

Loss-of-Function Mutations in *FRRS1L* Lead to an Epileptic-Dyskinetic Encephalopathy

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Glutamatergic neurotransmission governs excitatory signaling in the mammalian brain, and abnormalities of glutamate signaling have been shown to contribute to both epilepsy and hyperkinetic movement disorders. The etiology of many severe childhood movement disorders and epilepsies remains uncharacterized. We describe a neurological disorder with epilepsy and prominent choreoathetosis caused by biallelic pathogenic variants in *FRRS1L*, which encodes an AMPA receptor outer-core protein. Loss of *FRRS1L* function attenuates AMPA-mediated currents, implicating chronic abnormalities of glutamatergic neurotransmission in this monogenic neurological disease of childhood.

Pediatric movement disorders encompass a heterogeneous group of neurodevelopmental and neurodegenerative disorders affecting movement and limiting activities of daily living.¹ The etiology of many pediatric movement disorders remains uncharacterized at the current time. Epileptic-dyskinetic encephalopathies² (MIM: 308350) are a recently described class of disorders. Although these were originally described in association with *ARX* (MIM: 300382) mutations,² mutations in *SCN8A*³ (MIM: 600702), *STXBPI*⁴ (MIM: 602926), and *FOXG1*⁵ (MIM: 164874) have also been found to cause a similar phenotype characterized by dystonia and/or choreoathetosis, epilepsy, and encephalopathy.

The pathophysiology of choreoathetosis is poorly understood, although a loss of striatal inhibition appears to be a common mechanism.⁶ Prior work has implicated abnormalities of glutamatergic neurotransmission in individuals with Huntington disease (MIM: 143100), the most common cause of chorea. This includes a loss of putaminal⁷ (NMDA [N-methyl-D-aspartate]) and fronto-

cortical⁸ (AMPA [α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid]) glutamatergic receptors in postmortem brain tissue in individuals with Huntington disease.

AMPA receptors (AMPA receptors) represent the most common receptor subtype in the brain and mediate most fast excitatory post-synaptic potentials. AMPARs also play important roles in learning and memory via their role in long-term potentiation. In the case of long-term potentiation, repetitive presynaptic glutamate release leads to enhanced intracellular sodium concentrations through AMPAR conduction. This in turn allows for NMDA receptor activation, which itself conducts inwardly directed calcium currents. Increased levels of intracellular calcium activate CAMKII (calcium-calmodulin kinase II) activity and lead to enhanced AMPAR function by both increasing cell-surface AMPAR tracking and phosphorylating AMPARs.

AMPA receptors are composed of four subunits, designated GluR1–GluR4. Functional AMPARs are composed of a dimer of dimers containing two GluR2 subunits plus two

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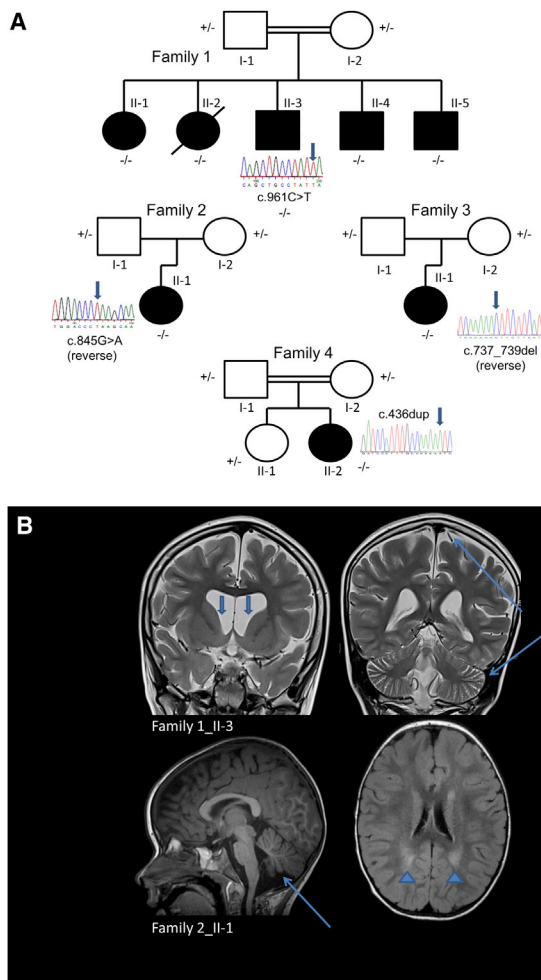


Figure 1. Families Affected by Choreoathetosis, Epilepsy, and Cerebral Volume Loss

(A) A total of eight individuals (one deceased) from four unrelated families are affected by this progressive neurological disease.

(B) Brain MRI shows diffuse cortical and cerebellar volume loss (long arrows), ex vacuo ventriculomegaly and flattening of the heads of the caudate nuclei (short arrows), and periventricular FLAIR (fluid attenuation inversion recovery) hyperintense signal (arrowheads).

of GluR1, GluR2, or GluR3. A variety of post-transcriptional mechanisms contribute to the finely orchestrated regulation of AMPAR function. Alternative splicing mediates the choice of isoforms (designed flip or flop), whereas RNA editing effectively limits Ca^{2+} permeability. In addition, a host of diverse auxiliary subunits and interacting proteins also modulate AMPAR properties.

A recent study identified *FRRS1L* (ferric chelate reductase 1-like) as an important component of the outer core of AMPAR accessory proteins,⁹ suggesting that loss of *FRRS1L* would affect AMPAR constituency and thus AMPAR function. Our studies suggest that this indeed is the case and that pathogenic variants in *FRRS1L* (aka *C9orf4* [MIM: 604574]) lead to an epileptic-dyskinetic encephalopathy.

In our study, the index family hails from southern Saudi Arabia. The parents are distant cousins (Figure 1A). All five

of the couple's children exhibit encephalopathy, epilepsy, and progressive choreoathetosis (Table 1). One child (individual 1_II-2) had already succumbed to the disease at the time of ascertainment. All children were born at term without complications. All had normal early development but began to regress between the ages of 18 and 22 months, when they ceased to walk and lost expressive language. This coincided with the onset of hemiclonic and tonic-clonic seizures, which showed some response to carbamazepine and/or valproate. Examinations disclosed no dysmorphic features, normal fundi, global developmental delay, diffuse hypotonia, and generalized chorea with a paucity of volitional movement. Routine laboratory screening was unremarkable, as was triplet repeat analysis of *HTT* (MIM: 613004). Brain MRI was initially normal, but repeat neuroimaging demonstrated diffuse cortical and cerebellar volume loss and flattening of the caudate heads (Figure 1B). All of the affected children became less responsive to their environment over time, and their hyperkinetic movement disorder gradually gave way to a rigid, akinetic state in late adolescence.

Given the family structure and putative autosomal-recessive nature of the disorder, we then applied tandem homozygosity mapping and whole-exome sequencing. Family members were enrolled in our institutional-review-board-approved protocol after written informed consent was obtained. Genomic DNA was extracted from whole blood, and homozygous regions shared by affected individuals but absent in unaffected individuals in family 1 were identified with Affymetrix Whole-Genome CytoScan HD SNP arrays according to the manufacturer's instructions. We identified six >3 Mb homozygous regions shared by affected family members alone (Table S1).

We performed whole-exome sequencing on individuals 1_II-1 to 1_II-4 and focused our analysis on homozygous stop-gain, stop-loss, or frameshift and splice-site or missense variants predicted to be deleterious. Exonic targets were enriched with the SeqCap EZ Human Exome Library v.2.0 (Nimblegen), and sequencing was performed on an Illumina HiSeq 2500 according to the manufacturer's instructions for the generation of paired-end reads. Average coverage depth was >70× within the targeted exome. Sequencing reads were aligned to the NCBI human reference genome (GRCh37) with the Burrow-Wheeler Aligner (0.7.10). Picard Tools (1.111) was subsequently used for sorting and marking duplicates. Local realignment, base-quality-score recalibration, and variant calling were performed with the Genome Analysis Toolkit (GATK 3.3.-0), and variant-call files were evaluated with NextCode or Cartagenia Bench Lab next-generation sequencing software. Filtering included removing low-quality calls (Illumina threshold < 20) and variants with a minor allele frequency > 0.01 in the Exome Aggregation Consortium (ExAC) Browser and NHLBI Exome Sequencing Project (ESP) Exome Variant Server while prioritizing homozygous variants predicted to be deleterious by SIFT, PolyPhen-2, MutationTaster, and CADD.

Table 1. Clinical Features of Individuals with *FRRS1L* Mutations

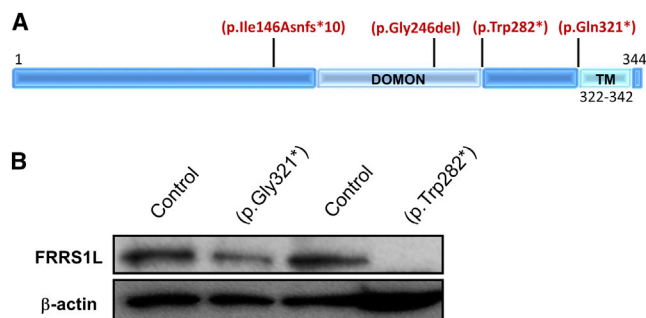
Individual	Mutation	Age at Onset	Regression	Intellectual Impairment	Movement Disorder	Epilepsy	Neuromotor Impairment	Other
1_II-1	c.961C>T (p.Gln321*)	18 months	yes	severe; no expressive speech	chorea; later rigidity, hypokinesia	generalized tonic-clonic seizures	spasticity; impaired volitional movement	-
1_II-2	c.961C>T (p.Gln321*)	22 months	yes	severe; no expressive speech	chorea; later rigidity, hypokinesia	hemiclonic seizures; later generalized	spasticity; impaired volitional movement	died at 16 years of age
1_II-3	c.961C>T (p.Gln321*)	22 months	yes	severe; no expressive speech	chorea; later rigidity, hypokinesia	hemiclonic seizures	spasticity; impaired volitional movement	-
1_II-4	c.961C>T (p.Gln321*)	22 months	yes	severe; no expressive speech	chorea; later rigidity, hypokinesia	generalized seizures; hemiclonic seizures	spasticity; impaired volitional movement	-
1_II-5	c.961C>T (p.Gln321*)	24 months	no	severe; no expressive speech	chorea	hemiclonic seizures	-	-
2_II-1	c.845G>A (p.Trp282*)	4 months	no	severe; no expressive speech	chorea; ballism	multifocal; intractable	hypotonia; impaired volitional movement	-
3_II-1	c.737_739del (p.Gly246del)	24 months	yes	severe; no expressive speech	chorea; cogwheel rigidity	juvenile spasms evolved to Lennox-Gastaut syndrome	hypotonia; impaired volitional movement	horizontal and vertical nystagmus
4_II-2	c.436dup (p.Ile146Asnfs*10)	6 months	yes	severe; no expressive speech	chorea; myoclonus	multifocal; clonic	hypotonia; no volitional movement	-

Using this strategy, we identified a homozygous c.961C>T (p.Gln321*) variant (GenBank: NM_014334.2) in *FRRS1L* within a prominent block of homozygosity (chr9: 104,622,396–119,149,440; UCSC Genome Browser hg19). We confirmed segregation within affected family members by Sanger sequencing (Figure 1A). This variant has not been reported in variant repositories and is predicted to lead to loss of a C-terminal hydrophobic motif that might contribute to *FRRS1L*'s membrane localization (Figure 2A).

We subsequently identified three additional, unrelated individuals with similar phenotypes and biallelic *FRRS1L* variants by whole-exome sequencing (Table 1) and confirmed sequence changes by Sanger sequencing (Figure 1A). None of these variants has been identified in public repositories. Individual 4_II-2 was the most severely affected.

Individual 2_II-1, an unrelated girl, was born to non-consanguineous parents of mixed European descent after an unremarkable pregnancy at 31 weeks of gestation. At 4 months of age, she started having choreoathetotic movements of her hands, opisthotonic posturing, and occasional hand tremors. She reached developmental motor and language milestones until the age of 13 months, when she began to have generalized tonic-clonic seizures. During periods of illness, her seizures were accompanied by chaotic eye movements and forced gaze deviation, frequent episodes of tone loss, fluctuating levels of alertness, and truncal hypotonia. Between 14 and 27 months of age, her seizures remained refractory to levetiracetam,

phenobarbital, clobazam, and the ketogenic diet. This was associated with developmental regression, and she remained nonverbal with diminished volitional movement. Her examination was significant for generalized chorea and ballism (Movie S1). Whole-exome sequencing identified a homozygous c.845G>A (p.Trp282*) change in *FRRS1L*.

**Figure 2. Variants in Human *FRRS1L* and Effects on Protein Abundance**

(A) The three variants leading to premature truncation codons are all predicted to lead to loss of the *FRRS1L* transmembrane (TM) domain, whereas p.Ile146Asnfs*10 and p.Gly246del are predicted to disrupt the predicted transmembrane and extracellular DOMON (dopamine beta-monooxygenase N-terminal) domain (InterPro: IPR005018).

(B) Immunoblot in affected fibroblasts demonstrates lower amounts of *FRRS1L* in individuals with premature stop variants than in age- and sex-matched control individuals (representative image); anti-*FRRS1L* (1:1,000, Atlas, Sigma-Aldrich) and anti- β -actin (1:1,000, Santa Cruz) served as loading controls.

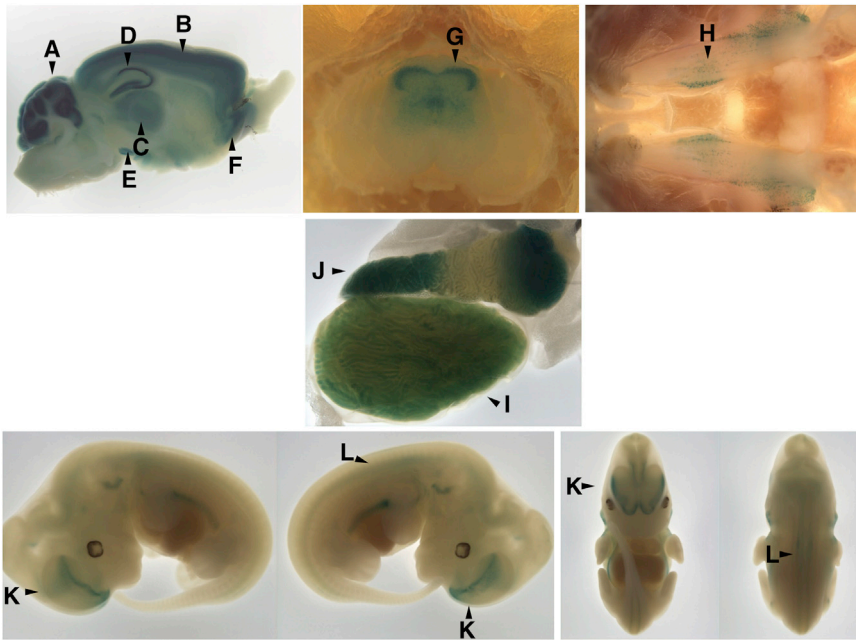


Figure 3. *Frrs1l* Expression Pattern

Murine *Frrs1l* expression detected by lacZ reporter. *Frrs1l* is expressed throughout the adult brain, predominantly in the cerebellum (A) and differentially throughout layers of the cortex (B), thalamus (C), hippocampus (D), substantia nigra (E), and anterior olfactory nucleus (F). There is expression in the dorsal horn of the lumbar spinal cord (G) and the trigeminal ganglion (H). Outside of the nervous system, *Frrs1l* is expressed in the epididymis (I) and seminiferous tubules (J) of the testis. During development (E12.5), *Frrs1l* is expressed in the ventral forebrain (K) and weakly in the spinal cord (L).

Individual 3_II-1, a Puerto Rican girl with no recognized consanguinity, was born at an estimated 37 weeks of gestation. She began to cruise with an uncoordinated gait at 11 months and used several words appropriately before experiencing a neurodevelopmental regression at the age of 24 months. She developed juvenile spasms and was later diagnosed with Lennox-Gastaut syndrome. She initially benefited from the ketogenic diet, but her seizures were never fully controlled. Examination revealed horizontal and vertical nystagmus with esotropia, generalized chorea and cogwheel rigidity of her upper limbs, hyperreflexia, hypotonia, and diminished volitional movement (she did not reach purposefully or have a means of locomotion). No expressive language was evident. A homozygous in-frame deletion (c.737_739del [p.Gly246del]) was identified by whole-exome sequencing. This variant was predicted to be deleterious by PROVEAN (score = -7.386).

Individual 4_II-1, an unrelated girl, was born at term by caesarean section to consanguineous Moroccan parents after a pregnancy complicated by gestational diabetes. Poor muscle tone was noted from early infancy, and she never attained good head control. She was delayed in reaching early motor milestones. At the age of 10 months, she could fix and follow objects, albeit with saccadic pursuit, and could grasp nearby objects and bring them to her mouth. She demonstrated choreoathetosis of her arms and hands. Her alertness fluctuated, putatively related to hypomotor seizures. These proved resistant to levetiracetam, valproic acid, and vigabatrin, and her eye contact and muscle tone deteriorated. At the age of 17 months, she developed status epilepticus. Phenobarbital led to satisfactory seizure control, and now she has mainly short, multifocal clonic seizures and intermittent myoclonus. She is currently fed via nasogastric tube.

Expressive speech is absent. A homozygous variant in *FRRS1L* (c.436dup [p.Ile146Asnfs*10]) was identified by whole-exome sequencing.

Targeted sequencing of *FRRS1L* in a cohort of 52 pediatric and adult individuals with early-onset Huntington-like disease and lacking triplet repeat expansions in *HTT* did not identify any predicted pathogenic alleles. Expanding our analysis to >500 in-house exomes of individuals with neurodegenerative disease of unknown etiology revealed four alleles predicted to be deleterious in the heterozygous form (Table S2). No biallelic *FRRS1L* variants predicted to be deleterious were detected in datasets from the Epi4K and EuroEPINOMICS-RES consortia.

Although *FRRS1L* is predominantly expressed in the brain, it is expressed at a lower level in fibroblasts (Figure 2B), as are AMPARs.¹⁰ Using affected fibroblasts and age- and sex-matched controls, we next determined that *FRRS1L* amounts were markedly reduced in both p.Gln321* and p.Trp282*-affected cells (Figure 2B). Given that the c.961C>T variant is found within the final exon of the transcript, we sought to determine whether this mRNA escapes nonsense-mediated decay. We found comparable levels of both control and mutant transcripts (Figure S1), indicating that the decreased abundance of *FRRS1L* in c.961C>T-affected cells is most likely related to post-transcriptional mechanisms.

Given the severe neurological phenotype in the affected individuals, we next evaluated *Frrs1l* expression in embryos and adult mice by using a lacZ reporter. In embryonic day 12.5 (E12.5) embryos, *Frrs1l* expression was evident in the ventral forebrain, but a lower level of expression was seen in the remainder of the embryos. *Frrs1l* expression was highest in the adult brain in the cortex, cerebellum, hippocampus, and basal ganglia (Figure 3). This is consistent with prior reports of robust expression in multiple brain regions, including the striatum, thalamus, and cortex.^{7,8} Developmentally, *FRRS1L* might play a role in neuronal maturation, given that AMPARs promote the formation and maturation of synapses during development.¹¹

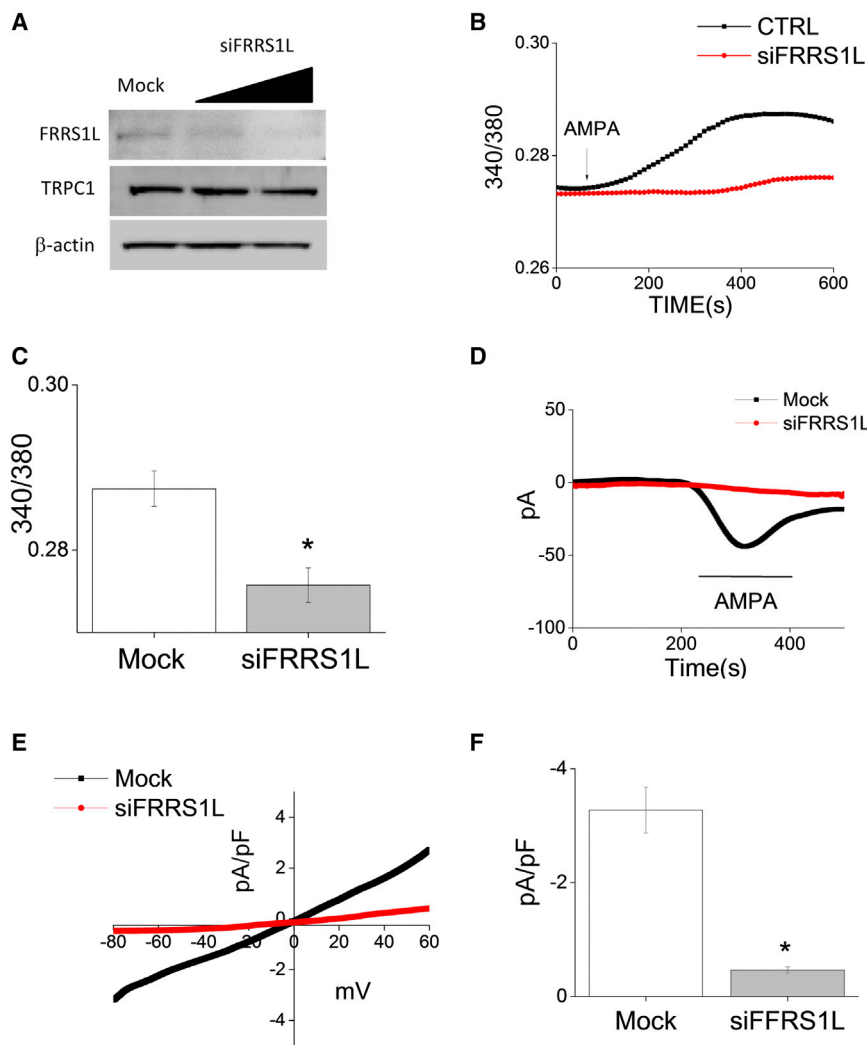


Figure 4. Effects of FRRS1L Loss on Electrophysiology

(A) siRNA knockdown of *FRRS1L* in neuro-nally differentiated SH-SY5Y cells led to *FRRS1L* amounts comparable to those seen in affected fibroblasts ($n = 3$); transient receptor potential channel 1 (1:500, Alomone) and β -actin served as loading controls.

(B) siFRRS1L-treated cells showed decreased Ca^{2+} influx after stimulation with 100 μ M AMPA (representative tracing).

(C) Mean Ca^{2+} influx was significantly decreased by *FRRS1L* knockdown (mean \pm SEM; $n = 30$ –50 cells per condition; $*p < 0.05$).

(D) A bath applied 100 μ M AMPA-induced inward currents to control and siFRRS1L cells at a holding potential of -80 mV (representative tracings).

(E) Current-voltage (I-V) curves under the conditions in (D).

(F) Mean current intensity at -80 mV (mean \pm SEM; $n = 8$ –10 recordings per condition; $*p < 0.05$).

Little is known about the biological function of FRRS1L. Despite its name (ferric chelate reductase 1-like), it lacks a ferrochelatase domain and so is unlikely to function in iron storage and processing. In a prior study, FRRS1L co-immunoprecipitated with GluR1, suggesting a direct interaction between the two proteins.⁹ We thus sought to determine whether the loss of FRRS1L in affected individuals could affect AMPAR function. The human sympathoadrenal cell line SH-SY5Y can be differentiated to a neuronal phenotype with the use of retinoic acid; these cell lines express subunits GluR1–GluR4 of AMPAR.¹² Using neuro-nally differentiated SH-SY5Y cells and siRNA, we knocked down *FRRS1L*, leading to reduced FRRS1L amounts analogous to those seen in affected cells (Figure 4A). We next performed a series of patch-clamp experiments with a focus on AMPA-mediated currents. We found that loss of FRRS1L significantly attenuated calcium influx (Figures 4B and 4C) and diminished AMPA-induced inward currents (Figures 4D–4F).

Our findings implicate FRRS1L as an important modulator of glutamate signaling with substantial implications in health and disease. AMPAR complexity is only now begin-

ning to be appreciated, given that dozens of auxiliary subunits govern ion-channel gating properties, and dynamic changes in AMPAR composition are likely to affect localization and abundance within the synapse.^{13–15} FRRS1L is an outer-core protein that is thought to function within the AMPAR and directly interact with inner-core components.⁹ The significant diminution of AMPA-mediated currents we observed with *FRRS1L*

knockdown points to an important role for the protein in normal AMPAR function, and its relevance has not previously been appreciated. Additional studies are needed to determine whether FRRS1L influences AMPAR function by modulating trafficking or by influencing channel gating properties more directly. FRRS1L could also have other roles outside of its interaction with glutamate receptors.

Our findings suggest that loss-of-function pathogenic variants might impair normal glutamatergic neurotransmission and thus lead to choreoathetosis, epilepsy, and persistent encephalopathy related to chronic disruption of normal neuronal-circuit function. Interestingly, pathogenic *STXBP1* variants, which also lead to an epileptic-dyskinetic encephalopathy, putatively impair normal SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor)-mediated neurotransmitter release, including glutamate exocytosis into the synaptic cleft.¹⁶ Pathogenic variants in another AMPAR outer-core component, *PRRT2* (MIM: 614386), have been shown to lead to seizures and choreoathetosis in affected individuals.¹⁷ Individuals with mutations in *PRRT2* generally have paroxysmal symptoms rather than a progressive course,

but such cases typically involve heterozygous pathogenic variants rather than the homozygous pathogenic variants we observed in our individuals. The recent identification of individuals with biallelic *PRRT2* mutations suggests a more severe phenotype in such individuals.¹⁸ The identification of additional individuals with pathogenic variants in *FRRS1L* or related AMPAR core proteins will help shed light on the role of these accessory proteins in modulating glutamatergic signaling in the CNS, whose complexities are now beginning to be unraveled.¹⁹

Accession Numbers

The accession numbers for the pathogenic *FRRS1L* variants reported in this paper are ClinVar: SCV000257326 (c.961C>T), SCV000257327 (c.845G>A), SCV000257328 (c.737_739del), and SCV000257329 (c.436dup).

Supplemental Data

Supplemental Data include one figure, two tables, and one movie and can be found with this article online at <http://dx.doi.org/10.1016/j.ajhg.2016.04.008>.

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Web Resources

1000 Genomes Project, <http://www.1000genomes.org/>
Burrow-Wheeler Aligner, <http://bio-bwa.sourceforge.net>
ClinVar, <http://www.ncbi.nlm.nih.gov/clinvar/>
Combined Annotation Dependent Depletion, <http://cadd.gs.washington.edu>
dbSNP, <http://www.ncbi.nlm.nih.gov/SNP/>
Ensembl, <http://www.ensembl.org/index.html>
Exome Aggregation Consortium (ExAC) Browser, <http://exac.broadinstitute.org/>

Genome Analysis Toolkit, <https://www.broadinstitute.org/gatk>
HGVS, <http://www.hgvs.org/>
International Mouse Phenotyping Consortium, <http://www.mousephenotype.org/>
Mouse Genome Informatics, <http://www.informatics.jax.org/>
MutationTaster, <http://www.mutationtaster.org/>
NCBI GRCh37 Assembly, http://www.ncbi.nlm.nih.gov/assembly/GCF_000001405.13/
NHLBI Exome Sequencing Project (ESP) Exome Variant Server, <http://evs.gs.washington.edu/EVS/>
OMIM, <http://www.ncbi.nlm.nih.gov/omim/>
PICARD, <http://picard.sourceforge.net/>
Phosphosite Plus, <http://www.phosphosite.org/>
PolyPhen-2, <http://genetics.bwh.harvard.edu/pph2/>
PROVEAN, <http://provean.jcvi.org>
RefSeq, <http://www.ncbi.nlm.nih.gov/refseq/>
SIFT, <http://sift.jcvi.org/>
UCSC Human Genome Browser (hg19), <http://genome.ucsc.edu/cgi-bin/hgGateway>
UniProt, <http://www.uniprot.org/>

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Supplemental Data

Loss-of-Function Mutations in *FRRS1L*

Lead to an Epileptic-Dyskinetic Encephalopathy

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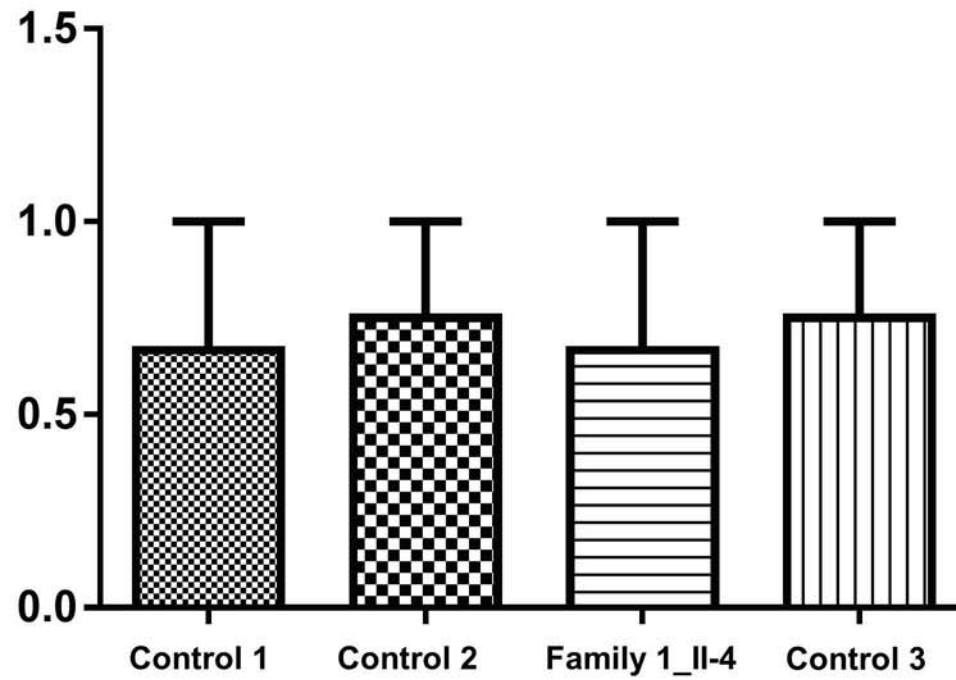


Figure S1. RT-PCR shows similar amounts of *FRRS1L* transcript in c.961C>T affected cells when compared to controls (mean \pm SEM from n = 3 experiments)

Table S1. Regions of homozygosity shared by index family members

Chromosome	Start	End	Size (Mb)
1	147,779,938	151,347,700	3.5
3	62,613	4,878,792	4.8
9	104,622,396	119,149,440	14.5
16	31,901,547	35,220,544	3.3
X	61,932,503	65,386,406	3.5

Table S2. Rare heterozygous *FRRS1L* sequence variants identified in a cohort of >500 individuals with neurodegenerative disease

<i>FRRS1L</i> variant	ESP6500	ExAc	Phenotype
c.469T>A; (p.C157S)	-	8.13E-06	Ataxia, optic nerve atrophy
c.469T>A; (p.C157S)	-	8.13E-06	Ataxia, intellectual disability
c.721delG; (p.A241fs)	-	8.13E-06	Multiple system atrophy
c.686T>C; (p.V229A)	0.000231	7.40E-04	Ataxia
c.885A>G; (p.I295M)	0.000077	1.63E-05	Multiple system atrophy