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Assessment of mutations in *KCNN2* and *ZNF135* to patient neurological symptoms

Vijeta Raghuram, PhD[1], **Sydney Weber, BA**[2], **Jacob Raber, PhD**[2], **Dong-Hui Chen**[3], **Thomas D. Bird, MD**[3], **James Maylie, PhD**[4], and **John P. Adelman, PhD**[1]

[1]Vollum Institute, Oregon Health & Science University, Portland, Oregon

[2]Department of Behavioral Neuroscience, Oregon Health & Science University, Portland, Oregon

[3]Neurology Department, University of Washington, Geriatric Research (GRECC), VA Puget Sound Health Care System, Seattle, Washington

[4]Department of Obstetrics and Gynecology, Oregon Health & Science University, Portland, Oregon

Abstract

Exome sequencing from a patient with neurological and developmental symptoms revealed two mutations in separate genes. One was a homozygous transition mutation that results in an in-frame, premature translational stop codon in the ZNF135 gene predicted to encode a transcriptional repressor. Another mutation was heterozygous, a single nucleotide duplication in the KCNN2 gene that encodes a Ca^{2+}-activated K^+ channel, SK2, and leads to a translational frame shift and a premature stop codon. Heterologous expression studies, brain slice recordings and coordination tests from a transgenic mouse line carrying the SK2 mutation suggest that it does not contribute to the patient's symptoms. ZNF135 is expressed in human brain and it is likely that the homozygous mutation underlies the human phenotype.

Keywords

SK potassium channel; Neuroscience; CRISPR-Cas system; apamin; transgenic mice; ataxia; human exome sequencing; ZNF135

Introduction

SK2 channels are tetrameric assemblies of subunits that are also co-assembled with the Ca^{2+} sensor, calmodulin that serves as the gating switch[1]. The SK channels are selectively blocked by the bee venom toxin apamin and affect neuronal excitability, synaptic

Corresponding author: John P. Adelman, Corresponding author's address: Vollum Institute, L474, Oregon Health & Science University, 3181 SW Sam Jackson Park Rd, Portland, OR 97239-3098, Corresponding author's phone: 503-494-5450, adelman@ohsu.edu.

Conflict of interest

Dr. Bird discloses two patents broadly related to this work (SCA14 and CMT1) for which he receives licensing fees. The other authors have no items to disclose.

transmission, and synaptic plasticity[2]. The c.581 dup A mutation in SK2 produces a truncated subunit. Neither parent carries the c.581 dup A in KCNN2.

The ZNF135 gene encodes a putative transcriptional repressor protein. The mutation found in the patient, (c.1156 C>T; R386X), would abolish the Zn^{2+} finger region and likely abrogate function. Both parents were heterozygous for the mutation, but did not present an obvious phenotype.

Methods

Exome sequencing

Exome sequencing was performed and analyzed by GeneDx (Gaithersburg MD).

SK2 knock in mice (Fig. 1)

To generate SK2 knock in mice, CRISPR/Cas9 gene editing was employed. An optimal target site for the CRISPR complex in the second exon of the mouse KCNN2 gene was identified using the online tool at crispr.mit.edu. The single guide RNA (sgRNA) design and synthesis using pX330 (Addgene) was performed as described in [3].

A 181 nt single-stranded oligodeoxynucleotide (ssODN) of the sequence:

gctgtattctttagctctgaaatgccttatcagtctctccacgatcatc

ctgcttggtctgatcatcgtgta**t**ca**t**gc**t**aga**a**gaaatacaAggtaacacaggctc

cactgttttctgaataaccagaagccatgcaggcagcataggagaaaagc aagacagcaaggggcctttaccaagc

was purchased as an Ultramer (Integrated DNA Technologies, Inc., Coralville, IA) and dissolved in RNase-free water to a concentration of 10 μM. An injection cocktail of Cas9 mRNA (100 ng/uL; Trilink Biotechnologies, Inc., San Diego, CA), SK2 sgRNA (50 ng/uL), ssODN (100 ng/uL) and SCR7(1mM; Xcess Biosciences, Inc., San Diego, CA) was injected into the cytoplasm of 218 zygotes of C57BL/6 mice. From them, 46 pups were born and 3 pups harbored the targeted mutation.

Genotyping was accomplished using the Surveyor assay and DNA sequencing. Mice harboring the desired mutation were bred to assure germ line transmission and offspring of the appropriate genotypes were used for experiments.

Motor coordination

Motor coordination in 10–12 weeks old wild type mice and SK2-L195VfsX10 heterozygotes was assessed on an accelerating rotarod. Mice were placed on an elevated rotating rod (diameter: 3 cm, elevated: 45 cm, Rotamex-5; Columbus Instruments, Columbus, OH), initially rotating at 5.0 rpm. The rod accelerated 1.0 rpm every 3 s. A line of photobeams beneath the rod recorded the latency to fall (s). Each mouse received 10 trials over 4 days - one trial on day 1 and three trials each on day 2, 3 and 4 with an average of 60 min delay between trials.

Molecular biology

The DNA coding for the hSK2-S-wild type and hSK2-S-L195VfsX10 mutation (SK2-WT and SK2-L195VfsX10), were purchased as synthetic gene fragments with codon optimization from IDT. These gene fragments were then cloned into pCAG-Ires-GFP, pCAG-Ires-Apple or pJPA5 vectors by gibson assembly with the help of the gibson assembly kit (New England Biolabs, Ipswich, MA). A triple myc epitope was incorporated between aa 246 and 247 of the wild type sequence (loop region between TM 3 and 4). A HA-tag was introduced between the aa 162 and 163 in the hSK2- L195VfsX10 protein (loop region between TM 1 and 2) by site-directed mutagenesis.

The rtPCR on human brain mRNA rtPCR on human brain mRNA used Poly A+ RNA from adult and fetal brains purchased from Clontech Laboratories, Inc. (Mountain View, CA) Expression of hZNF135 mRNA was tested using gene specific primers and AccessQuick RT-PCR system (Promega Corp. Madison, WI) followed by DNA sequencing of the PCR product.

Transfection and immunostaining

Transfections into HEK293 cells used Lipofectamine (Thermo Fisher Scientific, Inc., Waltham, MA) on poly-D-lysine-coated microscopic cover glasses in 12-well tissue culture plates. Immunocytochemistry was performed 24–48 h post-transfection[4].

Slice preparation and electrophysiology

All procedures were performed in accordance with the IACUC guidelines of Oregon Health and Science University (Portland, OR). Animals were anesthetized with isoflurane and decapitated. Transverse hippocampal slices were prepared from 4–5-week-old SK2-L195VfsX10 heterozygous mice and wild type littermates[5]. Whole-cell, patch-clamp recordings were obtained from CA1 pyramidal cells using a HEKA EPC 10 plus patch clamp, digitized using the built-in ITC-18 analog-to-digital converter, and transferred to a computer using Patchmaster software (Heka Instruments, Inc., Bellmore, NY). Patch pipettes (open pipette resistance, 2–4 M Ω) were filled with (in mM) 133 K-gluconate, 4 KCl, 4 NaCl, 1 $MgCl_2$, 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 4 MgATP, 0.3 Na2 guanosine triphosphate (Na_2GTP), and 10 phosphocreatine (pH 7.2). Series resistance was electronically compensated to greater than 70%. All the recordings were performed at room temperature (22–24 °C). CA1 neurons were clamped at #x02212;55 mV and stepped to 20 mV for 200 ms; tail currents were elicited upon stepping back to −55 mV. Data were not corrected for a junction potential of ~18 mV.

For HEK293 cell recordings, cells were transfected with hSK2S-wild type pCAGIG and L195VfsX10 pCAGIA. Recordings were performed according to at room temperature (22–24 °C) 1–3 days after transfection[4]. Transfected cells were identified by the presence of dual fluorescence from eGFP and mApple. Currents were evoked using 2s voltage ramps from #x02212;100 mV to 60 mV. Apamin-sensitive current was measured at #x02212;100 mV as the difference current before and after the addition of 100 nM apamin to the bath through a perfusion pencil. Data were analyzed using IGOR (WaveMetrics, Lake Oswego, OR). Data are expressed as mean ± SEM. Non-parametric Wilcoxon Mann-Whitney two-

sample rank test was used to determine significance between groups of data; $p<0.05$ was considered significant.

Results

Patient description

The subject is presently a 43-year old woman. She was born one month premature and had an initial Apgar score of zero. She was quickly resuscitated and spent two weeks in the neonatal ICU. Her developmental milestones were moderately slow, crawling at 10 months and walking at 16 months. She had tonic-clonic seizures starting at age 5 that have been well controlled on medication. She was able to ride a bicycle and graduated from high school with average grades. Her intellectual abilities were thought to be normal or low-normal. Because of mild persistent incoordination she was considered to have cerebral palsy. At age 17 because of stiff legs and brisk reflexes a neurology consultant considered dopa-responsive dystonia and placed her on levodopa/carbidopa with mild but incomplete improvement in her walking. In her 20s she began to use ankle foot orthoses, in her early 30s she was using a 4-wheel walker and in her late 30s she had to use a wheelchair. At age 40 her positive findings were marked ataxia of gait, dysarthria, bilateral finger to nose dysmetria, decreased tendon reflexes in her arms, increased tone and exaggerated tendon reflexes in her legs with ankle clonus but down going plantar responses. The best clinical diagnosis is spastic ataxia with seizures. Mental status, eye movements and sensory testing were normal. She did not have Parkinsonian features. Brain MRI showed normal cerebellum, brainstem and cerebral cortex with mild non-specific white matter intensities on T2 imaging in posterior regions. At age 42 she developed persistent vomiting, was found to have gastric paresis and required a feeding tube. The spasticity in her legs worsened, she became confined to a bed and a baclofen pump lessened the stiffness in her lower limbs. Her parents were normal with no family history of neurologic disorders.

Identification of mutations by whole exome sequencing

Exome sequencing revealed a homozygous mutation in ZNF135 (c.1156 C>T; Accession number: P52742) that results in a premature translational stop codon (p.R386X. The SK2 mutation is heterozygous, a single nucleotide duplication (c.581dupA) in the KCNN2 gene (NM_021614.3) (Accession numbers: NP_067627.2, XP_006714676.1), and results in a translational frame shift and a premature translational stop codon (p.L195VfsX10). No other mutation was found in the exome.

SK2- L195VfsX10 do not act as dominant negative subunits

The L195VfsX10 mutation predicts an SK2 subunit containing the intracellular N-terminal domain and the first two TMs. To test whether the mutant subunits confer a dominant negative effect, the SK2-L195VfsX10 subunit was expressed alone or together with wild type subunits in HEK293 cells. When SK2-L195VfsX10 subunits (or the empty vector) were expressed alone, whole cell recordings with an internal pipet solution containing 111 μM free Ca^{2+} failed to detect SK channel currents in response to voltage ramp commands (−0.1 ± 0.0 nA, n = 6; Fig. 2A). When expressed together with wild type SK2, the ramp currents reversed near the predicted K^+ reversal potential of −38 mV and the apamin sensitive SK

current amplitudes measured at −100 mV (−4.7 ± 1.3 nA, n = 5; Fig 2B) were not different from those recorded from HEK293 cells expressing wild type SK2 alone (−4.1 ± 0.9 nA, n = 6; p = 0.75; Fig. 2C). In addition, live cell staining for an external epitope tag (see methods) failed to detect the L195VfsX10 subunit in the plasma membrane, whether expressed alone or together with wild type SK2 subunits (Fig. 2E). Rendering the cells permeable revealed intracellular L195VfsX10 expression (Fig. 2F). Thus, in a heterologous system, SK2-L195VfsX10 subunits do not exert a dominant negative effect.

KCNN2 L195VfsX10 mice

To test whether SK2-L195VfsX10 resulting in haploinsifficiency contributes to symptoms consistent with the human patient carrying the heterozygous mutation, CRISPR-Cas9 gene editing was used to knock in the mutation in the mouse KCNN2 gene (c.566 dupA). Cohorts of 10 to 12 weeks old, adult SK2-L195VfsX10 heterozygous mice or wild type mice were tested for coordination performance using the accelerating rotarod test. No differences in performance were seen between SK2-L195VfsX10 heterozygous mice and wild type littermates (Fig. 3A). Similar to $SK2^{-/-}$ mice, SK2-L195VfsX10 homozygous mice displayed a tremor and were not studied further since the patient is heterozygous.

SK2 channels are expressed in CA1 pyramidal neurons in the hippocampus[6]. To determine whether expression of L195VfsX10 in mice altered SK2 channel function, whole cell recordings from CA1 pyramidal neurons were performed on hippocampal brain slices. Tail currents evoked following a depolarizing command yielded apamin-sensitive SK component that was not different between wild type (95.0 ± 10.3 pA, n = 12) and heterozygous L195VfsX10 mice (78.2 ± 12.8 pA, n = 14; p = 0.37) (Fig. 3B-C). Taken together with the results described above, it is unlikely that the SK2-L195VfsX10 mutation is the primary cause of the symptoms displayed by the human patient.

The ZNF135 gene

ZNF135 is predicted to encode a transcriptional repressor proteins[7]. To determine whether ZNF135 is expressed in human brain, rtPCR was performed on mRNA isolated from human fetal and adult brain. PCR product size and DNA sequencing confirmed the identity of the band. These results show that ZNF135 is expressed in both fetal and adult human brain (Fig. 4). Testing whether the homozygous mutation in ZNF135 underlies the human patient disorder using mouse model proved problematic as database searches did not reveal the existence of a ZNF135 ortholog in mouse or rat. However, ZNF135 expression in human brain, taken together with the lack of effect of L195VfsX10 in KCNN2 when present in a mouse model suggests that the human patient's symptoms are caused by homozygous c. 1156 C>T mutation in ZNF135.

Discussion

The c.581dupA mutation appears to be a de novo mutation, as neither parent carries the mutation. Moreover, the c.581dupA mutation was not observed in approximately 6,500 individuals of European and African American ancestry and was not reported in any SNP database including ExAc, indicating it is not a common benign variant in these populations.

Examining heterologously co-expressed mutant and wild type subunits indicated that the mutant subunit does not act as a dominant negative. In addition, heterozygous c.581dupA (L195VfsX10) mice showed no change in the SK-mediated component of tail currents or behavioral performance in the rotarod test. Thus it is unlikely that the c.581dupA mutation is responsible for the symptoms in the human patient.

The other mutation was in the ZNF135 gene that is expressed in human fetal and adult brain. The mutation is a single nucleotide transition c.1156 C>T is homozygous in the patient and each parent carries one allele with the mutation. However, neither parent exhibited symptoms. While the patient is the first known homozygote, the R386X variant was observed with a frequency of 0.19% (17/8600 allele) in individuals of European ancestry and thus may underlie neurological deficits in other patients for which the specific mutation has not been established. The most parsimonious conclusion from these results is that the homozygous c.1156 C>T mutation in ZNF135 is responsible for the symptoms in the human patient.

Acknowledgments

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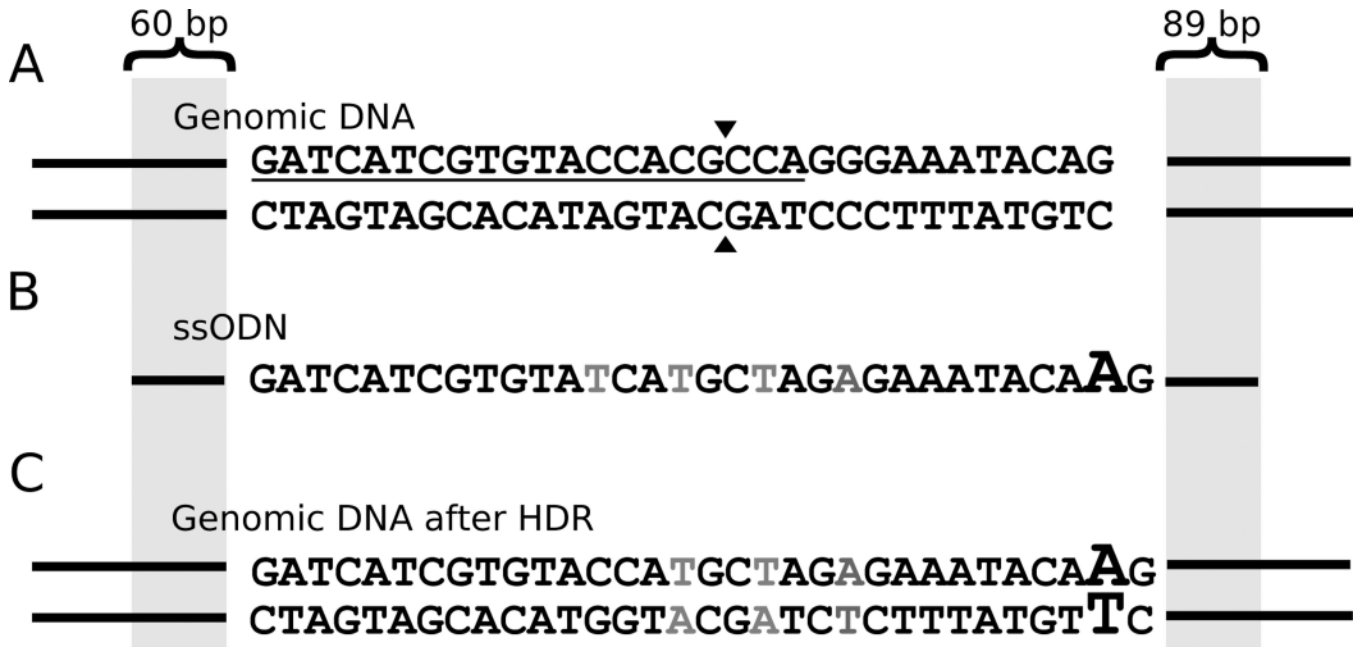

Figure 1.

Design for *KCNN2 L195VfsX10 mice* A. The region of interest in the exon 2 of the mouse SK2 gene is shown. The 20bp region against which the CRISPR guide was designed is underlined and the predicted site of the double-stranded break is shown by the solid arrow heads. B. The 181 nt single-stranded oligo deoxynucleotide (ssODN) is shown. Functioning as a repair template in the homology-dependent repair mechanism (HDR), the ssODN contained the desired single base insertion (large font) flanked by regions of homology to the genomic DNA (grey bars) and 4 silent base substitutions (grey font) to prohibit repeated attacks on the target site by the CRISPR complex. C. SK2 gene after successful targeting and repair is shown. The silent base substitutions as well as the single base insertion from the repair oligo were successfully incorporated into the gene as a result of a homology-dependent repair mechanism.

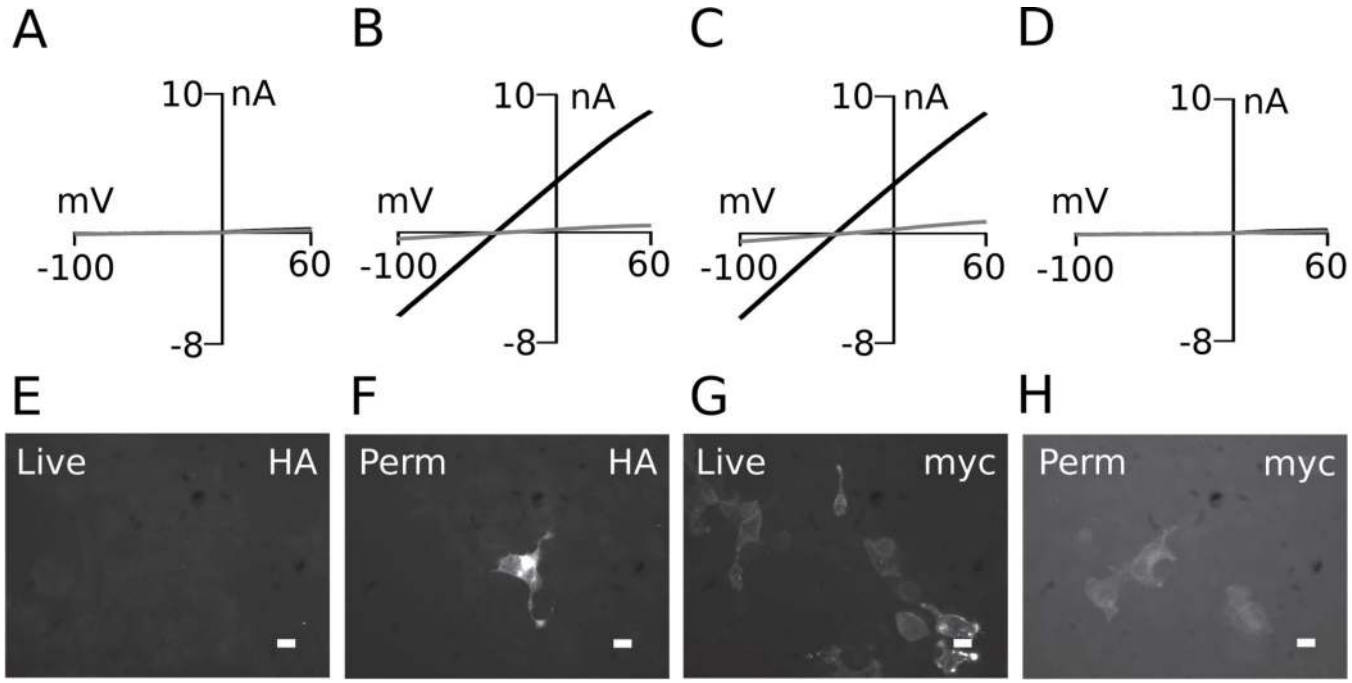

Figure 2.

hSK2-L195VfsX10 does not express functional channels by itself or with hSK2-wild type. A-D: Whole cell voltage clamp current recordings expressing (A) hSK2-L195VfsX10, (B) hSK2-L195VfsX10 and hSK2-wild type, (C) hSK2-wild type alone or (D) empty vectors before (black) and after (gray) the extracellular application of 100 nM apamin. E-H: The L195VfsX10 protein does not reach the plasma membrane in HEK293 cells. Live (E,G) or permeabilized (F,H) staining of HA-tagged hSK2-L195VfsX10 (E,F) and triple-myc-tagged hSK2-wild type (G,H). Scale bar, 10μm.

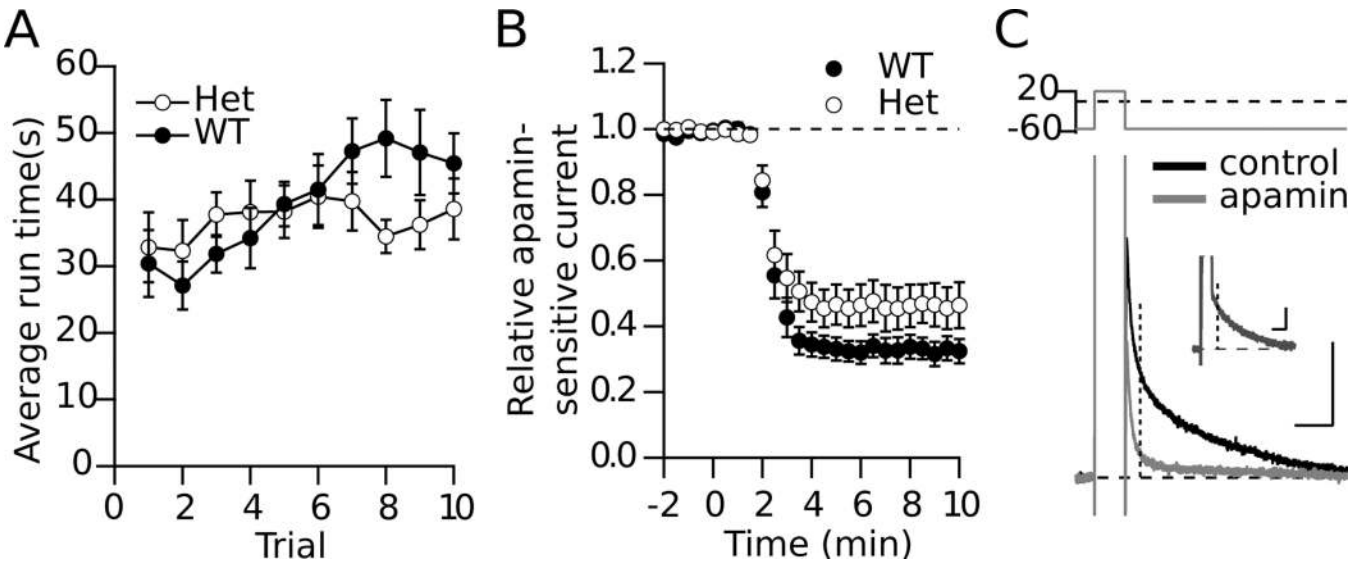

Figure 3.

Heterozygous SK2-L195VfsX10 mice do not display motor coordination deficits or loss of functional SK currents. A. Plot of average run time on rotarod in each trial for heterozygous SK2-L195VfsX10 mice (empty circles; n = 16) and wild type littermates (filled circles; n = 12). B-C. Heterozygous SK2-L195VfsX10 mice and wild type littermates show similar amplitudes of apamin-sensitive SK2 currents in CA1 pyramidal neurons. B. Time course of apamin block (100 nM) of tail current measured at 100 ms (indicated as a vertical dashed line in panel C) from heterozygous SK2-L195VfsX10 mice (empty circles; n = 14) and wild type littermates (solid circles; n = 12). C. Representative voltage-clamp recordings of tail currents in a wild type CA1 pyramidal neuron. Apamin (light gray trace) blocks a component of the tail current. Inset: subtraction of the traces before and after apamin application yielded the apamin-sensitive I_{SK}.

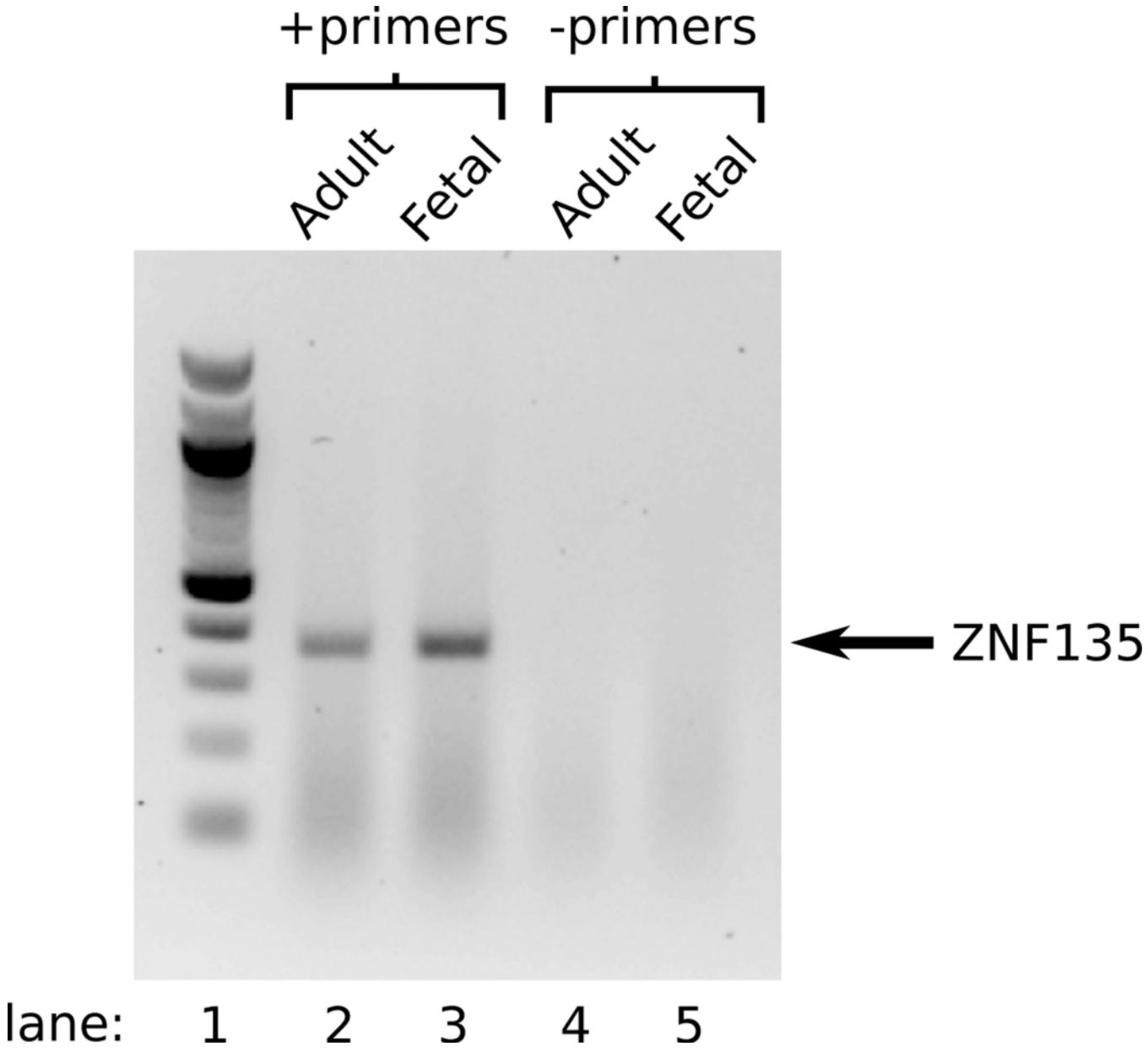

Figure 4.

ZNF135 is expressed in fetal and adult human brain. rtPCR products of the expected size for ZNF135 from human adult (lane 2) and fetal (lane 3) whole brain mRNA. Lanes 4 and 5 were duplicate reactions without adding primers. Lane 1 is MW marker.