A RAT MODEL FOR LGI1-RELATED EPILEPSIES

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ABSTRACT

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 (ADLTE). Furthermore, LGI1 is the main antigen present in sera and cerebrospinal fluids of patients with limbic encephalitis and seizures, highlighting the importance of LGI1 in a spectrum of epileptic disorders. *LGI1* codes for a neuronal secreted protein, which brain function is still largely unknown. Here, we generated *Lgi1*-mutant rats carrying a missense mutation (L385R) by ENU (N-ethyl-N-nitrosourea) mutagenesis. We found that the L385R mutation prevents secretion of Lgi1 protein by COS7 transfected cells. However, the L385R-Lgi1 protein was found at low levels in the brains and primary cultured neuron lysates of *Lgi1*-mutant rats, suggesting that mutant protein may be destabilized *in vivo*. Studies on the behavioral phenotype and intracranial electroencephalographic signals from homozygous and heterozygous *Lgi1*-mutant rats recalled several features of the human genetic disorder. We found that homozygous *Lgi1*-mutant rats generated early-onset spontaneous epileptic seizures from P10 and died prematurely. Adult heterozygous *Lgi1*-mutant rats were much 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, that are commonly used to treat partial seizures, but not by the prototypic absence seizure drug, ethosuximide. Our findings provide the first rat model for a missense mutation in the *Lgi1* gene, a model complementary to knockout mice in studies on *Lgi1*-related epilepsies, and reveal a new concept that *LGI1* disease-causing mutations might cause haploinsufficiency, and not only a failure of secretion of the protein.

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 (ADPEAF) (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 reported, and seizures can be triggered by noises or voices (5, 6). This seizure semiology tends to indicate a neocortical origin in the lateral temporal lobe. *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 antibody-mediated disruption of LGI1 causes increased excitability, which results in seizures and other symptoms of limbic encephalopathy.

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 (11). To the exception of R407C (12), all tested *LGI1* missense mutations tend to suppress protein secretion in *in vitro* overexpression systems (11, 13-17), indicating that extracellular levels of LGI1 may be critical to its pathophysiological effects. Lgi1 protein is expressed in the developing brain during embryogenesis and increases until adult, suggesting that Lgi1 may have a role during brain development (18-20). While its functions remain unclear, Lgi1 interacts with the presynaptic Kv1.1 voltage-gated potassium channel (21), ADAM22/ADAM23 (disintegrin and metalloproteinase domain 22 and 23)(22), ADAM11 (23) and NogoR1 (24). Insights into

the role of Lgi1 in epilepsy have emerged from recent studies on *Lgi1* knockout in mice. There is a consensus that in homozygous *Lgi1*$^{-/-}$ mice, the constitutive deletion of *Lgi1* induces early-onset spontaneous seizures with a premature death (19, 25, 26). Heterozygous *Lgi1*$^{+/-}$ mice do not generate seizures spontaneously, but they are more susceptible to seizure induction by pentylenetetrazole (25) and auditory stimuli (19). Suppression of *Lgi1* may be epileptogenic by modulating signaling in glutamatergic but not GABAergic synaptic transmissions (25, 26) as well as controlling postnatal maturation and pruning of glutamatergic synapses in the hippocampus (20) and retinogeniculate afferents in the thalamus (27).

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 mammalian cells and primary neurons in culture. Electroencephalographic (EEG) monitoring was then 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 (28). 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 (EAR) (Figure 1A), a residue highly conserved among vertebrates and invertebrates (Figure 1B). It was predicted to be "not tolerated" by the SIFT prediction tool of functional effects of variants (http://blocks.fhcrc.org/sift/SIFT.html). Interestingly, a well-documented ADLTE-causing mutation, p.Glu383Ala/E383A, was located close to the Rat mutation (3, 11, 13). 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/NSIc inbred background to eliminate mutations potentially induced by ENU-mutagenesis elsewhere in the genome (mean mutation frequency was ~1 in 4 x 10^6 base pairs). Backcrossed *Lgi1*$^{+/L385R}$ rats were then intercrossed to obtain wild-type (WT), heterozygous (+/L385R) and homozygous (L385R/L385R) animals. They were born in expected Mendelian ratios (+/+, n=17; homozygous, n=18; heterozygous, n=29; $\chi^2 = 0.59$, not significant) and sex ratios (*Lgi1*$^{L385R/L385R}$ rats: 28 females, 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 (wild-type Lgi1). Using an antibody directed against amino acids 200-300 of Lgi1 (ab30868), Western blot analysis revealed the presence of wild-type Lgi1 and both mutants in the cell lysates. However, while wild-type Lgi1 protein was predominantly present in the cell culture medium,

we did not detect the Flag-Lgi1-L385R or the Flag-Lgi1-E383A mutants (Figure 2A). This shows that the L385R mutation prevents secretion of Lgi1 into the culture medium of COS7 cells.

We next examined endogenous Lgi1 secretion in isolated neurons of primary cortical cultures from embryonic day 19 Lgi1$^{L385R/L385R}$, Lgi1$^{+/L385R}$ and *Lgi1*$^{+/+}$ littermates. 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 (Figure 2B).

L385R mutation affects *in vivo* stability of Lgi1 protein

We then compared the endogenous L385R-Lgi1 protein level by Western blot in whole brain homogenates of P12 *Lgi1*$^{L385R/L385R}$, *Lgi1*$^{+/L385R}$ and wild-type (*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; Figure 3A). The low abundance of L385R-Lgi1 protein in brain was confirmed with a second antibody generated to the C-terminus of Lgi1 (sc-9583, Santa Cruz) in whole brain lysates from P9 rats (Figure 1S, supplementary data). These findings were replicated in 3 additional litters of wild type, heterozygous and homozygous rats aged P5 and P9 (before onset of seizures) and P12 (after onset of seizures), indicating that shortened half-life of L385R-Lgi1 was not due to seizures-induced damage (data not shown). We next analyzed synaptic fractions of hippocampal and cortical lysates by preparing Triton X100-soluble crude fractions. In *Lgi1*$^{+/+}$ and *Lgi1*$^{+/L385R}$ rats, a strong signal was detected, indicating Lgi1 protein was present at the synapse. In contrast, similarly to whole brain homogenates, L385R-Lgi1 protein was not detected in the synaptic membrane fraction (Figure 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 L385R-*Lgi1* transcript. We extracted total RNAs from 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 RT-PCR. Relative levels of the L385R-Lgi1 transcript were not significantly different from levels of the $Lgi1^{+/+}$ transcript (Kruskal-Wallis test, P non significant; Figure 3C). These results suggest that low neuronal levels of L385R-Lgi1 protein may result from a rapid turnover of the protein rather than the transcript.

L385R mutation has no major effect on *in vitro* neuronal growth

We asked whether this L385R mutation modified the growth of primary neurons plated on poly-L-lysine coated culture vessels. 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 $Lgi1^{L385R/L385R}$ and $Lgi1^{+/+}$ rats (Image J measurement of total neurite network length, Kruskal-Wallis test, P non significant) (Figure 4A). No major morphological differences were detected between hematoxylin-stained brains of P12 $Lgi1^{L385R/L385R}$ rats and their $Lgi1^{+/L385R}$ and $Lgi1^{+/+}$ littermates (Figure 4B).

Homozygous *Lgi1*-mutant rats are epileptic and die prematurely

At birth, we detected no differences in appearance or behavior of homozygous $Lgi1^{L385R/L385R}$ rats and heterozygous $Lgi1^{+/L385R}$ and $Lgi1^{+/+}$ littermates. During the second postnatal week, $Lgi1^{L385R/L385R}$ pups began to exhibit spontaneous seizures (Figure 5A, movie 1). They occurred at a mean frequency of 8±2.8 per hour (mean± SD, n=8) from P10. Ictal epileptic discharges (n=11 electro-clinical seizures) were recorded by intracranial electroencephalography (EEG) in two homozygous $Lgi1^{L385R/L385R}$ pups (Figure 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 *Lgi1*$^{+/L385R}$ (*n*=7) or *Lgi1*$^{+/+}$ littermates (*n*=7).

As seizures emerged, *Lgi1*$^{L385R/L385R}$ rat pups began to lose body weight. At P15, the body weight of *Lgi1*$^{L385R/L385R}$ rats was significantly (*P*<0.009, t-test) lower than that of *Lgi1*$^{+/L385R}$ or *Lgi1*$^{+/+}$ rats (Figure 6A), and development slowed dramatically (Figure 6B). All homozygous *Lgi1*$^{L385R/L385R}$ rats died prematurely and the Kaplan-Meier curve revealed a mean lifetime of 13 days (*n*= 10). No homozygous *Lgi1*$^{L385R/L385R}$ rat survived beyond P17, while no *Lgi1*$^{+/L385R}$ or *Lgi1*$^{+/+}$ littermates had died at this age (Figure 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 one year. Spontaneous clinical seizures have never been observed either in 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 (AGS). A single 120 dB sound stimulus at 10 kHz never induced a seizure in *Lgi1*$^{+/L385R}$ or *Lgi1*$^{+/+}$ rats at 3, 5, 8 or 12 weeks of age, possibly due to this rat strain resistance. 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 (29). 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 at 8 weeks (Table 1). Following wild running, we noted that auditory stimulation yielded generalized tonic-clonic seizures (GTCS) in all *Lgi1*$^{+/L385R}$ rats, but only in 28 % of *Lgi1*$^{+/+}$ rats (100 % versus 28 %, χ^2=36, *P* =1.9 10^{-9}) (Figure 7A, movie 2). 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}; Figure 7B). The duration of wild

running was also shorter in *Lgi1*$^{+/L385R}$ than in *Lgi1*$^{+/+}$ rats, probably since wild running was more rapidly replaced by a GTCS in *Lgi1*$^{+/L385R}$ rats (Student's t test, P=6.8 10^{-5}; Figure 7C). The duration of GTCS did not differ significantly in *Lgi1*$^{+/L385R}$ and *Lgi1*$^{+/+}$ rats (Student's t test, P=4.5 10^{-1}; Figure 7D).

We compared cortical and hippocampal EEG signals generated by *Lgi1*$^{+/L385R}$ (n=3) and *Lgi1*$^{+/+}$ rats (n=1) during auditory stimuli (Figure 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 and ethosuximide intraperitoneally (20 mg/kg) at 30 min before the auditory stimuli in 8 week-old rats (Table 1, movie 3). 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 3 of 4 *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 to express a missense mutation in the *Lgi1* gene. We generated and characterized a *Lgi1*-mutant rat with a missense mutation (L385R) and studied its functional consequences *in vivo*. We first examined the impact of the mutation using *in vitro* overexpression paradigm. Our results showed that this mutation

prevented Lgi1 secretion in transiently transfected COS7 cells. This variant is thus deleterious and apparently shares common effects with ADLTE-causing missense mutations which nearly all decrease protein secretion, except one (30). 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 animals than of wild-type 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* computational models predicting that a number of disease-causing mutations alter protein folding (30). Thus a physiopathological loss-of-function may emerge not only due to a failure of protein secretion 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) (22), rather than cytoplasmic (through the modulation of Kv1.1 channel) (21). Here, the *Lgi1*-mutant rat carrying a missense mutation located nearby a well-characterized naturally occurring missense mutation found in ADLTE patients (3, 11, 13), lacks both cytoplasmic and extracellular Lgi1. While cortical tissue from patients is not available, we speculate that ADLTE-associated 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 immune-mediated disruption of LGI1 results in hyperexcitability (31).

While focal epilepsies are often associated with brain lesions, we detected no major abnormality in brain morphology of *Lgi1*-mutant rats. Since Lgi1 may promote neurite outgrowth *in vitro* via ADAM23 (32) and NogoR1 (24), we examined neuron growth in co-cultures of cortical and hippocampal neurons from *Lgi1*-mutant rats. We detected no obvious

defect in neuritic outgrowth or neuronal life-span suggesting that neuronal network activity, rather than dendritic architecture may contribute to hyperexcitability in this rat.

The phenotype of 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 homozygous animals around P13. *Lgi1*$^{+/L385R}$ rats, which carry a heterozygous missense mutation similar to ADLTE patients, did not generate seizures spontaneously but were highly susceptible to audiogenic seizures, as patients with *LGI1* mutations are susceptible to auditory-induced seizures (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 via 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 anti-epileptic molecules. As SV2A, Lgi1 may point towards novel antiepileptic therapies for drug-resistant patients.

The *Lgi1*-mutant rat is therefore a relevant animal model for modeling Lgi1-related epilepsy. It shares several features with the phenotype of *Lgi1* knockout mice (19): (i) a natural history of severe early onset spontaneous seizures (age at onset, semiology of seizures, EEG pattern and premature death associated with loss of body weight), (ii) an absence of morphological abnormalities, (iii) audiogenic seizures in heterozygous rats recapitulating the genetic cause and mimicking the auditory triggering of seizure in the human. We note the similar age of seizure onset (around P10) in *Lgi1*-null mice and *Lgi1*-mutant rats that might be

consistent with the timing of Lgi1 expression and maturation of glutamatergic synapses similar to both species. We were initially surprised to define a similar phenotype in homozygous *Lgi1*-mutant rats (L385R-Lgi1) and *Lgi1*-null mice (absence of Lgi1). However, our discovery of a rapid degradation of Lgi1-L385R suggests the point mutation induces a haploinsufficiency that is equivalent to a gene knockout.

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 protein, revealing a major finding that L385R-Lgi1 protein is unstable *in vivo*. 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 pharmacological studies on their auditory triggered-seizures which are close to those occurring in ADLTE patients.

MATERIAL AND METHODS

ENU mutagenesis in rats

ENU mutagenesis and screening protocols using MuT-POWER in rats have been described (28). 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 (supplemental Table 1). 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-$Lgi1^{m1kyo}$) 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 14h light/10h dark cycle. Animal care and

experiments 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 (supplemental Table 1) 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 pups aged postnatal day 9 (P9) and P12 were decapitated; whole brains were quickly removed and lysed in 3M urea, 2.5% SDS, 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 EDTA, 120 mM NaCl with complete inhibitor cocktail, spun for 1h at 165,000 × g and pellets resuspended with 1% Triton X-100. Total protein concentrations were determined by the BCA method (Pierce). Twenty-five µg of each sample were separated on 10 % Tris-glycine polyacrylamide gels were analyzed by Western blot with the following antibodies: rabbit polyclonal anti-Lgi1 antibody (ab30868; 1ug/ml; Abcam), goat polyclonal anti-Lgi1 antibody (sc-9583; 1ug/ml; Santa Cruz) and rabbit anti-actin antibody (1/1000, Sigma Aldrich), and detected with the SuperSignal Chemiluminescent Substrate (Pierce).

Cell culture and transfection

Drs. K. Senechal and J. Noebels kindly provided the mouse wild-type Lgi1 cDNA with a Flag tag at the N-terminus. Lgi1-E383A and Lgi1-L385R were generated using the QuikChange® Site-Directed Mutagenesis Kit. COS7 cells were cultured in DMEM containing 10% fetal bovine serum, penicillin and streptomycin. Transient transfections were performed using Lipofectamine™ 2000 according to instructions (Invitrogen), followed by a 14-16 hour incubation in serum-free media. Cells and media were analyzed 24-36 hours after transfection.

Cell lysates and conditioned media were prepared as described (13) and analyzed by Western blot.

Neuronal cultures

Cortex and hippocampus were removed from ten rat embryos aged embryonic day 19 (E19) and dissociated using the Nerve-Cell culture system (Sumitomo Bakelite co). Neurons were plated at 10^5 cells/ml in neuron culture medium (Sumitomo Bakelite co) on 35mm poly-L-Lysine coated dishes. They were cultured for 12 days and then lysed as previously described in COS7 cells. Neuronal outgrowth was imaged and measured automatically using ImageJ.

Quantitative reverse transcription PCR

Whole brains were removed from P9 rats (*n*=6) and stored in RNA*later*® solution (Applied Biosystems). Total RNA was isolated with RNeasy 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 PCR were performed as triplicates using the QuantiFast Multiplex PCR Kit (Qiagen) using predesigned probes for Lgi1 (QuantiFast Probe Assays) and peptidyl prolyl isomerase A (PPIA) as a reference gene included in all multiplex reaction. The error bars of the quantitative PCR represent SDs of triplicates.

Brain histochemistry

Lgi1$^{+/+}$ littermates (*n*=1), *Lgi1*$^{+/L385R}$ (*n*=2) and *Lgi1*$^{L385R/L385R}$ (*n*=1) 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 homozygous P10 rats (during 3 continuous hours) and heterozygous rats aged 8 weeks. Rats were anesthetized with an intraperitoneal injection of

sodium pentobarbital (40 mg/kg) and the heads were fixed in a stereotaxic 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-hour 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 cm^3) 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 recording.

Acoustic stimulation

The testing apparatus consisted of a 17×25×13 cm 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*$^{+/L385R}$ and *Lgi1*$^{+/+}$ littermate rats were exposed individually to intense auditory stimulation after 1-minute 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 10 kHz for 1 min at 8 weeks (36). The onset, latency, and duration of wild running and GTCS were measured from video records.

Antiepileptic drugs administration

Antiepileptic drugs (Sigma-Aldrich) were administrated intraperitoneally 30 minutes 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.

FIGURE LEGENDS

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 2 structural domains: four N-terminal leucine-rich repeats (LRR in blue) and seven epilepsy-associated repeats (EAR in yellow) in the C-terminal half of the protein. (**B**) Multiple protein alignments of Lgi1 protein showing strong conservation of L385 residue in both vertebrates and invertebrates using the Alamut® Mutation Interpretation Software.

Figure 2. Lack of secretion of L385R-Lgi1 mutant protein in COS7 cells and cortical neurons. (**A**) COS7 cells were transiently transfected with the wild-type (WT) or indicated mutant Flag-Lgi1-expressing plasmids. Cell lysates and cell media were analyzed by Western blot with anti-Lgi1 antibody (ab30868). Lgi1-WT was detected in both the lysate and the culture media, while Lgi1-L385R and Lgi1-E383A mutants were only detected in the cell lysates. (**B**) Cortical and hippocampal neurons from embryonic day 19 (E19) $Lgi1^{+/+}$, $Lgi1^{+/L385R}$ *and* $Lgi1^{L385R/L385R}$ littermate rats were cultured. Cell lysates and media were analyzed by Western blot with anti-Lgi1 antibody (ab30868). L385R-Lgi1 mutant protein was weakly detected in both the lysates and the culture media, in contrast to the wild-type Lgi1 protein. Ponceau staining indicated equal loading in each well.

Figure 3. Instability of the L385R-Lgi1 mutant protein. (**A**) We assessed Lgi1 protein expression by Western blot of whole brain lysates of $Lgi1^{+/+}$ (n=1), $Lgi1^{+/L385R}$ (n=1) and $Lgi1^{L385R/L385R}$ (n=5) littermate rats aged postnatal day 12 (P12). Wild-type Lgi1 protein was detected, but not L385R-Lgi1. Equal amounts of proteins were loaded as shown by the actin control. (**B**) Lysates from synaptic fractions of $Lgi1^{+/+}$ (n=1), $Lgi1^{+/L385R}$ (n=1) and $Lgi1^{L385R/L385R}$ (n=1) littermate rats aged P12 were loaded. L385R-Lgi1 mutant protein was not

detected. (**C**) Quantitative PCR on total cDNAs of *Lgi1*$^{+/+}$ (*n*=5), *Lgi1*$^{+/L385R}$ (*n*=6) and *Lgi1*$^{L385R/L385R}$ (*n*=6) rat brains. Data are means +/- SDs of triplicates (*P* non significant) corresponding to expression of Lgi1 transcript in relation to the housekeeping gene PPIA.

Figure 4. **No major effect of L385R mutation on neuronal growth**. (**A**) Photography of cortical neurons from E19 *Lgi1*$^{+/+}$ (*n*=2) and *Lgi1*$^{L385R/L385R}$ (*n*=6) littermate rats after 4 or 7 days in culture. We observed no major difference on the length of neurites or survival in *Lgi1*$^{L385R/L385R}$ rats compared to *Lgi1*$^{+/+}$ littermates. Background was subtracted using ImageJ. (**B**) Hematoxylin -staining of coronal brain sections show similar morphology of dentate gyrus in *Lgi1*$^{+/+}$ (*n*=1), *Lgi1*$^{+/L385R}$ (*n*=1) and *Lgi1*$^{L385R/L385R}$ (*n*=1) littermates aged P12.

Figure 5. Spontaneous epileptic seizures in homozygous *Lgi1*$^{L385R/L385R}$ rats. (A) Photography during a spontaneous seizure in a P10 *Lgi1*$^{L385R/L385R}$ rat showing asymmetric clonie of the four limbs. **(B)** Epidural EEG recording in a P10 *Lgi1*$^{L385R/L385R}$ rat showing the onset and end of an electroclinical seizure (trace corresponds to left cortex). Behavioral modifications are correlated with EEG event; (i) Tonic attack, (ii) Jerking.

Figure 6. Premature death and reduced body weight in homozygous *Lgi1*$^{L385R/L385R}$ rats. (**A**) Body weight was comparable for *Lgi1*$^{+/+}$ (*n*=6), *Lgi1*$^{+/L385R}$ (*n*=7) and *Lgi1*$^{L385R/L385R}$ (*n*=5) animals at birth. The body weight of *Lgi1*$^{L385R/L385R}$ rats became reduced with respect to *Lgi1*$^{+/L385R}$ and *Lgi1*$^{+/+}$ rats after P10. (**B**) Photography shows *Lgi1*$^{L385R/L385R}$ rats smaller than *Lgi1*$^{+/L385R}$ and *Lgi1*$^{+/+}$ littermates at P14. (**C**) Kaplan-Meier survival curves of *Lgi1*$^{+/+}$ (*n*=10), *Lgi1*$^{+/L385R}$ (*n*=10) and *Lgi1*$^{L385R/L385R}$ (*n*=10) rats from P0 to P17. All *Lgi1*$^{L385R/L385R}$ rats had died at P17.

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. Generalized tonic-clonic seizures (GTCS) 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 time of GTCS was not significantly different between *Lgi1*$^{+/L385R}$ (*n*=22) and *Lgi1*$^{+/+}$ (*n*=4). ** denotes $P < 0.01$ and * denotes $P < 0.05$ by student's t-test.

Figure 8. EEG recording in *Lgi1*$^{+/L385R}$ rat during audiogenic seizure. Traces of typical cortical and hippocampal EEG responses to auditory stimuli from an 8-week-old *Lgi1*$^{+/L385R}$ rat. Behavioral changes were correlated with EEG events; 1: Wild running, 2: Tonic attack, 3: Clonic convulsions, 4: Immobility, 5: Alternative knee bending exercise. Movement artefacts are present on the EEG signal during the wild running phase and mostly suppressed in the second tonic phase. Rhythmic low-voltage slow activities were observed in the third clonic phase, in cortex and hippocampus. They decreased in the fourth immobility phase, and were suppressed in the final phase until the end of the stimulus. Cx, cortex; Hp, hippocampus.

MOVIE LEGENDS

Movie 1. Video recording of a postnatal day 12 *Lgi1*$^{L385R/L385R}$ pup during a spontaneous epileptic seizure.

Movie 2. Video recording of a *Lgi1*$^{+/L385R}$ rat aged 8-weeks during an audiogenic seizure.

Movie 3. Video recording during auditory stimulus of a *Lgi1*$^{+/L385R}$ rat aged 8-weeks injected with carbamazepine.

SUPPLEMENTARY FIGURE LEGENDS

Figure 1S. Endogenous Lgi1 expression. We assessed Lgi1 protein expression by Western blot of lysates from brain samples of *Lgi1*$^{L385R/L385R}$, *Lgi1*$^{+/L385R}$ and *Lgi1*$^{+/+}$ littermate rats aged P9. Wild-type Lgi1 protein was detected, but not L385R-Lgi1 using the sc-9583 antibody against the C-terminus of Lgi1 (**A**) and the ab30868 antibody directed against amino

acid 200-300 (**B**). Asterisk indicates non-specific band. Equal amounts of proteins were loaded as shown by the actin control.

FUNDINGS

This work was supported by Japan Society of the Promotion of Sciences [to SB and SI]; FP6 Integrated Project EPICURE; Ministry of Education, Culture, Sports, Science and Technology Grant-in-Aid for Scientific Research (grant N°16200029); and Industrial Technology Research Grant Program in 2008 from New Energy and the Industrial Technology Development Organization of Japan [to TM].

ACKNOWLEDGMENTS

We would like to thank Philippe Couarch and Yayoi Kunihiro for technical help, Aurélien Dauphin for imaging, Sophie Rivaux-Pechoux for statistical analysis, and Richard Miles, Vincent Navarro and Yukihiro Ohno for helpful discussion.

AUTHOR CONTRIBUTIONS

SB and SI conceived the study and wrote the manuscript. TM designed and coordinated the study. MB performed Western blot assay to investigate Lgi1 expression level. NF assisted with EEG recording and animal breeding. MK performed histological studies. YO assisted with EEG recording and pharmacological trial. AT, TA, MU performed ICSI technology. AI, RT, TS participated in interpreting the results and revised the manuscript. ELG participated to the writing and revision of the manuscript. All authors read and approved the final manuscript.

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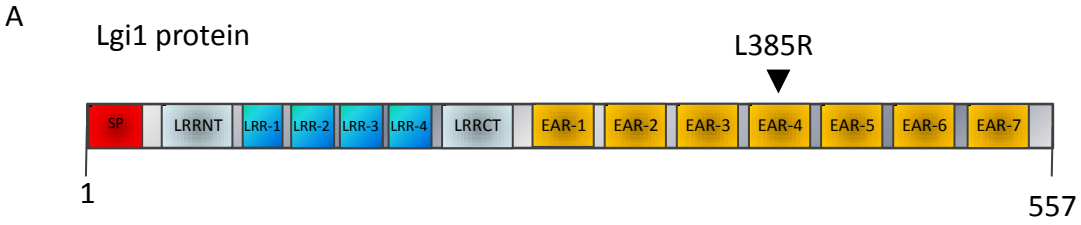
Genotype	Prime stimulation	Target stimulation	Antiepileptic drugs	Number of rats			
				Total	No seizure	Wild running only	Wild running + GTCS
+/+	-	+	-	11	11	0	0
+/+	+	+	-	14	0	10	4
+/ L385R	-	+	-	15	15	0	0
+/ L385R	+	+	-	22	0	0	22
+/+	+	+	Carbamazepine	3	3	0	0
+/+	+	+	Phenytoin	2	2	0	0
+/ L385R	+	+	Carbamazepine	6	6	0	0
+/ L385R	+	+	Phenytoin	4	4	0	0
+/ L385R	+	+	Levetiracetam	4	3	1	0
+/ L385R	+	+	Ethosuximide	4	0	0	4
+/ L385R	+	+	Saline solution	5	0	0	5

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

Lgi1 exons	Forward Primer	Reverse Primer	Amplicon size (bp)
Exon1	gctcccatctcagcacctc	tccaccagctggaattcttt	552
Exon2	cggattaacgtaagctctgt	tccaaatacatgccatatca	272
Exon3	tgagcgtgtaactgttctca	aacaacatcctcatttggtc	275
Exon4	ctgtctgaccaaatgaggat	tcaattaacccaaatcaacc	275
Exon5	ttgaattttcactacatttttgt	agccagtgatttcttaggtc	272
Exon6	tactgcaaatgcagcagac	tgctgtagaaatggttttca	370
Exon7	catcttcttttcctatttttgc	ccctctgtcaaagcagttta	366
Exon8-1	ccacacatctaatgtctcatctgtt	aggatcaaatgaggtgttctgag	495
Exon8-2	tgatgtggaatacctagaaatagcc	gaaaattacgcttgttaatggacac	508
Exon8-3	aagtttcaggagttaaatgttcagg	aacatgtttataaattatgggaaatca	687

Supplemental Table 1: Primers for *Lgi1* sequencing

Figure 1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L385R
SP
LRRNT
LRR-1
LRR-2
LRR-3
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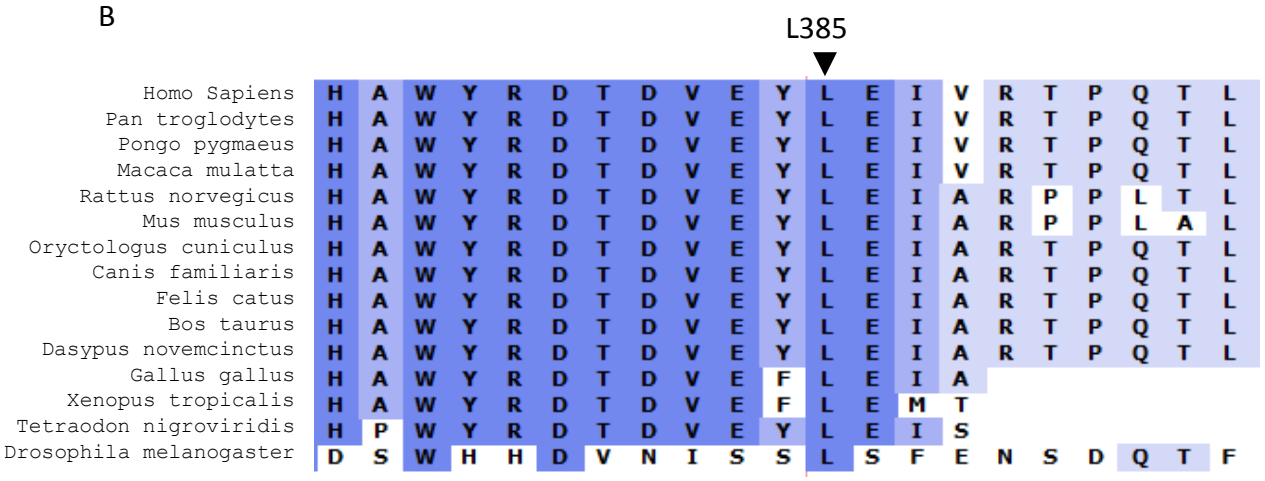
EAR-4
EAR-5
EAR-6
EAR-7
1
557


B
L385
Homo Sapiens H A W Y R D T D V E Y L E I V R T P Q T L
Pan troglodytes H A W Y R D T D V E Y L E I V R T P Q T L
Pongo pygmaeus H A W Y R D T D V E Y L E I V R T P Q T L
Macaca mulatta H A W Y R D T D V E Y L E I V R T P Q T L
Rattus norvegicus H A W Y R D T D V E Y L E I A R P P L T L
Mus musculus H A W Y R D T D V E Y L E I A R P P L A L
Oryctologus cuniculus H A W Y R D T D V E Y L E I A R T P Q T L
Canis familiaris H A W Y R D T D V E Y L E I A R T P Q T L
Felis catus H A W Y R D T D V E Y L E I A R T P Q T L
Bos taurus H A W Y R D T D V E Y L E I A R T P Q T L
Dasypus novemcinctus H A W Y R D T D V E Y L E I A R T P Q T L
Gallus gallus H A W Y R D T D V E F L E I A
Xenopus tropicalis H A W Y R D T D V E F L E M T
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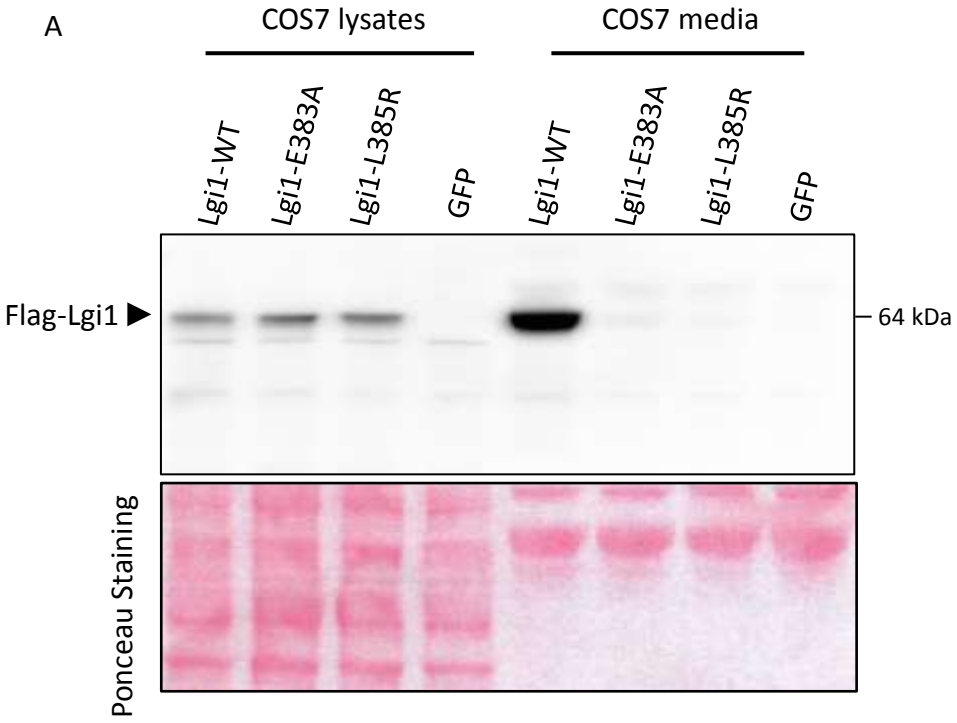
Drosophila melanogaster D S W H H D V N I S S L S F E N S D Q T F

Figure 2


A
COS7 lysates
COS7 media
Lgi1-WT
Lgi1-E383A
Lgi1-L385R
GFP
Lgi1-WT
Lgi1-E383A
Lgi1-L385R
GFP
Flag-Lgi1
64 kDa
Ponceau Staining

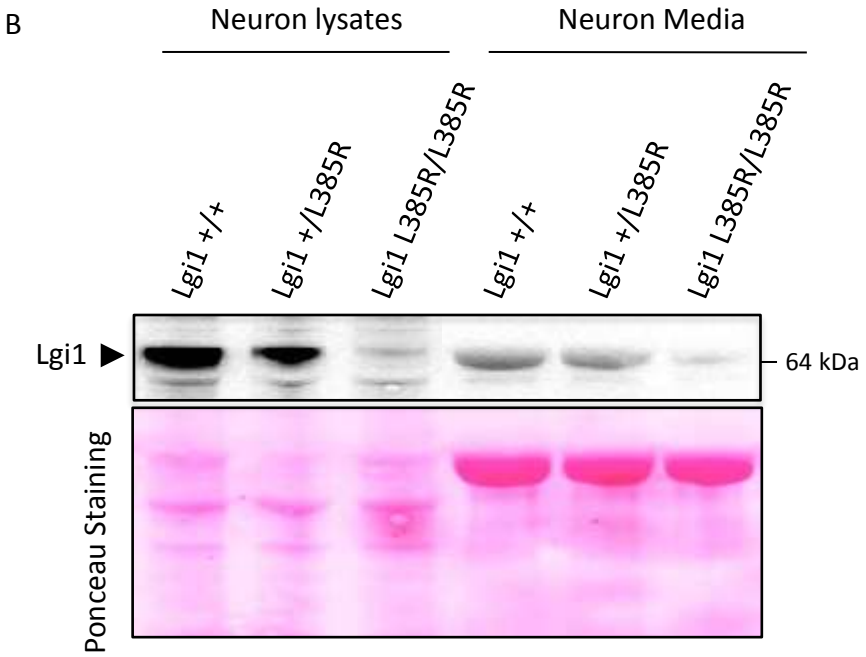
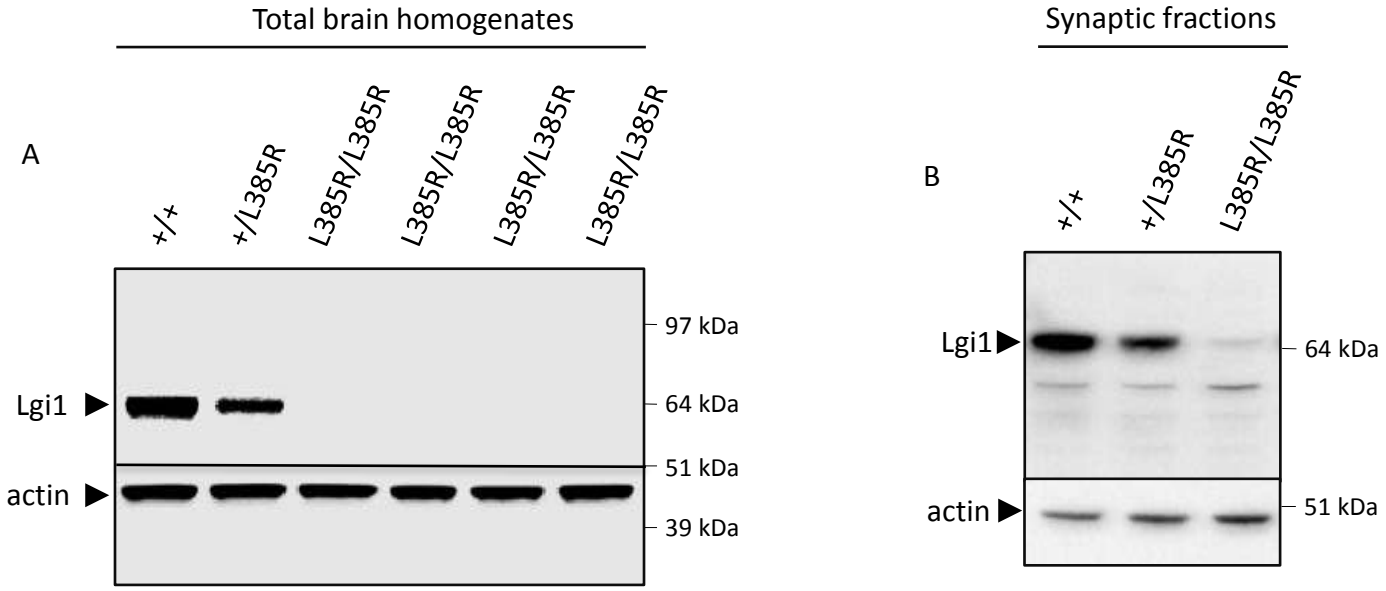

B
Neuron lysates
Neuron Media
Lgi1 +/+
Lgi1 +/L385R
Lgi1 L385R/L385R
Lgi1 +/+
Lgi1 +/L385R
Lgi1 L385R/L385R
Lgi1
64 kDa
Ponceau Staining

Figure 3


Total brain homogenates
A
+/+
+/L385R
L385R/L385R
L385R/L385R
L385R/L385R
L385R/L385R
Lgi1
actin
97 kDa
64 kDa
51 kDa
39 kDa
Synaptic fractions
B
+/+
+/L385R
L385R/L385R
Lgi1
actin
64 kDa
51 kDa

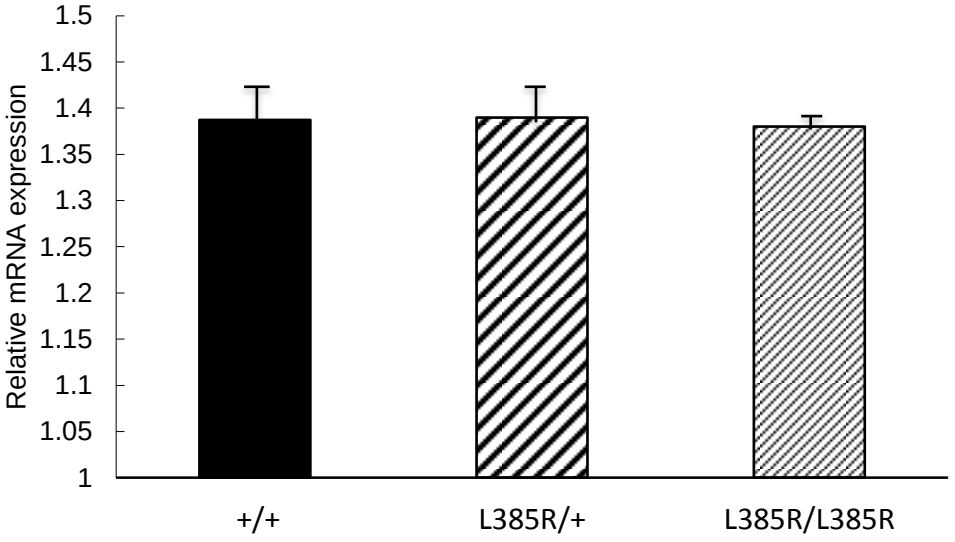
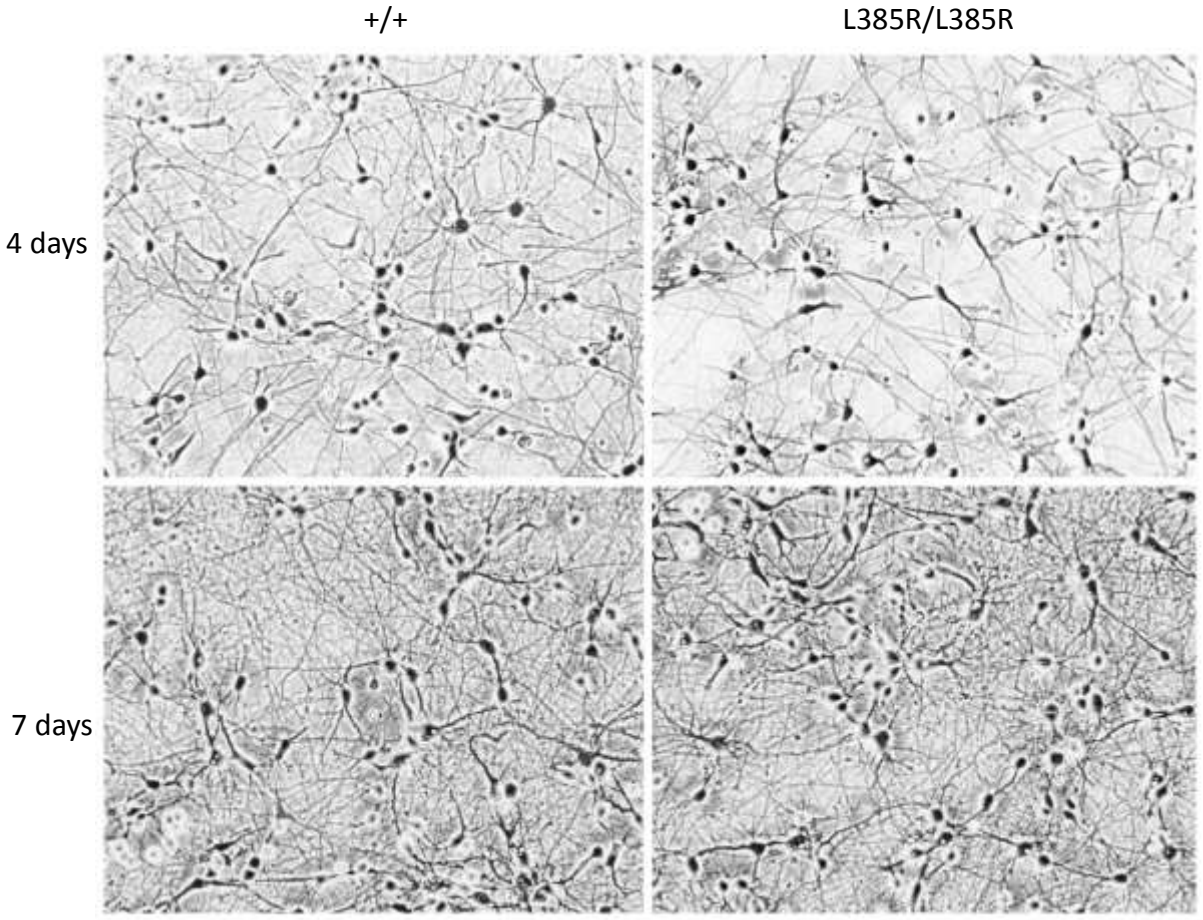

C
Relative mRNA expression
1.5
1.45
1.4
1.35
1.3
1.25
1.2
1.15
1.1
1.05
1
+/+
L385R/+
L385R/L385R

Figure 4

A



B

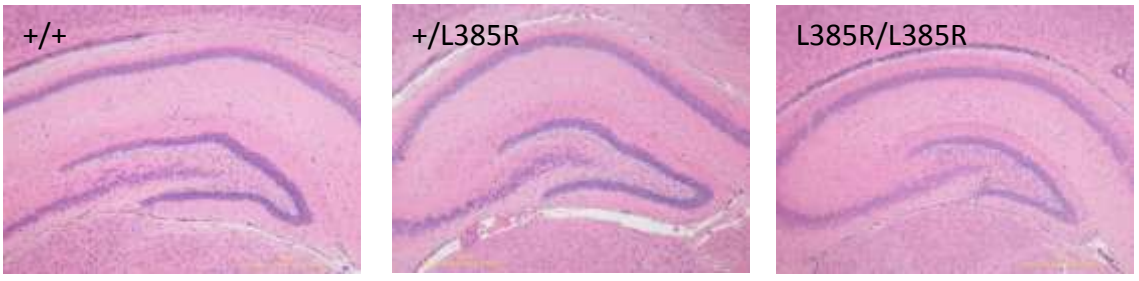


Figure 5

A

B

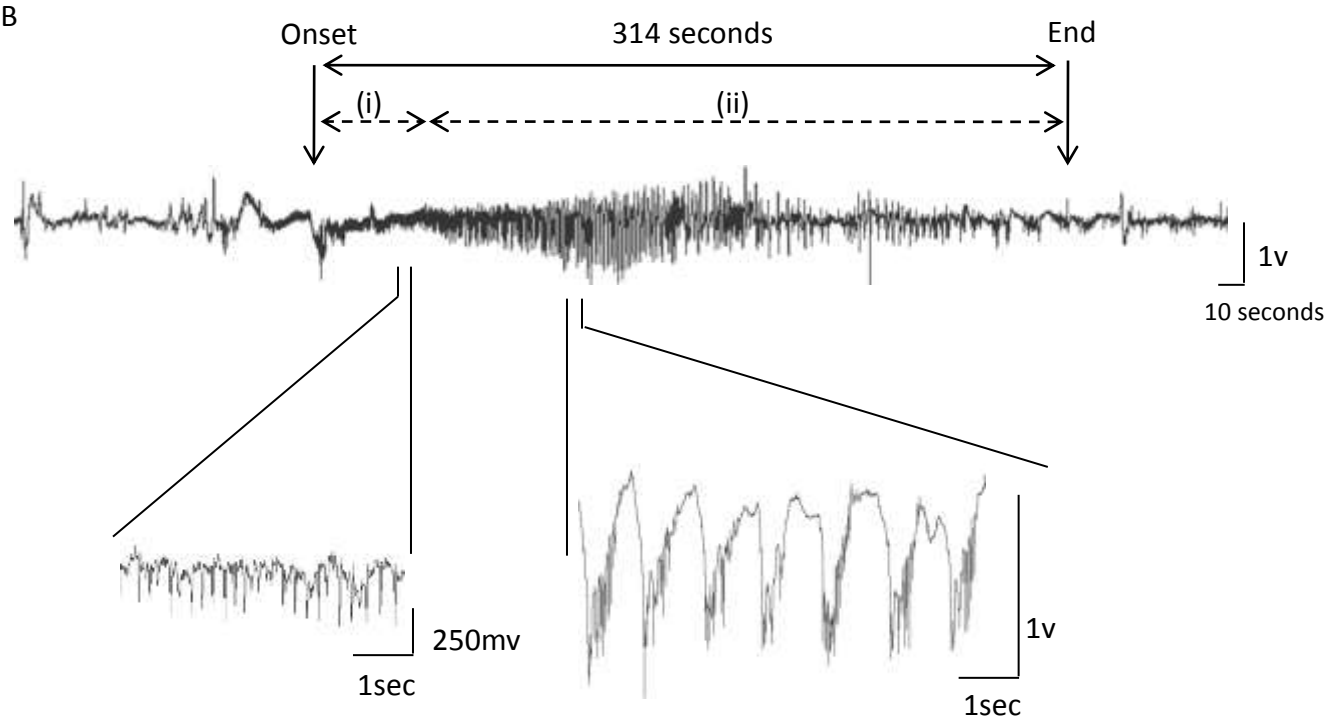
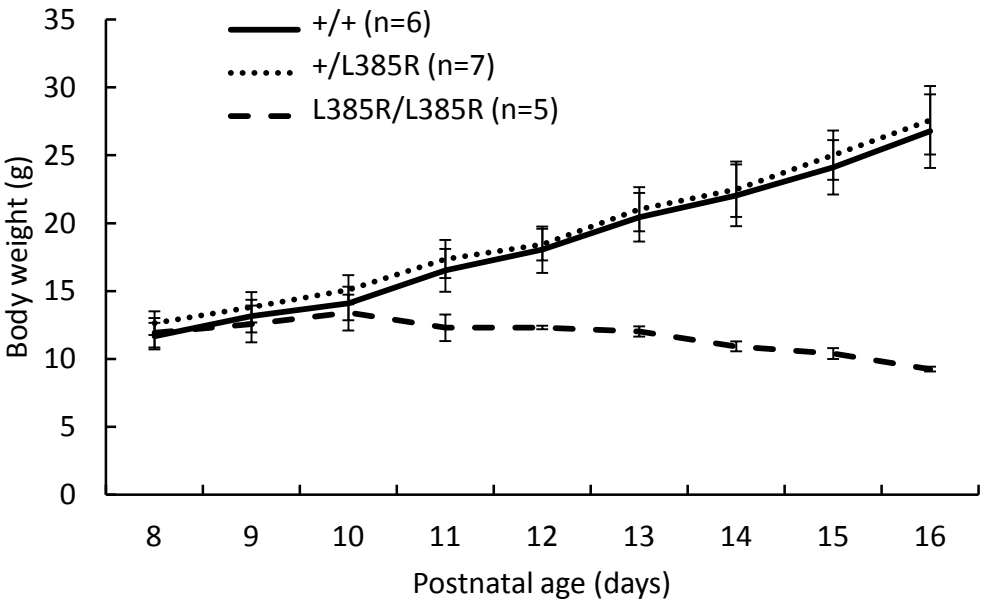
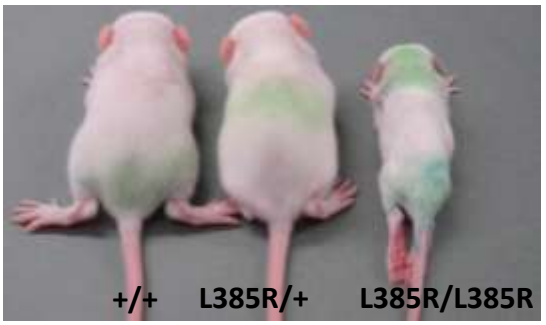


Figure 6

A


+/+ (n=6)
+/L385R (n=7)
L385R/L385R (n=5)
Body weight (g)
0
5
10
15
20
25
30
35
8
9
10
11
12
13
14
15
16
Postnatal age (days)

B


+/+
L385R/+
L385R/L385R

C

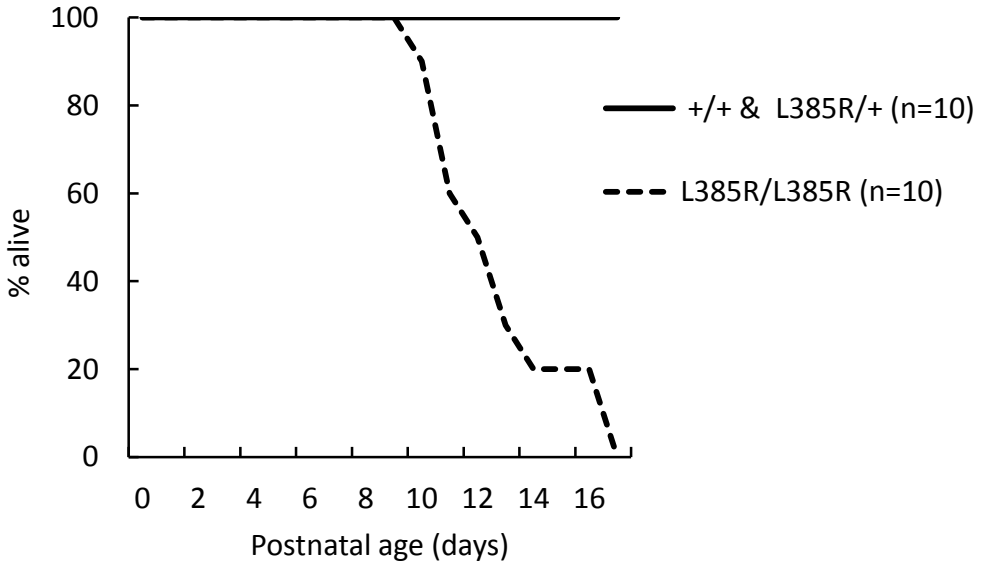

100
80
60
40
20
0
% alive
0
2
4
6
8
10
12
14
16
Postnatal age (days)
+/+ & L385R/+ (n=10)
L385R/L385R (n=10)

Figure 7

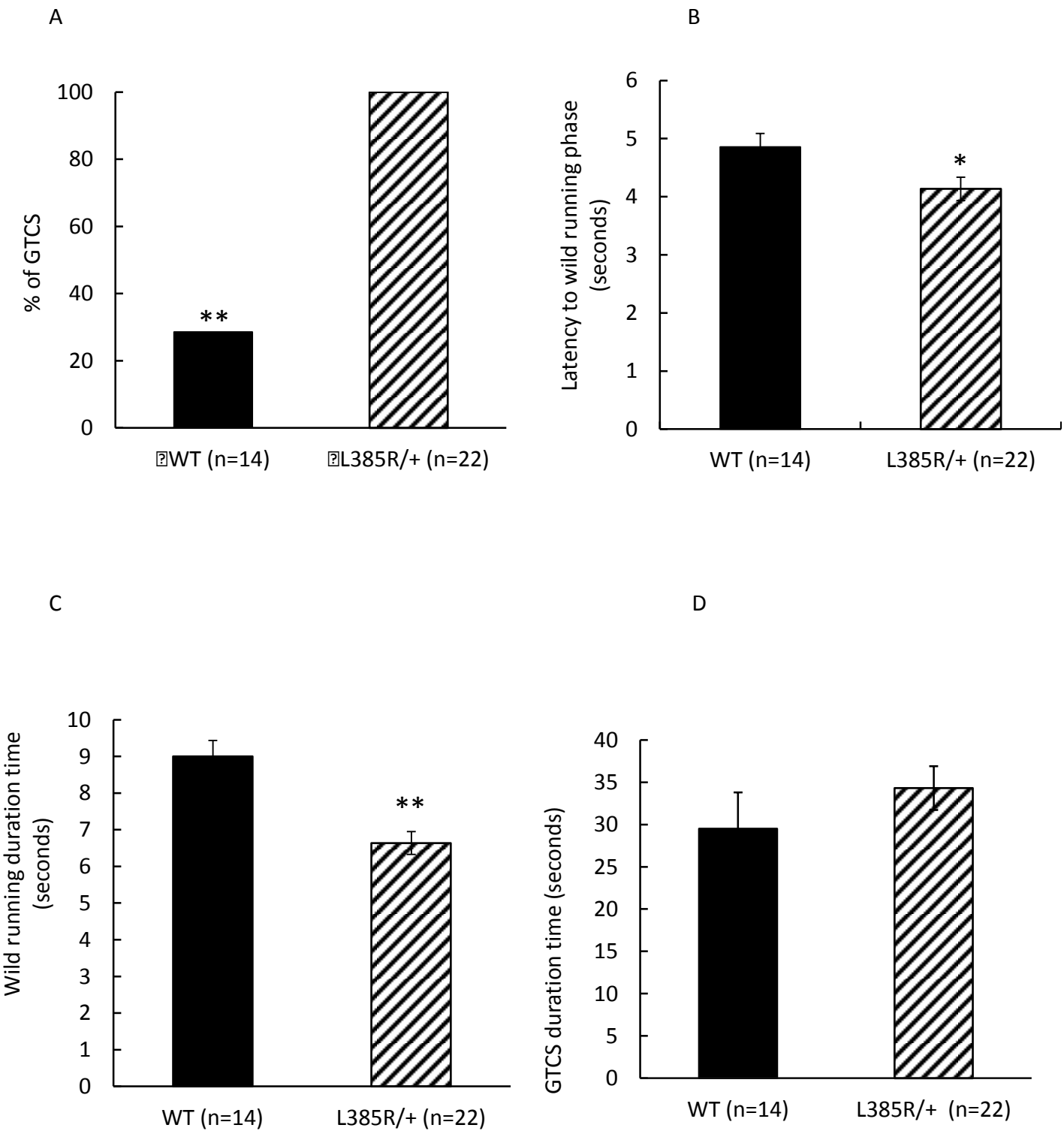
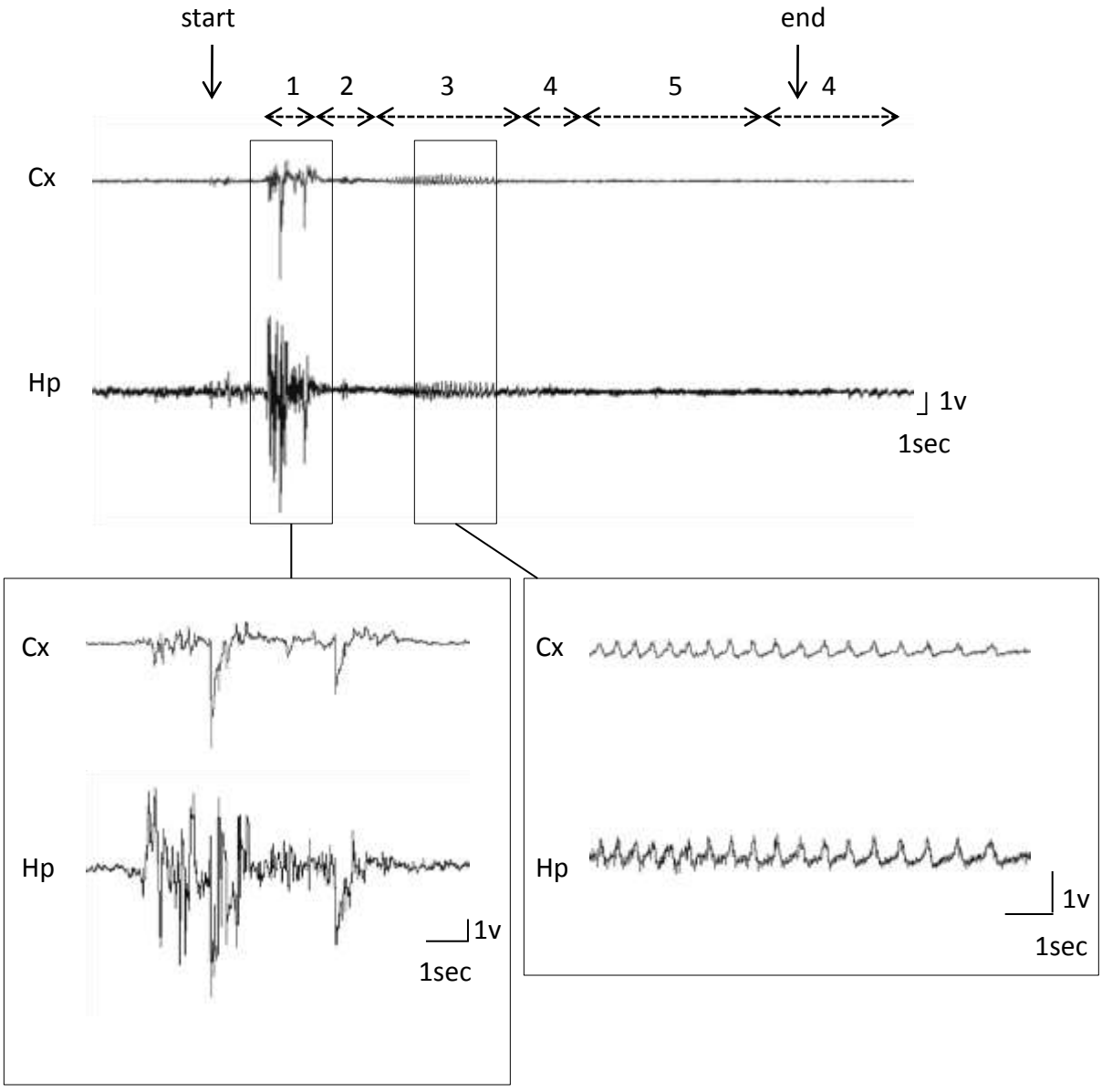

A
100
80
60
40
20
0
% of GTCS
**
WT (n=14)
L385R/+ (n=22)
B
6
5
4
3
2
1
0
Latency to wild running phase (seconds)
*
WT (n=14)
L385R/+ (n=22)
C
10
9
8
7
6
5
4
3
2
1
0
Wild running duration time (seconds)
**
WT (n=14)
L385R/+ (n=22)
D
40
35
30
25
20
15
10
5
0
GTCS duration time (seconds)
WT (n=14)
L385R/+ (n=22)

Figure 8



Supplementary figure 1