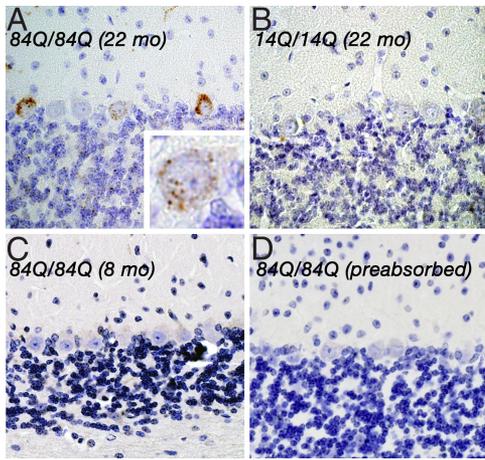


Fig. 5. NI formation in *Sca6*^{84Q/84Q} Purkinje neurons. (A) Immunohistochemistry for A6RPT-polyQ revealed NI formation in *Sca6*^{84Q/84Q} PCs (22-month-old). (Inset) Individual PC at higher magnification. Note that these NIs were seen mainly in the cytoplasm of these neurons. Similar NI-harboring PCs were scattered in the cerebellum. (B and C) In contrast, only weak immunoreactivities were detected in the cerebellum of a 22-month-old *Sca6*^{14Q/14Q} (B) or an 8-month-old *Sca6*^{84Q/84Q} mouse (C). (D) The immunoreactivity was entirely blocked in the cerebellum of the 22-month-old *Sca6*^{84Q/84Q} mouse when A6RPT-polyQ was preincubated with its polypeptide antigens. (Magnification of original photographs, $\times 400$.) Parallel staining undertaken with control human specimen also revealed similar NI formation in SCA6 PCs (data not shown).

phenotype in the relatively short life span of the mouse (17, 20–22). The absence of motor phenotype in heterozygous or homozygous *Sca6*^{30Q} mice also appears to support the notion that SCA6 is not a simple channelopathy because previously described loss-of-function *Cacna1a* mutants often show early-onset motor phenotypes associated with distinct alterations of Ca²⁺ channel properties (23, 24).

One critical genetic feature of SCA6 is that the mutation is located in exon 47, where alternative splicing produces two major isoforms, MPI and MPc. Our data revealed a reduced MPI/MPc ratio in Purkinje cell layers of the cerebella of all KI alleles. We speculate that the single *loxP* sequence left in the intron and/or the human nucleotide sequence in chimeric exon 47 contributed to the altered splice patterns at this locus. It is interesting, however, that the relative abundance of MPI transcripts increased as the CAG repeat length increased. This finding is in good agreement with the observation that the cerebellar transcripts containing 28 CAG repeats, obtained from the cerebellar cortex of an SCA6 patient, had a higher relative abundance of the GGCAG-containing variant compared with those containing 13 CAG repeats (18). It is not clear how CAG repeat length affects splicing, but we propose that the CAG repeat itself or its flanking sequence may act as an exonic *cis*-element contributing to correct splice-site identification. Indeed, online searches through ESEfinder (25) predicted the location of several putative exonic splicing enhancer motifs that include part of the CAG repeat (data not shown). Expansion of the repeat may alter the binding of specific serine/arginine-rich proteins to the motif(s) and thereby influence the splicing patterns at this locus.

Whether SCA6 arises from direct alteration of the channel function or a cytotoxic gain-of-function effect caused by the polyglutamine stretch has been a hotly debated topic (26). Our *Sca6* KI animals gave us an opportunity to assess the effect of the SCA6 mutation on the function of voltage-gated calcium channels in PCs. Contrary to the previous reports (8–11), our results clearly indicate that the hyperexpanded *Sca6*^{84Q} mutation does not alter voltage sensitivity of activation or inactivation in the acutely dissociated PCs. The *Sca6*^{84Q/84Q} PCs

showed reduced Ca_v2.1 immunoreactivity and a corresponding reduction of whole-cell current density compared with WT PCs. However, this reduction was not caused by repeat length because a similar reduction was observed for both the *Sca6*^{14Q/14Q} and *Sca6*^{30Q/30Q} channels, suggesting that the reduced current density seen in the *Sca6*^{84Q/84Q} PCs is not the key factor that accounts for the neurological phenotypes unique to these animals. Saegusa *et al.* (27) recently reported KI mice expressing a cDNA encoding a single MPI isoform of the human Ca_v2.1 channel. Electrophysiological analysis on their homozygous mutants also indicated that the repeat length did not affect the basic properties of the channel, although the expression levels of the human Ca_v2.1 channel were greatly reduced in these mice.

Decreased protein turnover and aggregation are common features of polyglutamine diseases, and accumulation of the polyglutamine-containing protein is thought to underlie a toxic gain-of-function mechanism (4). Thus, our observations that the mutant Ca_v2.1 channel did not lose its function and that its aggregation occurred in *Sca6*^{84Q} cerebella in both an age- and dosage-dependent manner support the notion that SCA6 is caused by a toxic gain-of-function mechanism. At least two forms of Ca_v2.1 accumulation (surface and cytoplasmic) have been implicated in the SCA6 pathogenesis (11, 18, 19). Although the Ab used here showed cytoplasmic mutant Ca_v2.1 aggregation in cerebellar PCs of *Sca6*^{84Q/84Q} mice, further study using mice with larger expanded alleles will be necessary to determine whether the mutant Ca_v2.1 also accumulates on the cellular surface or in the presynaptic terminals over time and ultimately changes the Ca²⁺ homeostasis in PCs of aged mutant mice. Such accumulation could lead to altered synaptic neurotransmission like that seen in flies expressing expanded full-length htt (28) and thereby induce motor dysfunction before the appearance of detectable neurodegeneration.

Kordasiewicz *et al.* (13) have found that the C-terminal region of Ca_v2.1 is capable of being cleaved from the protein, translocating to the nucleus, and causing toxicity to cells if it contained an expanded polyglutamine tract. Kubodera *et al.* (29) also reported the production of similar fragment in HEK293T cells overexpressing Ca_v2.1. Interestingly, the C terminus of the L-type Ca_v1.2 Ca²⁺ channel can also be cleaved and translocate to the nucleus, where it may act as a transcription factor (30). In our *Sca6* KI mice, we could not detect such a small C-terminal fragment in cerebellar extracts. It is conceivable that the cleaved product is sequestered with the large aggregated channel. Alternatively, the polyglutamine-containing cleavage product may be produced only when Ca_v2.1 is overexpressed in the heterologous context. Finally, it is possible that chimeric Ca_v2.1 protein produced in the KI brains may be no longer susceptible to such processing. Irrespective of the reason for the lack of aberrant accumulation of the cleaved product, it seems that such an event is not necessary to cause the neurological impairment seen in *Sca6*^{84Q/84Q} mice.

In conclusion, introducing the expanded CAG tract into Ca_v2.1 did not alter the basic properties of P/Q-type calcium channels but led to motor impairment coupled with aggregation of mutant Ca_v2.1. Both occurred in a dosage- and age-dependent manner as typically found in human SCA6. Also similar to human SCA6, increasing CAG repeat length increased the proportion of MPI transcripts in the cerebellum. Thus, the *Sca6* mutation not only gives rise to transcripts encoding glutamine-expanded channel proteins but also up-regulates their expression, possibly by regulating splicing events. We hypothesize that SCA6 pathogenesis must involve a toxic gain-of-function mechanism caused by altered activities or interactions of the C-terminal fragment in addition to possible partial loss-of-function due to decreased levels of specific isoforms.

Materials and Methods

Further details are described in *SI Materials and Methods*.

Generation of Sca6 KI Mice. Clones obtained from the 129/SvEv mouse genomic DNA library were used to construct a targeting vector. A SacI fragment that includes part of the mouse *Sca6* exon 47 was replaced with the homologous fragment prepared from human Cav2.1 cDNAs (12). A selectable cassette containing the neomycin resistance gene (*Neo*) and the thymidine kinase gene (*Tk*) flanked by two *loxP* sites were inserted into intron 45. The 72 CAG repeats were expanded to 84 during the ligation steps. The resultant targeting constructs were electroporated into ES cells derived from 129/SvEv strain, and subsequent electroporation of Cre recombinase expressing plasmids allowed the excision of *Neo/Tk* selection cassette. Correctly targeted ES cells were injected into C57BL/6J blastocysts to generate chimeras.

Laser Microdissection. Sagittal cerebellar tissue cryosections 8 μ m in thickness were mounted on polyethylene membrane slides (Leica). Each cryosection was lightly fixed in 95% ethanol and 5% acetic acid followed by a brief rinse in ice-cold water before staining with HistoGene staining solution (Arcturus). Laser microdissection was performed by using an AS LMD system (Leica). Total RNA from these samples was extracted by using the RNeasy micro kit following the manufacturer's instructions. The quality of RNA was checked with RNA 6000 Pico LabChip Kit (Agilent).

Semiquantitative RT-PCR. The reverse-transcription reaction was carried out with Sensiscript reverse transcriptase (Qiagen) following the manufacturer's instructions.

Primers MuEx46F3 (5'-AAGCATCGGCCACACCACCACCA-3') and TAARev (5'-TTAGCACCAGTCCTCGCTCTCGTGAC-3') were used to amplify the part of the cDNA containing both the junction of exon 46/47 and the CAG repeat.

Amplified products were cloned into pCRII-TOPO vector (Invitrogen). Clones were picked up for each RNA sample and subjected to sequencing.

Protein Expression. Protein extracts of mouse cerebellum were obtained by using NE-PER nuclear and cytoplasmic extraction reagents (Pierce). Fifty micrograms of protein was loaded on SDS gel. For detection of aggregated forms of Cav2.1, samples were lysed in buffer containing 8 M urea and 4% SDS. Nitrocellulose blots were probed with CT-2 Ab (1:1,000).

Rotarod Test. Animals were placed on an accelerated rotating rod (Ugo Basile) in four trials every day for a period of 4 days, as described in ref. 31.

PC Preparation. Neurons were prepared from P12–P14 cerebella with a protocol modified from Swensen and Bean (32). The cells were plated on coverslips precoated with poly-D-lysine and laminin (BD Biosciences) in a 24-well plate containing Neurobasal complete medium (containing sodium pyruvate, L-glutamine, B27, and N-acetylcysteine).

Electrophysiology. Whole-cell currents were recorded at room temperature by using an Axopatch 200B patch-clamp amplifier (Axon Instruments). Pipette resistance was \approx 2–3 M Ω when filled with an internal solution. Cells were voltage-clamped at -80 mV, and pulse depolarizations were applied at 10-s intervals. Data were passed through a 4-pole low-pass Bessel filter at 1–2 kHz, digitized at 5–10 kHz by using a Digidata 1320A (Axon Instruments). Patch-clamp data were acquired and analyzed by using Clampex 8.2 and Clampfit 8.2, respectively (Molecular Devices).

Immunohistochemistry. Formalin-fixed, paraffin-embedded, 6- μ m-thick sections were deparaffinized, exposed to microwave in citrate buffer, and treated in formic acid before incubation with human Cav2.1-specific custom Abs A6RPT-polyQ raised against MERRVPGPARSESPRA (Fig. S1D). Detailed characterization of the Abs will be published elsewhere.

Statistical Analysis. Where applicable, data are presented as mean \pm SEM, and statistical significance was tested by using a two-tailed Student's unpaired *t* test for the electrophysiological analysis and semiquantitative measurements of Cav2.1 immunofluorescence. For the Rotarod test, the time the mice spent on the rod without falling was recorded and subjected to statistical analysis by using one-way ANOVA with repeated measures.

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