A rat model for LGI1-related epilepsies

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Mutations of the leucine-rich glioma-inactivated 1 (LGI1) gene cause an autosomal dominant partial epilepsy with auditory features also known as autosomal-dominant lateral temporal lobe epilepsy. LGI1 is also the main antigen present in sera and cerebrospinal fluids of patients with limbic encephalitis and seizures, highlighting its importance in a spectrum of epileptic disorders. LGI1 encodes a neuronal secreted protein, whose brain function is still poorly understood. Here, we generated, by ENU (N-ethyl-N-nitrosourea) mutagenesis, Lgi1-mutant rats carrying a missense mutation (L385R). We found that the L385R mutation prevents the secretion of Lgi1 protein by COS7 transfected cells. However, the L385R-Lgi1 protein was found at low levels in the brains and cultured neurons of Lgi1-mutant rats, suggesting that mutant protein may be destabilized in vivo. Studies on the behavioral phenotype and intracranial electroencephalographic signals from Lgi1-mutant rats recalled several features of the human genetic disorder. We show that homozygous Lgi1-mutant rats (Lgi1L385R/L385R) generated early-onset spontaneous epileptic seizures from P10 and died prematurely. Heterozygous Lgi1-mutant rats (Lgi1+/L385R) were more susceptible to sound-induced, generalized tonic-clonic seizures than control rats. Audiogenic seizures were suppressed by antiepileptic drugs such as carbamazepine, phenytoin and levetiracetam, which are commonly used to treat partial seizures, but not by the prototypic absence seizure drug, ethosuximide. Our findings provide the first rat model with a missense mutation in Lgi1 gene, an original model complementary to knockout mice. This study revealed that LGI1 disease-causing missense mutations might cause a depletion of the protein in neurons, and not only a failure of Lgi1 secretion.

INTRODUCTION

Epilepsy, with a lifetime prevalence of 3%, is a frequent neurological disorder. Studies on familial idiopathic epilepsies have identified multiple disease-causing genes (1). For example, mutations in the leucine-rich glioma-inactivated 1 (LGI1) gene cause an inherited epilepsy syndrome designated either autosomal dominant lateral temporal epilepsy (ADLTE) (2) or autosomal dominant partial epilepsy with auditory features (3). Focal seizures, with prominent auditory auras in about two thirds of patients, emerge in adolescence (4). Aphasic symptoms and other aberrant perceptions of a visual, vertiginous, epigastric or psychogenic nature are also

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reported, and seizures can be triggered by noises or voices (5,6). LGI1 mutations (36 published to date) (7) have been found in up to 50% of ADLTE families and 2% of sporadic cases (8). The role of LGI1 in neurological diseases was further expanded with the recent discovery that a subset of patients with limbic encephalitis (an autoimmune disorder associated with seizures in the majority of patients) (9,10) has serum antibodies against LGI1. It is speculated that the antibody-mediated disruption of LGI1 causes increased excitability, which results in seizures and other symptoms of limbic encephalopathy (11).

In contrast to other genes linked to idiopathic epilepsies, LGI1 does not encode an ion channel subunit, but rather a secreted leucine-rich repeat (LRR) protein (12). Except R407C (13), all tested LGI1 missense mutations tend to suppress protein secretion in in vitro overexpression systems (12,14–18), indicating that extracellular levels of LGI1 may be critical to its pathophysiological effects. Lgi1 protein is expressed during mouse embryogenesis and increases until adult, suggesting that it may have a role during brain development (19–21). While its functions remain unclear, Lgi1 interacts with the presynaptic Kv1.1 voltage-gated potassium channel (22), ADAM22/ADAM23 (disintegrin and metalloproteinase domains 22 and 23) (23), ADAM11 (24) and NogoR1 (25). Insights into the role of Lgi1 in epilepsy have emerged from three recent studies on Lgi1 knockout (Lgi1−/−) in mice. There is a consensus that in homozygous Lgi1−/− mice, the constitutive deletion of Lgi1 induces early-onset spontaneous seizures followed by premature death (20,26,27). Heterozygous Lgi1+/− mice do not generate seizures spontaneously, but they are more prone to seizure induction by pentylenetetrazole (26) and auditory stimuli (20). Lgi1 may cause epilepsy by modulating signaling in glutamatergic (21,26,27) but not GABAergic synaptic circuits (21,27). However, the two studies of Lgi1−/− brain slices contradict each other, suggesting that the loss of Lgi1 either reduces (26) or increases (27) alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate-mediated miniature excitatory post-synaptic currents in CA1 hippocampal neurons. An alternative pathophysiological mechanism might be that Lgi1 controls the postnatal maturation of glutamatergic synapses in the hippocampus (21) and retinogeniculate thalamic afferents (28).

In the present study, we generated and characterized Lgi1-mutant rats carrying a missense mutation (L385R). We deciphered the mechanisms by which this mutation led to a loss of function in transfected mammalian cells and primary neurons in culture. Electroencephalographic (EEG) monitoring was used to define how the L385R-Lgi1 protein affected the phenotype of homozygous and heterozygous Lgi1-mutant rats in vivo. Finally, we examined actions of antiepileptic drugs on the audiogenic seizures in heterozygous Lgi1+/-L385R rats.

RESULTS

Generation of Lgi1-mutant rats

The ENU (N-ethyl-N-nitrosourea)-mutagenized F344/NSlc rat archive (KURMA: Kyoto University Rat Mutant Archive) was screened for mutations in the Lgi1 gene by a high-throughput screening assay (29). A missense mutation (c.1154 T > G) in exon 8 of Lgi1 was found in one DNA sample of KURMA. This mutation resulted in the p.Leu385Arg/L385R amino acid substitution in the fourth epilepsy-associated repeat

**Figure 1.** The L385R mutation. (A) Schematic representation of the Lgi1 protein showing its domain organization and location of the L385R mutation. The protein is composed of two structural domains: four N-terminal LRR (in blue) and seven EAR (in yellow) in the C-terminal half of the protein. (B) Multiple protein alignments of Lgi1 protein showing the strong conservation of L385 residue in both vertebrates and invertebrates using the Alamut® Mutation Interpretation Software.
(EAR; Fig. 1A), a residue highly conserved among vertebrates and invertebrates (Fig. 1B). Four bioinformatics prediction programs of functional effects of variants were queried for L385R. All predicted a significant effect of this amino acid change on protein function: ‘not tolerated’ by SIFT (http://sift.bii.a-star.edu.sg/), ‘disease causing’ by Mutation taster (http://www.mutationtaster.org/), ‘probably damaging’ by PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2) and a P-deleterious of 0.991 by Panther (http://www.pantherdb.org/).

Interestingly, a well-documented ADLTE-causing mutation, p.Glu383Ala/E383A, was located close to the Rat mutation (3,12,14). The heterozygous Lgi1-mutant rat (Lgi1\(^+/L385R\)) was recovered from the corresponding frozen sperm by intracytoplasmic sperm injection. Nine generations were backcrossed on the F344/NSc inbred background to eliminate mutations potentially induced by ENU mutagenesis elsewhere in the genome (mean mutation frequency was \(\approx 1 \times 10^{-6}\) bp). Backcrossed Lgi1\(^{+/L385R}\) rats were then intercrossed to obtain wild-type (WT; Lgi1\(^{+/+}\)), heterozygous (Lgi1\(^{L385R/L385R}\)) and homozygous (Lgi1\(^{L385R/L385R}\)) animals. They were born in expected Mendelian ratios (Lgi1\(^{+/+}\), \(n = 17\); Lgi1\(^{L385R/L385R}\), \(n = 18\); Lgi1\(^{L385R/L385R}\), \(n = 29\); \(\chi^2 = 0.59\), not significant) and sex ratios (Lgi1\(^{L385R/L385R}\) rats: 28 females and 36 males; \(\chi^2 = 1\), not significant).

### L385R mutation impairs Lgi1 secretion in COS7 cells and cortical neurons

We asked whether the rat L385R mutation impaired Lgi1 secretion, by transiently transfecting COS7 cells with Flag-Lgi1-L385R, Flag-Lgi1-E383A or Flag-Lgi1-WT. Using an antibody directed against amino acids 200–300 of Lgi1 (ab30868), western blot analysis revealed the presence of WT Lgi1 and both mutants in the cell lysates. However, while WT Lgi1 protein was predominantly present in the cell culture medium, we did not detect the Flag-Lgi1-L385R or the Flag-Lgi1-E383A mutants (Fig. 2A). This shows that the L385R mutation prevents the secretion of Lgi1 into the culture medium of COS7 cells.

We next examined endogenous Lgi1 secretion in isolated neurons of primary cortical cultures from rat embryonic day 19 (E19) Lgi1\(^{L385R/L385R}\), Lgi1\(^{+/L385R}\) and Lgi1\(^{+/+}\) littersmates. Western blot analysis revealed only weak signal of 65 kDa in the neuron lysate of homozygous Lgi1\(^{L385R/L385R}\) rats, as well as in the neuron medium, indicating a low level of L385R-Lgi1 protein in neurons (Fig. 2B).

### L385R-Lgi1 is unstable in vivo

We then compared the endogenous L385R-Lgi1 protein level by western blot in whole brain homogenates of postnatal day 12 (P12) Lgi1\(^{L385R/L385R}\), Lgi1\(^{+/L385R}\) and WT (Lgi1\(^{+/+}\)) littermate rats. Immunoblot revealed a single band of 65 kDa in the lysate of Lgi1\(^{+/+}\). It was reduced by about half in Lgi1\(^{+/L385R}\) and was absent in Lgi1\(^{L385R/L385R}\) (\(n = 5\); Fig. 3A). The low abundance of L385R-Lgi1 protein in the brain was confirmed with a second antibody generated to the C terminus of Lgi1 (sc-9583, Santa Cruz; Supplementary Material, Fig. 1B). These findings were replicated in three additional litters of WT, heterozygous and homozygous rats aged P5 and P9 (before onset of seizures) and P12 (after onset of seizures), indicating that lack of L385R-Lgi1 was not secondary to seizures (data not shown). We next analyzed synaptic fractions of hippocampal and cortical lysates by preparing Triton X-100 crude fractions. A strong signal was detected in Lgi1\(^{+/+}\) and Lgi1\(^{L385R/L385R}\) lysates, indicating that WT Lgi1 protein was present in the synapse-enriched fraction. In contrast, only a very weak band was detected for L385R-Lgi1 protein in the Triton X-100-insoluble membrane synaptic fraction (Fig. 3B), suggesting that it is probably unstable and thus not delivered to the synapse.

We next asked whether low levels of L385R-Lgi1 protein resulted from a preferential degradation of the L385R-Lgi1 transcript. We extracted total RNAs from the whole brains of Lgi1\(^{L385R/L385R}\) (\(n = 5\)), Lgi1\(^{+/L385R}\) (\(n = 7\)) and Lgi1\(^{+/+}\) (\(n = 5\)) littermate rats and analyzed Lgi1 transcript expression by quantitative reverse transcription (RT)–polymerase chain reaction (PCR). Relative levels of the L385R-Lgi1 transcript were not significantly different from levels of the Lgi1\(^{+/+}\) transcript (Kruskal–Wallis test, not significant; Fig. 3C).
These results suggest that low neuronal levels of L385R-Lgi1 protein may result from a shortened half-life of the protein rather than the transcript.

L385R mutation has no major effect on in vitro neuronal growth

We asked whether this L385R mutation affected the growth of primary neurons plated on poly-L-lysine coated culture plates. Cortical and hippocampal neurons from E19 rats were co-cultured for 3 weeks and examined daily. No differential effect on life span or neurite outgrowth was detected between \( \text{Lgi1}^{L385R/L385R} \) and \( \text{Lgi1}^{+/-} \) rats (ImageJ measurement of total neurite network length, Kruskal–Wallis test, not significant; Fig. 4A). In addition, no major morphological differences were detected between hematoxylin-stained brains of P12 \( \text{Lgi1}^{L385R/L385R} \) rats and their \( \text{Lgi1}^{+/-} \) littermates (Fig. 4B).

Homogenous \( \text{Lgi1} \)-mutant rats are epileptic and die prematurely

At birth, we detected no differences in appearance or behavior of homozygous \( \text{Lgi1}^{L385R/L385R} \) rats and heterozygous \( \text{Lgi1}^{+/-} \) littermates. During the second postnatal week, \( \text{Lgi1}^{L385R/L385R} \) pups began to exhibit spontaneous seizures (Fig. 5A, Supplementary Material, Movie S1). They occurred from P10 at a mean frequency of 8 ± 2.8 per hour (mean ± SD, \( n = 8 \)) with a mean duration of 83 ± 4.9 s. Ictal epileptic discharges (\( n = 11 \) electroclinical seizures) were recorded by intracranial EEG in two homozygous \( \text{Lgi1}^{L385R/L385R} \) pups (Fig. 5B). Seizures typically consisted of sequences of (i) hypertonic, often asymmetric, trunk, limb and tail postures, (ii) clonies of all limbs or jerking. EEG records began with rhythmic 5–7 Hz spike activity, which increased in amplitude. It was replaced by polyspike-and-wave complexes at 1 Hz during jerking episodes which slowed (0.5 Hz) as the seizure terminated. Seizures were sometimes associated with motor automatisms, such as chewing. Such spontaneous epileptic activity was never observed in age-matched heterozygous \( \text{Lgi1}^{+/-} \) rats (\( n = 7 \)). As seizures emerged, \( \text{Lgi1}^{L385R/L385R} \) rat pups failed to gain weight. At P15, the average body weight of \( \text{Lgi1}^{L385R/L385R} \) rats was significantly (pairwise Student’s \( t \)-test, \( P < 0.009 \)) lower than that of \( \text{Lgi1}^{+/-} \) rats (respectively, 10.4, 25 and 24 g; Fig. 6A), and development slowed dramatically (Fig. 6B). All homozygous \( \text{Lgi1}^{L385R/L385R} \) rats died prematurely and the Kaplan–Meier curve revealed a mean lifetime of 13 days (\( n = 10 \)). No homozygous \( \text{Lgi1}^{L385R/L385R} \) rat...
survived beyond P17, while no Lgi1+/L385R or Lgi1+/+ littermates had died at this age (Fig. 6C). Possibly, this early mortality results from a failure to feed due to seizures.

Heterozygous Lgi1-mutant rats display increased audiogenic seizure vulnerability

Heterozygous Lgi1+/L385R rats appear normal, are fertile and live for at least 1 year. Spontaneous clinical seizures have never been observed in either pups or adults. Since partial seizures can be triggered by audiogenic events in ADLTE patients, we tested the susceptibility of heterozygous Lgi1-mutant rats to audiogenic seizures. A single 120-dB sound stimulus at 10 kHz never induced seizures in Lgi1+/L385R or Lgi1+/+ rats at 3, 5, 8 or 12 weeks of age, possibly due to this rat strain resistance to audiogenic seizures. Acoustic priming (5 min, 10 kHz, 120 dB) was thus applied to rat pups aged P16, corresponding to the critical period when rats become seizure prone (30). Primed rats were then tested for audiogenic seizures at 8 weeks of age. Auditory stimulus first induced wild running, a typical behavior of audiogenic seizures, in all Lgi1+/L385R (n = 22) and Lgi1+/+ (n = 14) rats (Table 1). Following wild running, auditory stimulation yielded generalized tonic-clonic seizures (GTCSs) in all Lgi1+/L385R rats, but only in 4 of 14 (28%) Lgi1+/+ rats (χ²= 22, P = 3 × 10⁻⁶; Fig. 7A, Supplementary Material, Movie S2). The latency from auditory stimulus to wild running was shorter in Lgi1+/L385R than Lgi1+/+ rats (Student’s t-test, P = 2.7 × 10⁻²; Fig. 7B). The duration of wild running was also shorter in Lgi1+/L385R than in Lgi1+/+ rats (Student’s t-test, P = 6.8 × 10⁻⁵; Fig. 7C), probably since wild running was more rapidly replaced by a GTCS in Lgi1+/L385R rats. The duration of GTCSs did not differ significantly in Lgi1+/L385R and Lgi1+/+ rats (Student’s t-test, P = 4.5 × 10⁻¹; Fig. 7D).

We compared cortical and hippocampal EEG signals generated by Lgi1+/L385R (n = 3) and Lgi1+/+ (n = 1) rats during auditory stimuli (Fig. 8). During wild running, movement artifacts tended to obscure EEG signals. After running terminated, EEG signals were strongly suppressed in the tonic phase of Lgi1+/L385R rats and the immobility phase of Lgi1+/+ rats. During the clonic seizure phase in Lgi1+/L385R rats, continuous rhythmic slow activity at 2–3 Hz was detected in cortex and hippocampus. EEG signals were then suppressed, as Lgi1+/L385R rats remained immobile until auditory stimuli ceased.
Effect of antiepileptic drugs on audiogenic seizures

Finally, we evaluated the efficacy of several antiepileptic drugs on audiogenic seizures in primed Lgi1+/+ and Lgi1+/L385R rats. We administered carbamazepine, phenytoin, levetiracetam or ethosuximide intraperitoneally (20 mg/kg) 30 min before the auditory stimuli in 8-week-old rats (Table 1, Supplementary Material, Movie S3). Both wild running and GTCS were completely inhibited by carbamazepine and phenytoin in Lgi1+/+ and Lgi1+/L385R rats. Levetiracetam prevented wild running and GTCS in three of four Lgi1+/L385R rats. No ictal EEG activity was detected during auditory stimulation (not shown). Ethosuximide, a prototypic generalized absence seizure drug, had no effect on seizures of Lgi1+/L385R rats (n = 4).

DISCUSSION

Here, we present the first genetically engineered animal model expressing a missense mutation in the Lgi1 gene as found in patients with ADLTE. We generated and characterized an Lgi1-mutant rat with the L385R mutation and studied its functional consequences in vivo. We first examined the impact of the mutation using an in vitro overexpression paradigm. Our results showed that this mutation prevented Lgi1 secretion in transiently transfected COS7 cells, indicating that it apparently shares common effects with ADLTE-causing missense mutations which nearly all, except one, decrease protein secretion (31). Testing endogenous expression levels of the mutated Lgi1 protein in cultured cortical neurons of Lgi1-mutant animals revealed very low levels of L385R-Lgi1 protein, both in extracellular medium from cultures and also in neuron lysates. Moreover, endogenous levels of Lgi1 protein were also substantially lower in the brain of Lgi1-mutant rats than of WT littermates. Probably, in vivo, the L385R mutation favors misfolding and so reduces Lgi1 protein stability, causing its degradation through protein quality-control mechanisms. This is consistent with in silico models predicting that a number of missense disease-causing mutations alter protein folding (31). Thus, a physiopathological loss of function may emerge not only due to a failure of protein secretion as suggested by in vitro experiments but also from a lack of correctly folded neuronal Lgi1. This new mechanism must be considered together with previous suggestions of a defective secreted extracellular Lgi1 (acting as a ligand for ADAM22/23 at the post-synaptic) (23), rather than cytoplasmic (through the modulation of Kv1.1 channel) (22). In this model, the Lgi1-mutant rat carrying a missense mutation located nearby a naturally occurring missense mutation found in ADLTE patients (3,12,14) lacks both cytoplasmic
We thus conclude that ADLTE pathogenesis due to Lgi1 mutations might also lead to instability in neuronal network activity rather than abnormal dendritic development. The phenotype of Lgi1-mutant rats possessed similarities to the ADLTE syndrome. Epileptic seizures, associated with cortical and hippocampal ictal epileptiform activity, emerged at P10 in homozygous Lgi1\(^{L385R/L385R}\) pups. Frequent and severe seizures led to death of the animals around P13. Lgi1\(^{L10/L385R}\) rats, which carry a heterozygous missense mutation recapitulating the human genetic cause, did not generate seizures spontaneously but were highly susceptible to audiogenic seizures, as patients with LGI1 mutations (seizures may be triggered by noises or voices) (6). In addition, we showed that rat audiogenic seizures responded to the same drugs as used in the human: they were suppressed by two antiepileptic drugs, carbamazepine and phenytoin, that target voltage-gated channels, but also by levetiracetam which anticonvulsant activity is mediated through interaction with the synaptic vesicle protein 2A (SV2A) (33,34). Since Lgi1 co-immunoprecipitates with several other neuronal vesicle-related proteins (35), this latter pathway involving SV2A might be promising for preventing seizures in this syndrome. As expected, ethosuximide, a first choice drug for absence seizures, did not prevent audiogenic seizures. This rat model thus permitted more detailed studies on audiogenic seizures and tests on antiepileptic molecules. As SV2A, Lgi1 may point toward novel antiepileptic therapies for drug-resistant patients.

The phenotype of Lgi1-mutant rats and Lgi1 knockout mice that we generated (20) were comparable with a clear gene dosage relation between Lgi1 and epileptic syndromes: severe early onset spontaneous seizures without gross brain anatomical abnormalities occurred in homozygous animals, and a high susceptibility to audiogenic seizures in adult heterozygous animals. Spontaneous seizures shared similar features: age at onset (P10), duration and behavioral manifestations consisting of wild running followed by tonic and/or clonic events, ictal epileptic activities present in both the hippocampus and the cortex and seizure severity causing animals to fail to gain weight by P10 and die prematurely a week after seizure onset. We note, however, that spontaneous events were more frequent in Lgi1-mutant rats (~8/h) than in Lgi1\(^{+/+}\) mice (~1–2/h). In our previous study, we concluded that seizures probably originated in the hippocampus of Lgi1\(^{+/+}\) mice since hippocampal ictal activity preceded cortical discharges. Seizures may also be initiated in the hippocampus of Lgi1-mutant rats, although we have no direct evidence on this point. We note the striking restricted time window (P10) when seizures emerge in both Lgi1\(^{+/+}\) mice and Lgi1-mutant rats. The timing of seizure onset was not exactly correlated with that of Lgi1 expression. Lgi1 expression preceded seizure onset both in mice, in which it was detected at E9.5 in the primitive eye (19) or E16 in white brain lysate (20), and in rats, in which we show an expression as early as P0 in brain lysate (Supplementary Material, Fig. 2S). Most probably, onset of seizures is related to the period of brain developmental changes, common to rats and mice, embracing maturation of excitatory synapses of the cortex and hippocampus and/or the switch in the polarity of GABAergic signaling by inhibitory interneurons (36). This natural history of seizures beginning within the 3 first weeks of life, followed by death shortly after, is common to several epileptic and extracellular Lgi1. While cortical tissue from patients is not available, we speculate that ADLTE-causing missense mutations might also lead to instability in vivo, causing a haploinsufficiency. We note such a deficiency in Lgi1 occurs in patients with limbic encephalitis and seizures, in which the immune-mediated disruption of LGI1 results in hyperexcitability (11).

While focal epilepsies are often associated with brain lesions, we observed no major abnormality in the brain morphology of Lgi1-mutant rats, although subtle changes could not be ruled out at this level of analysis. Moreover, we found no obvious defect in neuritic outgrowth or neuronal life-span in cortical cultures from Lgi1-mutant rats, contrary to previous reports, suggesting that Lgi1 may promote neurite outgrowth (25,32). We thus conclude that ADLTE pathogenesis due to the L385R mutation in Lgi1 may involve a hyperexcitability due to altered neuronal network activity rather than abnormal dendritic development.
mice models, including ADAM23−/− (32), ADAM22−/− (37), Kv1.1−/− (38) and Scna1−/− (39) mice.

In conclusion, we report a unique and original rat model of Lgi1-related epilepsies, which is complementary to knockout mice. It gave us the opportunity to better understand the consequences of missense mutations on the fate of the mutant Lgi1 protein, revealing a major finding that L385R-Lgi1 protein is unstable in vivo, explaining the severe epileptic phenotype of Lgi1-mutant rats. We speculate that the overall similar phenotype observed in homozygous Lgi1-mutant rats (L385R-Lgi1) and Lgi1−/− mice (the absence of Lgi1) is due to the rapid degradation of Lgi1-L385R leading to a

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Prime stimulation at age P16 was applied as follow: 120 dB, 10 kHz for 5 min. Target stimulation at 8 weeks of age was performed as follow: 120 dB, 10 kHz for 1 min. Antiepileptic drugs (20 mg/kg) were injected intraperitoneally 30 min before auditory stimulation.

**Figure 7.** Susceptibility to audiogenic seizures. (A) Primed rats were tested at 8 weeks for audiogenic seizures with a stimulus of 10 kHz, 120 dB applied for 1 min. GTCSs were induced in all Lgi1+/−,L385R rats but in only 28% of Lgi1+/+ rats. (B) The time to onset of wild running was shorter in Lgi1+/−,L385R rats than in Lgi1+/+ rats. (C) The duration of wild running was shorter in Lgi1+/−,L385R than in Lgi1+/+ rats. (D) The duration of GTCS was not significantly different between Lgi1+/−,L385R (n = 22) and Lgi1+/+ (n = 4). **P < 0.01 and *P < 0.05 by Student’s t-test.
haploinsufficiency equivalent to a gene knockout. Thanks to this model, we also investigated the consequences of Lgi1-deficiency on the neuronal and neurite outgrowth. Finally, the heterozygous Lgi1"L385R" rats allowed us to initiate pharmaco-logical studies on their sound-induced seizures, which replicate the auditory triggering of seizure in the human.

MATERIALS AND METHODS

ENU mutagenesis in rats

ENU mutagenesis and screening protocols using MuT-POWER in rats have been described (29). The sperm archive KURMA has been deposited in the National BioResource Project-Rat in Japan (NBRP-Rat: www.anim.med.kyoto-u.ac.jp/nbr). Primers were designed to amplify by PCR the exonic region of the rat Lgi1 gene from ~50 bp flanking each intron (Supplementary Material, Table S1). Sequencing was performed with BigDye terminator mix, followed by the protocol for the Applied Biosystems 3100 DNA Sequencer. Lgi1-mutant rats were recovered from frozen sperm by intracytoplasmic sperm injection.

Animals

Lgi1-mutant rats (strain name, F344-Lgi1"L385R") were deposited in NBRP-Rat (N° 0656). They were kept and bred at the Institute of Laboratory of Animals, Graduate School of Medicine, Kyoto University, in air-conditioned rooms under a 14 h light/10 h dark cycle. Animal care and experiments were conformed to the Guidelines for Animal Experiments and were approved by the Animal Research Committee of Kyoto University.

Genotyping of Lgi1-mutant rats

Exon 8 of Lgi1 was amplified by PCR with Ex8-1 primers (Supplementary Material, Table S1) using the Ampdirect Plus® PCR buffer (Shimadzu) and FTA® card for blood samples. PCR products were then sequenced with BigDye terminators mix.
Western blots

Littermate rat P5, P9 and P12 pups were decapitated; whole brains were quickly removed and lysed in 3 M urea, 2.5% dodecylsulfate de sodium, 50 mM Tris, 30 mM NaCl buffer (total brain homogenates). For synaptic fractions, brains of littermate rats were homogenized in 50 mM Tris, 5 mM acide éthyle`ne diamine tétraace´tique 120 mM NaCl with complete inhibitor cocktail, spun for 1 h at 165 000 × g and pellets resuspended with 1% Triton X-100. Total protein concentrations were determined by the bichinonic acid assay method (Pierce). Twenty-five micrograms of each sample was separated on 10% Tris-glycine polyacrylamide gels were analyzed by western blot with the following antibodies: rabbit polyclonal anti-Lgi1 antibody (ab30868; 1 μg/ml; Abcam), goat polyclonal anti-Lgi1 antibody (sc-9583; 1 μg/ml; Santa Cruz) and rabbit anti-actin antibody (1/1000, Sigma Aldrich).

Cell culture and transfection

Drs K. Senechal and J. Noebels kindly provided the mouse WT Lgi1 cDNA with a Flag-tag at the N terminus. Lgi1-E383A and Lgi1-L385R were generated using the Quik-Change® Site-Directed Mutagenesis Kit. COS7 cells were cultured in Dulbecco modified Eagle’s minimal essential medium containing 10% fetal bovine serum, penicillin and streptomycin. Transient transfections were performed using LipofectamineTM 2000 according to instructions (Invitrogen), followed by a 14–16-h incubation in serum-free media. Cells and media were analyzed 24–36 h after transfection. Cell lysates and conditioned media were prepared as described (14) and analyzed by western blot.

Neuronal cultures

Primary cortical and hippocampal cultures were prepared from the brains of 10 individual rat embryos at E19. Neurons were dissociated using the Nerve-Cell culture system (Sumitomo Bakelite Co.) on 35-mm poly-L-lysine coated dishes. Each culture was derived from a single embryo. Cultures were daily observed for 21 days. Neuronal outgrowth was imaged and measured automatically using ImageJ.

Quantitative RT–PCR

Whole brains were removed from P9 rats (Lgi1+/+, n = 5; Lgi1+/L385R, n = 7; Lgi1L385R/L385R, n = 5) and stored in RNA-later® solution (Applied Biosystems). Total RNA was isolated using RNaseasy Miniprep columns (Qiagen) and contaminating DNA was depleted using RNase-free DNase. First-strand cDNA was synthesized from 5 μg of total RNA by oligo dT-primed reverse transcription (ThermoScript™ Reverse Transcriptase, Invitrogen). Quantitative PCRs were performed as triplicates using the Quantifast Multiplex PCR Kit (Qiagen) with pre-designed probes for rat Lgi1 (Quantifast Probe Assays) and peptidylprolyl isomerase A (PPIA) as a reference gene included in all multiplex PCRs. The error bars of the quantitative PCR represent SDs of triplicates.

Brain histochemistry

Lgi1+/+ (n = 1), Lgi1+/L385R (n = 2) and Lgi1L385R/L385R (n = 1) littermates aged P12 were deeply anesthetized with sodium pentobarbital (50 mg/kg by intraperitoneal injection). Brains were removed, fixed in Bouin’s fixative and embedded in paraffin. Morphological changes were evaluated from hematoxylin and eosin-stained, 4-μm thick paraffin sections.

Animal surgery and intracranial EEG recordings

Cortical EEG was recorded from Lgi1L385R/L385R P10 rats (during 3 continuous hours) and Lgi1+/+ and Lgi1L385R/L385R rats aged 8 weeks. Rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (40 mg/kg) and the heads were fixed in a stereotoxic instrument. One-mm-diameter screw electrodes were implanted into the epidural space of the left frontal cortex. A reference electrode was fixed on the frontal cranium. For hippocampal EEG, 0.2-mm-diameter stainless-steel electrodes were implanted in the hippocampus (3.8 mm caudal, 2.0 mm lateral to the bregma and 2.2 mm from the cortex surface). A miniature plug was positioned and fixed on the midline of the skull to provide electrical connections. After 1-h recovery period for P10 rats and 1-week recovery period for 8-week-old rats, animals were placed in a shielded box (40 × 40 × 40 cm3) and the EEG signals were amplified with a sampling rate of 0.5–100 Hz with a 8-channel system (MEG-6108; Nihon Kohden) and recorded (RTA-1100; Nihon Kohden) under free-moving conditions. The signals were stored in a computer for analysis (ML845; PowerLab). Behavioral changes were simultaneously observed with video records.

Acoustic stimulation

The testing apparatus consisted of a 17 × 25 × 13-cm³ plastic cage placed inside a larger sound-proof box. Acoustic stimulation was administered from a loudspeaker (JBL Professional) centrally placed on the cover of the cage. Tone bursts were delivered by a sound stimulator (DPS-725, Dia Medical System Co.) and the signal was amplified using a power amplifier (D75-A, Amcron). Lgi1+/+ and Lgi1L385R/L385R littermate rats were exposed individually to intense auditory stimulation after 1-min habituation. Priming stimulation was performed in P16 rats with a sound stimulus of 120 dB at 10 kHz for 5 min. Target stimulation consisted of a 120-dB sound stimulus at 0.5–100 Hz with a 8-channel system (MEG-6108; Nihon Kohden) and recorded (RTA-1100; Nihon Kohden) under free-moving conditions. The signals were stored in a computer for analysis (ML845; PowerLab). Behavioral changes were measured from video records.

Antiepileptic drugs administration

Antiepileptic drugs (Sigma-Aldrich) were administered intraperitoneally 30 min before target stimulation with therapeutic range (20 mg/kg). Carbamazepine and ethosuximide were first dissolved in polyethylene glycol 400 then in water. Phenytoin
was first dissolved in 0.5 N NaOH and then diluted with saline solution. Levetiracetam was dissolved in saline solution.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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