LG1 tunes intrinsic excitability by regulating the density of axonal Kv1 channels

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Antibodies that immunoprecipitate a VGKC complex labeled with 15N). This subset was initially identified by the presence of serum from patients with limbic encephalitis (LE), who display epilepsy and psychiatric symptoms (14, 15). LGI1 autoantibodies occur in a subset of patients with limbic encephalitis, including those with the CA3 region of the hippocampus, a classical focus for epileptogenesis. Our data indicate that LGI1 is expressed at the axonal initial segment and regulates action potential firing by setting the density of the axonal Kv1.1 channels that underlie dendrotoxin-sensitive D-type potassium current. LGI1 deficiency incurs a >50% down-regulation of the expression of Kv1.1 and Kv1.2 via a posttranscriptional mechanism, resulting in a reduction in the capacity of axonal D-type current to limit glutamate release, thus contributing to epileptogenesis.

In autosomal dominant epilepsy with auditory features (ADEAF), lateral temporal seizures are accompanied by auditory aura or are triggered by auditory stimuli. ADEAF results from mutations in leucine-rich glioma-inactivated 1 (LG1), a soluble glycoprotein secreted by neurons. Animal models of LG1 depletion display spontaneous seizures, however, the function of LG1 and the mechanisms by which deficiency leads to epilepsy are unknown. We investigated the effects of pure recombinant LG1 and genetic depletion on intrinsic excitability, in the absence of synaptic input, in hippocampal CA3 neurons, a classical focus for epileptogenesis. Our data indicate that LG1 is expressed at the axonal initial segment and regulates action potential firing by setting the density of the axonal Kv1.1 channels that underlie dendrotoxin-sensitive D-type potassium current. LG1 deficiency incurs a >50% down-regulation of the expression of Kv1.1 and Kv1.2 via a posttranscriptional mechanism, resulting in a reduction in the capacity of axonal D-type current to limit glutamate release, thus contributing to epileptogenesis.

Significance

Leucine-rich glioma-inactivated 1 (LG1) is a secreted neuronal protein associated in a multiprotein complex with Kv1 channels. Natural mutations in this protein are found in the early-adulthood-onset disorder “autosomal dominant epilepsy with auditory features” and animal models with LG1 genetic deletion display spontaneous seizures. We investigated the potential link between LG1 depletion and intrinsic neuronal excitability. We found that LG1 determines neuronal excitability in CA3 pyramidal neurons through the control of axonal Kv1-channel expression. Genetic deletion of LG1 induces a robust diminution in Kv1 channels, especially in axons where they colocalize with LG1, very likely contributing to epileptogenesis. These results strongly suggest that LG1 controls neuronal excitability by regulating Kv1-channel expression.


The authors declare no conflict of interest.

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0.009 vs. 0.14
72 h displayed a robust decrease in excitability (Fig. 2)
clamp by counting the number of action potentials generated by
excitability of CA3 neurons was assessed under current
nant LGI1 (rLGI1) modified intrinsic excitability. Organotypic
clamp recording was performed to determine whether recombi-

53
100 nM LGI1, vs. a control value with heat-denatured LGI1
C
with a significant increase in rheobase (Fig. 2
E
<

3

P
< 0.01) and decrease in gain (Fig. 2
D
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2

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A
/

2

Fig. 1. Recombinant expression of LGI1. (A) Schematic representation of recombinant glycosylated LGI1 with signal peptide SP, His6, and T7 tags, leucine-rich region (LRR), and epitope domain. (B) SDS-PAGE of purified LGI1: Coomassie blue-stained LGI1 (lane 1; 1 μg) and Western blot (0.1 μg) probed with anti-LGI1 (lane 2). (C) Western blots of 0.1 μg of purified LG1 (lane 1) incubated in PNGase buffer (lanes 2 and 3) in the absence (lane 2) or presence of PNGase (lane 3) probed with anti-T7 antibody. To optimize protein resolution, the gel in C was run for a longer time than in B.

Exogenous LGI1 Reduces Intrinsic Excitability in CA3 Neurons. Patch clamp recording was performed to determine whether recombinant LGI1 (rLGI1) modified intrinsic excitability. Organotypic rat hippocampal slice cultures were incubated with rLGI1 and the excitability of CA3 neurons was assessed under current clamp by counting the number of action potentials generated by current injections of increasing intensity, in the presence of blockers of glutamatergic (kynurenate) and GABAergic (picro-

Current clamp recordings (Fig. 3) revealed a robust increase in excitability in Lgi1+/− neurons. Evoked spikes in WT neurons were preceded by a slowly depolarizing ramp (Fig. 3A), which is the signature of D-type current. In contrast, action potentials in Lgi1−/− neurons (Fig. 3B) were elicited at lower current intensities and often triggered at the beginning of the pulse. Analysis confirmed first that there was no significant difference in capacitance or input resistance in WT vs. Lgi1−/− neurons (Fig. S2A and B). However, a strong increase in the intrinsic excitability of CA3 neurons was measured in Lgi1−/− mice (Fig. 3C), with an almost threefold

Increased Intrinsic Neuronal Excitability in Lgi1−/− Animals. We next asked whether genetic depletion of LGI1 leads to a reduction in D-
type current. The intrinsic excitability of CA3 neurons was compared in hippocampal slice cultures from Lgi1-null vs. WT mice. Current clamp recordings (Fig. 3) revealed a robust increase in excitability in Lgi1+/− neurons. Evoked spikes in WT neurons were preceded by a slowly depolarizing ramp (Fig. 3A), which is the signature of D-type current. In contrast, action potentials in Lgi1−/− neurons (Fig. 3B) were elicited at lower current intensities and often triggered at the beginning of the pulse. Analysis confirmed first that there was no significant difference in capacitance or input resistance in WT vs. Lgi1−/− neurons (Fig. S2A and B). However, a strong increase in the intrinsic excitability of CA3 neurons was measured in Lgi1−/− mice (Fig. 3C), with an almost threefold

reduction in rheobase (Fig. 3D) (Lgi1+/− = 68 ± 12 pA vs. WT = 195 ± 51 pA) and an approximately twofold increase in gain (Fig. 3E) (Lgi1+/− = 0.11 ± 0.01 spike/pA vs. WT = 0.06 ± 0.01 spike/pA). We next determined the contribution of D-type currents to these parameters. While DTX-K reduced the rheobase by about 50% in WT littermate mice (Fig. 3F), it had no significant effect in Lgi1-null mice (Fig. 3G). However, DTX-K application induced an 

Modification of D-Type Potassium Conductance in Lgi1−/− Background. To determine the effects of LGI1 depletion on D-type current more directly, CA3 neurons were recorded in the voltage-clamp mode, and potassium currents were evoked by 10-mV steps from −80 to +10 mV, before and after the addition of DTX-K (Fig. 4). WT neurons displayed slowly inactivating D-type currents (188 ± 51 pA, n = 6, at −30 mV), that were robustly blocked by DTX-K (Fig. S2D). In contrast, outward currents in Lgi1-null neurons (Fig. S2D) were significantly weaker (41 ± 11 pA, n = 8, P < 0.05), but still revealed a small DTX-sensitive

Fig. 2. Exogenous LGI1 reduces the intrinsic excitability of hippocampal CA3 neurons. (A) Representative neurons from rat hippocampal slice cultures, treated for 72 h with 100 nM heat-denatured (Top) or native (Bottom) LGI1, recorded under current clamp. (Right) Input–output curves illustrating the number of action potentials elicited plotted against injected current for each neuron. Gray and pink traces represent maximal injected currents that did not elicit an action potential and the corresponding responses upon treatment with native and denatured LGI1, respectively. Black and red arrows indicate threshold currents. (B) Averaged input–output curves. (C) Rheobase; arrows indicate representative neurons in A. (D) Gain in WT and Lgi1−/−. (E) Rheobase of neurons treated ±100 nM LGI1 for 24 h. (F) In some cells DTX-K (100 nM) was bath applied and the effects on rheobase were determined. Error bars represent SEM.
In tau inactivation. Furthermore, the D-type current inactivated more rapidly than in WT cells (Fig. 4). Using HPRT and HCN4 as stable normalizing genes, Kv1.1 and Kv1.2 transcript levels showed a limited however significant (REST 2009 software) increase (FC = 1.535; P < 0.001 and FC = 1.276; P < 0.001, respectively) in Lgi1-null mice compared with WT controls (Fig. 4B). Thus, the observed decrease in Kv1.1 and Kv1.2 protein expression is not a consequence of transcriptional down-regulation.

Axonal Distribution of Kv1.1 and Lgi1. Lgi1 is a soluble protein that interacts with membranes by binding to its receptors ADAM22, ADAM23 (22), and NogoR (11). Previous investigation underlined a postsynaptic localization for ADAM22 (22), difficult to reconcile with the view that Lgi1 sets the density of axonal Kv1.1 channels (25). However, recent studies in myelinated axons indicated that ADAM22 associates with Kv1 channels at axonal initial segments and juxtaparanodes (20). To clarify the site of action of Lgi1, we analyzed its subcellular distribution in hippocampal CA3 sections from Lgi1+/− and Lgi1−/− mice. Fig. 6A illustrates colocalization of immunostaining for Lgi1 (green) and βIV spectrin (red), a marker of axonal initial segments (AISs). In Lgi1−/− mice (Fig. 6B), only background staining for Lgi1 was detected. Similar results were obtained in cortex (Fig. S5A). Lgi1 is thus principally expressed in AISs. In contrast, in the CA3 of Lgi1+/− mice, Kv1.1 (green) was present in both neuronal soma, ankyrin G-positive (red) AISs, and along the axons (Fig. 6C). In Lgi1-null mice, the axonal expression of Kv1.1 was strongly reduced and mainly detected in the soma (Fig. 6D).

Component Analysis. Analysis of the biophysical properties of the DTX-sensitive component of the outward current in Lgi1−/− vs. WT neurons did not reveal significant difference in the steady-state activation or inactivation parameters (Fig. 4B and Fig. S2 E and F). In contrast to tetraethylammonium (TEA)-sensitive currents that did not significantly change between WT and Lgi1−/− neurons (Fig. S3), DTX-sensitive conductance at −30 mV was significantly reduced in Lgi1-null neurons (Fig. 4A, from 2.5 ± 0.2 nS, n = 15–1.2 ± 0.3 nS, n = 14, Mann–Whitney U test P < 0.01). Furthermore, the D-type current inactivated more rapidly than in WT cells (Fig. 4C), displaying an approximately twofold decrease in tau inactivation.

Decrease in Kv1 Protein Expression in Lgi1−/− Neurons. To confirm the hypothesis that Lgi1−/− mice show reduced Kv1-channel expression at the cell membrane, 125I-DEK binding experiments were carried out on crude synaptosomal (P2) membrane fractions from the brains of Lgi1+/+, −/−, and −/− mice (Fig. 5A). Membranes were incubated with 0.1 nM 125I-DEK alone or in the presence of 0.1 μM DTX-K. Membranes from Lgi1-null mice showed a 75% reduction in specific binding, whereas heterozygotes displayed an ~40% decrease. Western blotting of brain homogenates (Fig. 5 B and C) was carried out for VGKC subunits and associated proteins, using GAPDH as a loading control. They were consistent with a marked decrease (~50%) in the total expression of Kv1.1 and Kv1.2 subunits. In contrast, the expression of Kv1 subunits, ADAM22, ADAM23, or the un-associated ion channel subunit HCN4, were unaffected. qPCR analysis of HCN4 mRNA expression [normalization with hypoxanthine phosphoribosyltransferase (HPRT)] showed no change in Lgi1 knockout mice (Fig. S4A). Using HPRT and HCN4 as stable normalizing genes, Kv1.1 and Kv1.2 transcript levels showed a limited however significant (REST 2009 software) increase (FC = 1.535; P < 0.001 and FC = 1.276; P < 0.001, respectively) in Lgi1-null mice compared with WT controls (Fig. 4B). Thus, the observed decrease in Kv1.1 and Kv1.2 protein expression is not a consequence of transcriptional down-regulation.
LGI1 depletion might induce loss of AMPA receptor function in inhibitory interneurons, thus increasing overall excitability in the hippocampus (6). However, inhibitory synaptic transmission is not affected in LGI1 mutant mice (7, 13) and conditional LGI1 knock out in inhibitory parvalbumin interneurons does not confer an epileptic phenotype (8).

We now report evidence that LGI1 determines neuronal excitability in CA3 pyramidal neurons through the control of Kv1-channel expression. We first showed that addition of recombinant LGI1 decreased neuronal excitability in rat CA3 neurons. Interestingly, the rheobase in the presence of DTX was similar in treated and control neurons, indicating that this effect was likely to be due to a change in Kv1-channel expression. Next, we showed that Lgi1−/− mice display increased excitability compared with the WT mice. To corroborate LGI1-dependent modifications in Kv1 current, we measured the effects of blocking Kv1 channels with DTX in WT and Lgi1−/− neurons. DTX reduced the rheobase in WT neurons, but failed to significantly change the action potential threshold in LGI1-null neurons. These results suggest that

Boillot et al. recently reported that LGI1 acts presynaptically as a negative modulator of excitatory synaptic transmission during early postnatal development (21). Pharmacological blockade of Kv1 channels leads to an increase of the spike duration and Ca2+ entry in CA3 presynaptic terminals (26). We therefore asked whether a loss of axonal Kv1 channels resulting from LGI1 deficiency might lead to increased glutamate release, by comparing the effects of DTX-K on both spontaneous and spike-evoked excitatory postsynaptic currents (EPSCs) from WT vs. Lgi1-null CA3 neurons.

Blocking Kv1 channels enhanced spike evoked EPSCs by about 64% in connected pairs of WT CA3 neurons (Fig. 6 E and F). However, in LGI1-deficient neurons, DTX-K failed to boost EPSC amplitude, indicating that in KO mice, glutamate release is no longer controlled by Kv1 channels. To examine the impact of this Kv1 loss on network dynamics, we recorded spontaneous activity in CA3 neurons. Spontaneous activity was stronger but less sensitive to boosting by Kv1 block in Lgi1−/− compared with WT mice (Fig. S5).

**Discussion**

Investigation into the precise mechanisms by which LGI1 depletion leads to epilepsy has so far emphasized a role in excitatory neurotransmission, but neglected its implication in intrinsic excitability. Furthermore, analysis of glutamatergic neurotransmission in hippocampal slices from Lgi1−/− mice has yielded conflicting evidence. Yu et al. (7) reported enhanced excitatory transmission in Lgi1−/− mice, which suggests a basis for the seizure phenotype. In contrast, Fukata and colleagues found reduced AMPA receptor function (6, 22, 23) and suggested that
LGII modulates neuronal excitability via presynaptic Kv1 channels. However, DTX treatment led to a 50% increase in the gain in LGII-deficient neurons, suggesting that DTX-sensitive current was not totally absent. Voltage clamp recordings in Lgi1−/− CA3 neurons indicated a substantial reduction in D-type conductance and confirmed the persistence of residual current with similar steady-state parameters.

The anti-LGI1-DTX binding to WT and Lgi1−/− synaptosomes indicated a robust decrease in Kv1-channel expression at the membrane. Kv1.2 has been shown to impart axonal and presynaptic targeting to Kv1.1 (7). LGII deletion decreased both total Kv1.1 and Kv1.2 protein expression levels by 50%, but did not induce any significant change in the expression levels of Kvβ1 subunit, the Kv1.1 binding partners ADAM22 and ADAM23, or of the unrelated HCN4-channel subunit. Thus, genetic deletion of LGII induced a strong elevation in neuronal excitability, due to a reduction in the density of Kv1 channels.

The decrease in Kv1.1 protein levels could be due to either transcriptional repression, reduced translation, or posttranslational degradation. Analysis by qPCR showed surprisingly a small but significant increase in Kv1.1 and Kv1.2 mRNA expression in Lgi1−/− animals (1.5- and 1.2-fold, respectively). The steady-state mRNA expression level was analyzed at P10, corresponding to peak LGII protein expression in WT animals. This result suggests that whereas genetic deletion of LGII impacts Kv1.1 and Kv1.2 mRNA expression, this cannot underlie the decrease in protein expression. Specific regulatory mechanisms must be taking place affecting the turnover and/or axonal targeting of Kv1 proteins.

Immunohistochemical analysis of Kv1.1 expression in the cortex and CA3 of WT animals revealed colocalization of LGII and Kv1.1 at the axonal initial segment. The colocalization of LGII with Kv1.1 channels at the AIS, is consistent with the observation that ADAM22, a membrane receptor for LGII, is a component of clustered Kv1 channels in certain distinct axonal domains, including the AIS in dissociated hippocampal cultures (20). The analysis of Lgi1−/− samples corroborated the decrease in Kv1.1 protein expression levels, as immunostaining of Kv1.1 showed a marked depletion especially in axons, compatible with perturbed excitability and the increase of rheobase in Lgi1−/− animals.

Interestingly, the absence of LGII accelerated D-current inactivation kinetics. This finding is consistent with oocyte expression studies showing that LGII prevents rapid inactivation of Kv1-channel current (19). In the study by Schulte et al. (19), LGII was thought to be an intracellular protein that antagonizes rapid inactivation mediated by Kvβ1 subunit, by either steric hindrance or a direct interaction of LGII with the inactivation domain of the channel. Our data indicate that Kv1 channels inactivate more rapidly in LGII-null neurons. It is not clear how this inactivation occurs but it is possible that the reduced expression of Kv1.1 subunits, although Kvβ1 levels are unaffected, leads to an increased Kvβ1/Kv1.1 ratio and hence to an increased probability that Kvβ1 binds to and inactivates residual channels.

LGII deficiency was also shown to result in a loss of the capacity of presynaptic D-type current to limit glutamate release; as in contrast to WT mice, release was not stimulated by DTX in Lgi1−/− mice. Our results support and extend reports of enhanced excitatory transmission in Lgi1−/− mice (7, 21). It is possible that conflicting evidence, suggesting reduced AMPA receptor function in Lgi1−/− mice (6, 22, 23), may be the consequence of homeostatic synaptic responses to increased excitatory transmission.

Inhibitory synaptic inputs at AISs can play a significant role in modulating neuronal excitability. Recent studies have shown that ADAM11 is involved in clustering Kv1 channels at cerebellar basket cell terminals, which encapsulate Purkinje cell AISs and exert inhibitory ephaptic control of output (27). Furthermore LGII binding to ADAM11 has been reported (28), which suggests that LGII may influence synaptic inhibition. However, selective deletion of LGII in parvalbumin-positive interneurons did not generate an epileptic phenotype, whereas knock out in glutamatergic neurons resulted in seizures (8), suggesting that loss of LGII in excitatory neurons is the predominant factor.

Do our present findings have implications for LE with LGII autoimmunity? Lalic et al. (29) have reported that in vitro application of serum from LE patients increased the release probability at mossy-fiber CA3 cell synapses, and this effect was mimicked by DTX. It will be interesting to determine whether the anti-LGII autoantibodies identified in LE patients have an impact on Kv1-channel expression.

In conclusion, our results are consistent with the conclusion that LGII is located in the axonal initial segment and buffers excitatory neurotransmission by tuning axonal Kv1.1-channel density. Loss of this process in Lgi1-null mice is likely to be a major contributor to epileptogenesis. Further work will be necessary to clarify this issue and dissect the posttranslational mechanisms by which LGII controls the expression of Kv1.1.

Materials and Methods

Antibodies and Other Reagents. Details are provided in SI Materials and Methods.

Production of Lgi1−/− Mice. Heterozygous Lgi1−/+ mice (5) were intercrossed to generate Lgi1−/+ and Lgi1−/− littersmates. All experiments were carried out according to the European and Institutional guidelines for the care and use of laboratory animals (Council Directive 86/609/EEC and French National Research Council).

Production of LGII. Recombinant LGII was produced using the Bac-to-Bac expression system (Life Technologies). LGII was purified using 6-histidine capture on HisTrap Excel columns and aliquots frozen at −80 °C. Details are provided in SI Materials and Methods.

Western Blotting. P10 brains were homogenized in 25 mM Tris-HCl pH 7.4, 150 mM NaCl, and homogenates were recovered in supernatants of a 900 × g centrifugation. Ten micrograms of proteins were resolved by SDS/PAGE and processed for Western blotting using the indicated antibodies. Samples from six different brains of both WT and Lgi1−/− were analyzed in triplicate.

RNA Extraction, cDNA Synthesis, and Relative Quantification Assays. qPCR quantification of mRNAs was performed on P9–11 mouse brains extracts. TaqMan gene expression assays were used for quantifying Hprt and HCN4 genes and a homemade designed assay was used for Kv1.1 and Kv1.2. Details are provided in SI Materials and Methods.

Hippocampal Slice Cultures. All experiments were carried out according to the European and Institutional guidelines for the care and use of laboratory animals and approved by the local health authority (D13055-08, Préfecture des Bouches du Rhône).

Young Wistar rats (P7-P8) or mice (P9-P11) were deeply anesthetized by i.p. injection of chloral hydrate, the brain was removed, and each hippocampus dissected. Hippocampal slices (350 μm) were placed on 20-mm latex membranes (Millicell) inserted into 35-mm Petri dishes containing 1 mL of culture medium and maintained for up to 21 d in an incubator at 34 °C, 95% O2–5% CO2. The culture medium contained (in milliliters) 25 mM Tris, 150 mM NaCl, and 0.1% BSA at pH 7.2 (TBSA). Samples were filtered over Whatman GF/C filters treated with 0.3% polyethyleneimine, washed three times with 1 mL TBS, and bound radioactivity was measured by gamma counting. Specific binding was the difference between total and nonspecific binding.

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Electrophysiology. Whole-cell recordings were obtained from CA3 neurons in organotypic cultures. The external solution contained (in millimoles): 125 NaCl, 26 NaHCO3, 3 CaCl2, 2.5 KCl, 2 MgCl2, 0.8 Na2HPO4, and 10 D-glucose, and was equilibrated with 95% O2–5% CO2. Patch pipettes (5–10 MΩ) were filled with a solution containing (in millimoles): K-glutamate 120, KCl 20, Hepes 10, EGTA 0.5, MgCl2 2, NaATP 2, and NaGTP 0.3 (pH 7.4). All recordings were made at 29 °C in a temperature-controlled recording chamber (Luigs & Neumann). Neurons were recorded in current clamp or voltage clamp with a Multiclamp 700B Amplifier (Axon Instruments, Molecular Devices). Only neurons with a resting membrane potential more negative than −60 mV were kept for final analysis. Excitability was measured by delivering a range of long (1 s) depolarizing current pulses (10–250 pA, by increments of 10 pA) and counting the number of action potentials. Ionotropic glutamate and GABA receptors were blocked with 2–4 mM kynurenate and 100 μM picrotoxin. Input–output curves were determined for each neuron and two parameters were examined; the rheobase (the minimal current eliciting at least one action potential) and the gain (measured on each cell as the linear fit of the spike number as a function of current pulse). Voltage-clamp protocols to measure Kv currents consisted of a family of voltage-step commands from a holding potential of −90 mV (−80–0 mV in 10-mV increments). To block voltage-dependent Ca2+ and Na+ currents, 200 μM Ni2+, 50 μM Cd2+, and 0.5 mM TTX were added to the saline. A万一quantitative isolation of the D-type and TEA sensitive currents were done by subtracting the currents evoked in the presence of the Kv1-blocker, DTX-K, or TEA (10 mM), respectively, from currents evoked in control solution. In all voltage-clamp experiments, leak subtraction was performed using a p(n/4) protocol. The voltage and current signals were low-pass filtered (3 kHz), and acquisition was performed at 10 kHz with pClamp10 (Axon Instruments) or Igor (Wave-metrics). Data were analyzed with ClampFit (Axon Instruments) and Igor (Wave-metrics). Pooled data are presented as mean ± SD and SEM and statistical analysis was performed using the Mann–Whitney U test or Wilcoxon rank-sum test.

30. Dolly JG, Parcej DN, Debinde D (2010) Voltage-dependent Ca2+ current pulses (100–200 μA) were depolarizing 15 mV above the resting membrane potential and recorded with an Axopatch 200B (Axon Instruments). Data were analyzed using Clampfit (Axon Instruments) or Igor (Wave metrics). Pooled data are presented as mean ± SD and SEM and statistical analysis was performed using the Mann–Whitney U test or Wilcoxon rank-sum test.