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FULL-LENGTH ORIGINAL RESEARCH

Epilepsia®

LGI1 downregulation increases neuronal circuit excitability

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Abstract

Objective: Leucine-rich glioma-inactivated 1 (LGI1) is a secreted transsynaptic protein that interacts presynaptically with Kv1.1 potassium channels and a disintegrin and metalloprotease (ADAM) protein 23, and postsynaptically influences α-amino-3-hydroxy-5-methylisoxazole-4-propionate receptors through a direct link with the ADAM22 cell adhesion protein. Haploinsufficiency of LGI1 or autoantibodies directed against LGI1 are associated with human epilepsy, generating the hypothesis that a subacute reduction of LGI1 is sufficient to increase network excitability.

Methods: We tested this hypothesis in ex vivo hippocampal slices and in neuronal cultures, by subacutely reducing LGI1 expression with shRNA.

Results: Injection of shRNA-LGI1 in the hippocampus increased dentate granule cell excitability and low-frequency facilitation of mossy fibers to CA3 pyramidal cell neurotransmission. Application of the Kv1 family blocker, α-dendrotoxin, occluded this effect, implicating the involvement of Kv1.1. This subacute reduction of LGI1 was also sufficient to increase neuronal network activity in neuronal primary culture.

Significance: These results indicate that a subacute reduction in LGI1 potentiates neuronal excitability and short-term synaptic plasticity, and increases neuronal network excitability, opening new avenues for the treatment of limbic encephalitis and temporal lobe epilepsies.

KEYWORDS

epilepsy, Kv1.1, LGI1, short-term plasticity

1 | INTRODUCTION

Leucine-rich glioma inactivated protein 1 (LGI1) is a secreted brain protein and is part of the synaptic extracellular matrix.[1] Although mutations in LGI1 were first described in association with malignant glioblastoma,[2] LGI1 mutations were later found to be highly associated with autosomal dominant lateral temporal lobe epilepsy with auditory hallucinations.[3,4] Mice with an embryonic knockout (KO) of *Lgi1* die within 3 weeks from birth due to severe generalized seizures.[5] Given its structure, with a lack of a transmembrane domain, enriched leucine repeats and cysteines at the N-terminal, and a beta-propeller at the C-terminal, LGI1 is an ideal protein-protein interactor.[6,7] LGI1 associates

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presynaptically with a disintegrin and metalloprotease (ADAM) protein 23, which is part of the macro complex formed by the Kv1.1 shaker potassium channel and Kvβ1 regulatory subunit.[8,9] It has been suggested that LGI1 regulates both the kinetics and density of Kv1.1 channels in the axon.[10] By trapping Kvβ1, LGI1 prevents the rapid closure of Kv1.1, changing its dynamics from fast opening–fast inactivation to fast opening–slow inactivation.[9] It has also been shown that in *Lgi1* KO mice, the density of Kv1.1 at the axon initial segment is greatly reduced (~50%), and this affects the intrinsic properties of CA3 neurons.[10] Postsynaptically, LGI1 binds to ADAM22, which is part of the complex that links α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptors to stargazin and postsynaptic density-95 scaffolding proteins.

Specific autoantibodies against LGI1 have also been detected in the serum of adult patients with limbic encephalitis, characterized by faciobrachial dystonic seizures, status epilepticus, and cognitive impairments.[11,12] Plasma exchange treatment in these patients ameliorates the cognitive symptoms and the severity of the seizures, providing evidence for a causative effect of the antibodies.[13] The LGI1 autoantibody targeting protein-ligand interactions between LGI1 and ADAM22/23 has been proposed to be the basis of LGI1 autoimmune encephalitis.[14] Chronic infusion of IgGs-LGI1 in the ventricles of wild-type (WT) mice altered the density of both AMPA receptors and Kv1.1 channels, and was associated with behavioral deficits.[15] However, it is unclear to what extent local inflammation rather than a direct protein interaction contributed to this.

Kv1.1 is highly expressed in dentate granule cells and in particular at mossy fiber (MF) boutons.[9,16,17] The dentate granule cells form a critical gate in the generation of seizure activity such that increasing dentate granule cell excitability promotes seizure activity, whereas hyperpolarizing dentate granule cells inhibits seizure activity.[18] Thus, this leads to the hypothesis that loss of Kv1.1. activity can promote seizure activity through increasing dentate granule cell and circuit excitability.

Conditional or total KO animals for Lgi1 have been previously employed to study the consequences of loss of LGI1, but these animals have developmental abnormalities that have been implicated in contributing to the phenotype.[1,5,19,20] In this study, we asked whether a subacute (7-14 day) local reduction in LGI1 was sufficient to increase cellular and network excitability. First, we used an RNA interference (shRNA) approach to silence LGI1 by injecting lentiviral particles expressing the shRNA against LGI1 in vivo to target the dentate granule cells and the MF-CA3 synapses. There, we examined short-term plasticity and neuronal excitability. We then investigated LGI1 shRNA effects on neuronal circuits by using a combination of calcium imaging and multielectrode arrays (MEAs) in vitro. Collectively, our results indicate a central role for LGI1 in regulating network excitability and provide a link between deficiency in LGI1 and the emergence of seizure activity.

Key Points

- LGI1 mutations have been associated with epilepsy, and LGI1 autoantibodies result in limbic encephalitis with seizures and cognitive defects
- Subacutely reducing LGI1 gene expression leads to enhanced short-term plasticity, and increased neuronal excitability and network activity
- This can be partly explained through LGI1's interaction with a presynaptic potassium channel, Kv1.1
- This result opens new avenues for the treatment of limbic encephalitis and temporal lobe epilepsies

2 | MATERIALS AND METHODS

All animal care and procedures were approved and carried out in line with the UK Animals (Scientific Procedures) Act, 1986 and under the Home Office License PLL/PIL 11035/13691. In vitro experiments (cloning, cell cultures, Western blot, live cell imaging, multielectrode arrays, whole patch clamp, and local field potential), in vivo procedures (stereotaxic injection of substances), and statistical analysis were conducted as described in Appendix S1.

3 | RESULTS

3.1 | Downregulation of LGI1 increases MF facilitation

We used an shRNA approach to subacutely decrease LGI1 expression, over the course of 7 days. To optimize the knockdown (KD) sequence, we initially evaluated three shRNA-LGI1 sequences (Table S1) in a mouse neuroblastoma cell line (Neuro2a). All three sequences resulted in ~80% KD efficiency compared with the effect of the scramble sequence (Figure S1). For further experiments, we used shRNA2 (Table S1, Figure S1) cloned in a lentiviral vector driven by a U6 promoter (LGI1-shRNA; Figure 1A). We transduced primary neuronal cultures at 1 day in vitro (DIV) with both LGI1-shRNA and Scr-shRNA, and collected them at 21 DIV for quantification of LGI1 protein. LGI1 levels were reduced on average by 38.2 ± 0.05% in LGI1-shRNA cultures compared

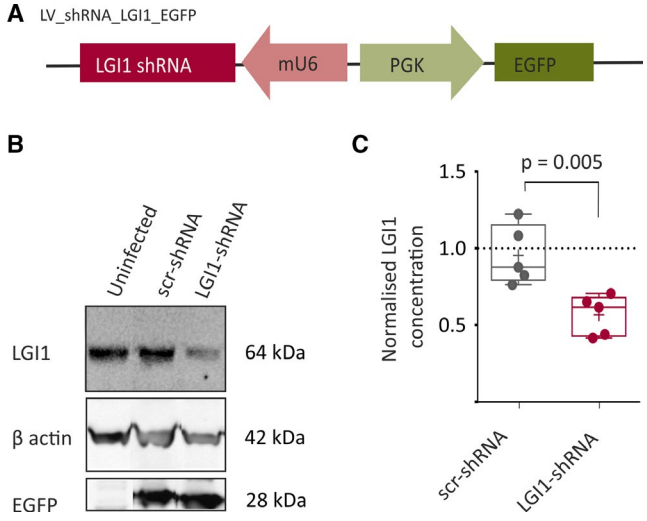

FIGURE 1 shRNA plasmid design and in vitro LGI1-shRNA downregulation. A, shRNA construct: LGI1-shRNA (shRNA2, Table S1, Figure S1) is under the promoter murine U6 small noncoding RNA (mU6); meanwhile, enhanced green fluorescent protein (EGFP) is under the independent promoter phosphoglycerate kinase (PGK). B, LGI1 expression in neuronal cultures. The first lane is the endogenous level of LGI1 in untreated primary cultures, the second lane shows the levels of LGI1 in cultures transduced with scramble virus, and the last lane shows the levels of LGI1 in cultures transduced with shRNA-LGI1. The β-actin and EGFP signals are also shown. EGFP intensity was detected to evaluate that the reporter gene was also expressed at the same time as the shRNA and hence could be a reliable source of transduction rate. C, Quantification of LGI1 concentrations detected by the Western blot method. $P = .005$ two-tailed Student t test (blots n = 5 from five distinct preparations)

to Scr-shRNA ones (Scr-shRNA mean = 0.955 ± 0.084, LGI1-shRNA mean = 0.573 ± 0.057; $P = .05$, two-tailed Student t test; Figure 1B).

We next asked whether the downregulation of LGI1 is sufficient to alter hippocampal excitability. Previous experiments on LGI1 KO have demonstrated a developmental role for LGI1 but do not directly address the impact of a subacute reduction. We tested the impact of LGI1 KD on dentate granule cell MF boutons to CA3 pyramidal cell synapses, as dentate granule cells strongly express LGI1 protein and they are highly involved in epileptogenic mechanisms, suggesting a key function of this protein in those circuits.[9,21]

We therefore injected either Scr-shRNA or LGI1-shRNA in the dentate gyrus (DG) region (Figure S2) and performed local field potential experiments stimulating granule cell layers and recording in the stratum lucidum of CA3 (Figure 2A,B). We used an established protocol (windup) to induce short-term MF plasticity.[22] The results show that MFs in LGI1-shRNA–injected hippocampi facilitated to a significantly greater degree than did Scr-shRNA–injected hippocampi (Figure 2C,D; Scr-shRNA mean = 1.89 ± 0.04, LGI1-shRNA mean = 3.17 ± 0.04; $P < .0001$, two-tailed Student t test). At the end of every stimulation, the specific metabotropic glutamate receptor 2 agonist, DCG-IV, was applied to check that the field potential recorded was mediated by MFs.[22,23] Scr-shRNA and LGI1-shRNA were blocked to similar degrees (Scr-shRNA mean = 0.05 ± 0.01%, ~95%; LGI1-shRNA mean 0.03 ± 0.01%, ~98%; $P > .4$, two-tailed Student t test; Figure 2E). As a further control, we determined that there was no difference in baselines slopes before and after the windup in control conditions (Scr-shRNA baseline #1 = 0.032 ± 0.008, Scr-shRNA baseline #2 = 0.039 ± 0.015; $P > .05$, two-tailed Wilcoxon test).

Lastly, short-term plasticity was also assessed by paired-pulse stimulation. This form of rapid plasticity in the DG is generally reported as facilitating and it is dependent upon presynaptic mechanisms such as calcium accumulation after the first stimulus, but also potassium channel activity.[24] Kv1 channels are a major player in the presynaptic modulation of many synapses, including MF synapses.[24–27] The results show that there was no significant difference in paired-pulse ratio (PPR) in LGI1-shRNA and Scr-shRNA at 50-millisecond delay (Scr-shRNA PPR median = 1.99, range = [0.86-8.33] 7.47, interquartile range [IQR] = 2.33; LGI1-shRNA median = 2.00, range = [0.85-4.66] 3.81, IQR = 1.46; $P > .5$ Mann-Whitney test; Figure S3).

3.2 | Blocking Kv1.1 potassium channels occludes increased facilitation due to LGI1 downregulation

Recent work has demonstrated that LGI1 expression can determine the number of Kv1 channels at the synapse and axon initial segment, and that mice heterozygous for LGI1 (±) expressed 40% fewer Kv1.1 channels compared to control mice.[10] Previous work has reported that nonsecreted LGI1 proteins are also able to change Kv1.1 kinetics through direct block of the Kvβ1 regulatory subunit.[9] The Kv1 family and LGI1 protein largely overlap in their expression in the medial perforant path, granule cell molecular layer, and axons and terminals of DG MFs.[9,16,17] To test whether the increased facilitation was due to changes in the activity of the Kv1.1 channel, we repeated the windup experiments applying a broad blocker of the Kv1 family, α-dendrotoxin (α-DTX; 1 μmol·L^{-1}).[27] In the Scr-shRNA condition, application of α-DTX increased the windup facilitation (Figure 3A-D), whereas in the LGI1-shRNA condition, it decreased facilitation (Figure 3A,B,E,F; Scr-shRNA α-DTX mean = 32.8 ± 5.3% larger than with artificial cerebrospinal fluid [aCSF], LGI1-shRNA α-DTX mean = 67.8 ± 6.2% smaller than with aCSF; $P < .0001$,

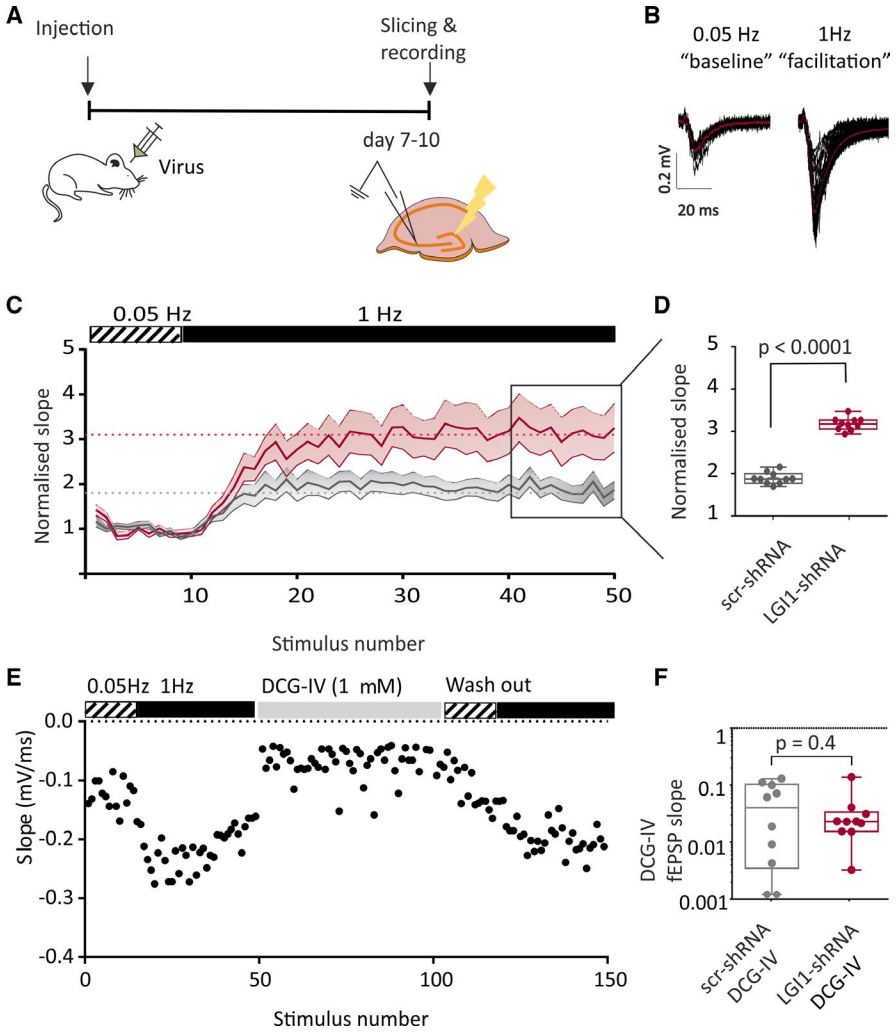

FIGURE 2 Mossy fiber (MF) to CA3 local field potential is increased in animals knocked down by shRNA-LGI1. A, Experimental timeline. B, Stimulation paradigm. On the right side are shown original representative trace of baseline and windup stimulation of MF-CA3 circuit. Red traces represent the averaged signal of 10 baselines traces and 40 facilitation traces. Black lines represent single recordings. C, Pooled timeline data showing the increase in slope following different stimulation frequencies. At 1-Hz stimulation, the amplitude in LGI1 conditions is 30% greater than in the scramble conditions. The bar graph represents the averaged last 10 traces of each recording. D, Scr-shRNA n = 27 slices from eight animals and LGI1-shRNA n = 20 slices from nine animals. E, Representative recording showing the effect of 1 μmol·L^{-1} DCG-IV in reducing field excitatory postsynaptic potential (fEPSP) slope. F, Bar graph summarizing the effects of 1 μmol·L^{-1} DCG-IV in Scr-shRNA and LGI1-shRNA slices

two-way analysis of variance [ANOVA] $F_{1, 20} = 18.24$, followed by Bonferroni correction for comparisons; Scr-shRNA n = 14 from six mice, LGI1-shRNA n = 11 from six mice). As a result, there was no significant difference between windup facilitation in Scr-shRNA–treated and LGI1-shRNA–treated hippocampi in the presence of α-DTX ($P = .7$). As a control, 0.1% bovine serum albumin (BSA) alone had no effect on the rate of facilitation observed in two slices (WT + 0.1% BSA = 7.85 ± 0.08%, n = 2 slices from one mouse, data not shown).

α-DTX had no significant effect on baseline excitatory postsynaptic potential (EPSP; baseline slope ACSF vs α-DTX, repeated measures two-way ANOVA, $P = .1$, $F_{1, 20} = 1.8$). Nevertheless, LGI1-shRNA showed a trend of having a smaller baseline fEPSP compared to control (Scr-shRNA n = 13 from six mice, LGI1-shRNA n = 9 from six mice; Figure S4A,B). The size of the fEPSP is dependent on release probability and number of fibers stimulated. To determine the latter, we analyzed the slope of the fiber volley, which is an indirect measure of axonal recruitment. The analysis of the fiber volleys showed that for both groups, after application of α-DTX, the slope of the fiber volley was reduced by 12.7% of the baseline for Scr-shRNA and 12.6% for LGI1-shRNA (Scr-shRNA n = 12 from five mice, LV-shRNA n = 7 from five mice), but this was not significantly different (fiber volleys slope aCSF vs α-DTX, repeated measures two-way ANOVA, $P = .18$, $F_{1, 17} = 1.9$; Figure S4A,C).

We have therefore shown that blocking the Kv1 family occludes the increased MF facilitation in LGI1-shRNA hippocampi (graphical representation in Figure S5).

3.3 | Paracrine effects of subacute LGI1 downregulation in DG granule cells

Because restricted downregulation of LGI1 is able to increase circuit excitability despite the low efficacy of LV transduction, we asked whether a paracrine effect could exist in the DG. Previous work has demonstrated that LGI1 is a secreted protein, probably released from the dendritic and axonal compartment of neurons.[28] The total KO of *Lgi1*

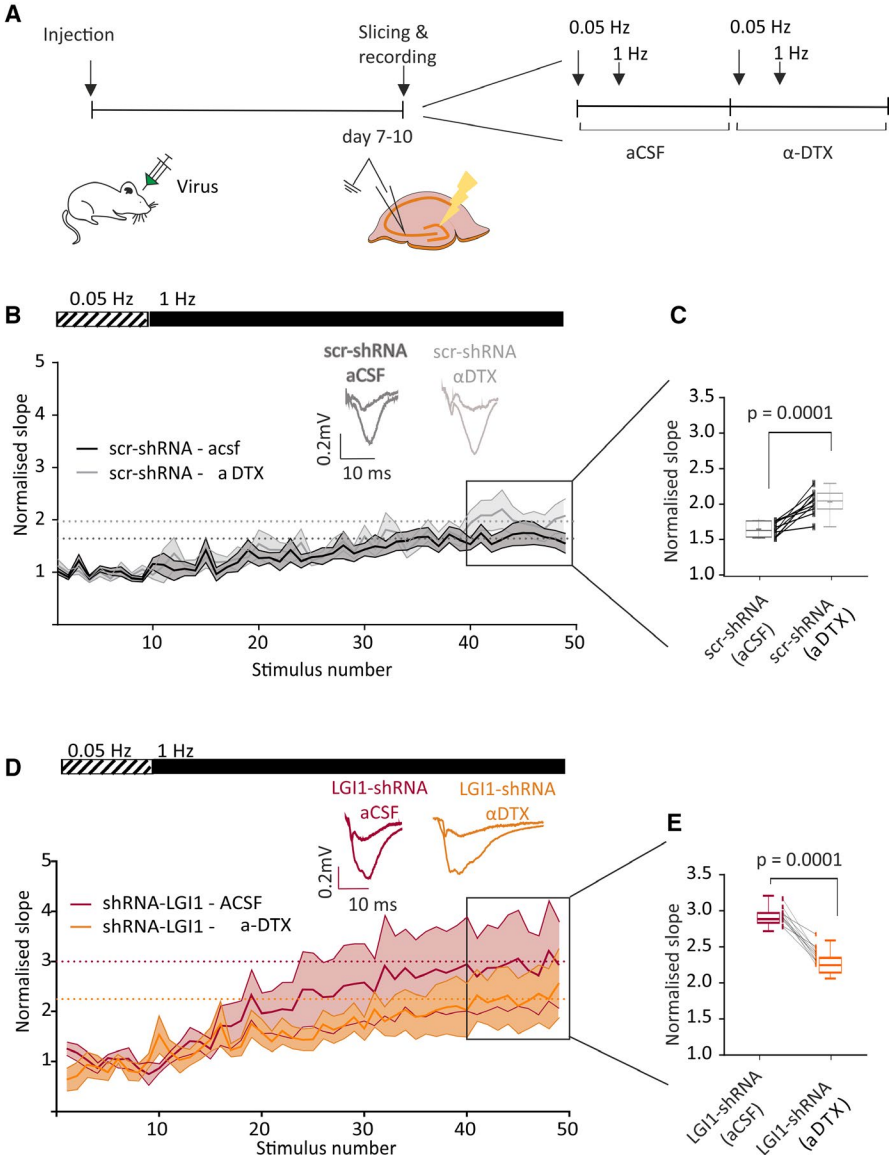

FIGURE 3 The overfacilitation observed in animals with subacute reduction of LGI1 in mossy fiber (MF) to CA3 circuits is due to early inactivation of Kv1.1 channels. A, Experimental timeline and paradigm. B-E, Representative trace and pooled timeline data of windup stimulation of MF-CA3 circuit before and after application of α-dendrotoxin (α-DTX) in scramble-shRNA–injected slices (B, C) and in shRNA-LGI1–injected slices (D, E). Bar graphs in C and E show the quantification of the slope of the last 10 windup stimuli normalized over baseline stimulation. Scr-shRNA, n = 14 from six mice; LGI1-shRNA, n = 11 from six mice

from the early embryonic stage has many different effects on neuronal physiological properties, spanning both pre- and postsynaptic features.[1,29,30] Interestingly, when LGI1 was reintroduced onto *Lgi1* KO neurons, the AMPA/N-methyl-D-aspartate ratio was rescued in both transfected positive neurons and in nearby cells.[28] It is well established that excitatory neurons deprived of LGI1 have a lower rheobase and increased excitability in KO animals, mutants, and conditional KO animals (CAMKIIα-*Lgi1* cKO). In these animal models, the cerebral space and the developmental effects of LGI1 deprivation affect the whole brain, not offering the correct tool for an analysis of the impact of LGI1 downregulation in a localized circuit.[20] Therefore, we aimed to test whether subacute downregulation of LGI1 had a paracrine effect on active and passive neuronal excitability properties in neighboring DG granule cells.[1,10,20,29] We assessed neuronal excitability of cells that were in close proximity to enhanced green fluorescent protein (EGFP)-positive neurons (Figure 4A-C). Neighboring LGI1-shRNA–positive DG neurons displayed a lower threshold to elicit the first action potential (AP), compared to neighboring Scr-shRNA neurons (Scr-shRNA vs LGI1-shRNA, $P = .001$, repeated measures two-way ANOVA with Bonferroni correction for comparisons $F_{1,\ 25} = 12.62$). The half-width of the AP measured at the current threshold was not significantly different between the two groups (Scr-shRNA mean = 1.64 ± 0.58 milliseconds, LGI1-shRNA mean = 1.67 ± 0.66 milliseconds; $P = .739$, two-tailed Student *t* test; Figure 4D); the latency to the first spike between the first four spikes >90pA injected current was shorter for LGI1-shRNA–neighboring neurons (Scr-shRNA vs LGI1-shRNA, $P < .0001$, repeated measures two-way ANOVA with Bonferroni correction for comparisons $F_{3,\ 57} = 25.88$; Figure 4E). Importantly, analysis of passive properties of

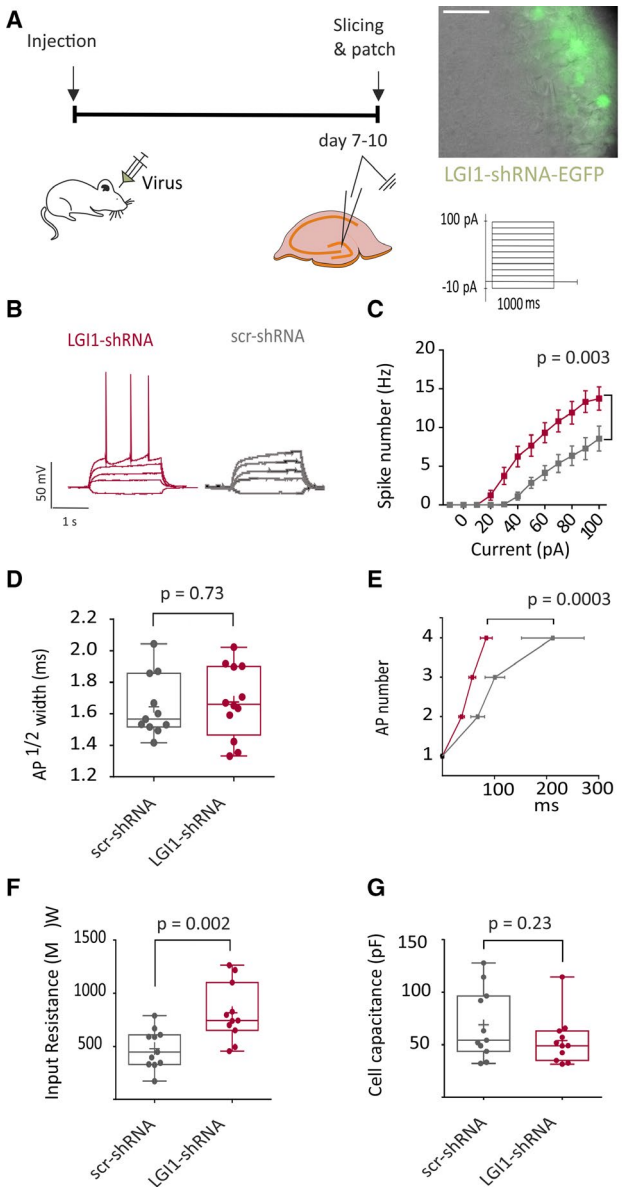

FIGURE 4 Subacute knockdown of LGI1 increases intrinsic neuronal excitability. A, Experimental timeline and paradigm. The image shows granule cells in an acute slice. The pipette is patching an untransfected neuron in the vicinity of neurons positive for LGI1-shRNA (scale bar = 50 μm). B, Current injection protocol and action potential (AP) shape. C, Excitability thresholds of neurons close to cells positive for LGI1-shRNA infection. Representative traces show the current injection from −10 pA to 40 pA for shRNA-LGI1 and scramble treatment. D, Bar graph showing the half-width of the first AP at rheobase. E, Spike latency between the first four APs at 90 pA; 90pA was chosen as the current step in which all the cells in the database were spiking at least four times. F, G, Bar graphs showing membrane input resistance and cell capacitance for neurons close to cells positive for LGI1-shRNA and Scr-shRNA. Scr-shRNA, n = 12; LGI1-shRNA, n = 13 in three mice for each group

patched neurons highlighted a significant difference in the input resistance (Scr-shRNA vs LGI1-shRNA, $P = .002$, two-tailed Student t test), leaving the membrane capacitance unaffected (Scr-shRNA mean = 1.158 ± 0.028 pF, LGI1-shRNA mean = 1.244 ± 0.03 pF; $P = .06$, two-tailed Student t test; Figure 4F,G). Resting membrane potential was not significantly different between treatments (Scr-shRNA, −66.4 ± 2.4 mV; LGI1-shRNA, −70.6 ± 2.5 mV; $P = .25$, two-tailed Student t test)

3.4 | LGI1 downregulation affects circuit network excitability

Thus, subacutely decreasing LGI1 expression has an impact on synaptic transmission and neuronal excitability. Because LGI1 is a secreted protein, it is challenging to study the effect of a spatially restricted KD on broader network excitability within the hippocampus. We therefore took advantage of the more diffuse KD obtainable in primary hippocampal neuronal cultures.[31] It has previously been shown that epileptic bursts and APs correlate with calcium transients in cultures.[32] Therefore, we performed live fluorescence measurement of calcium activity in high-density cortical primary cultures (Figure 5A,B). We showed that neurons treated with LGI1-shRNA were spontaneously more active than the control group (Scr-shRNA mean percentage of active neurons = 43.08 ± 3.87%, LGI1-shRNA mean = 58.46 ± 5.16%; $P = .02$, two-tailed Student t test; Figure 5C). There was also a trend for a higher spike frequency in the LGI1-shRNA group (Scr-shRNA mean = 0.08 ± 0.009, LGI1-shRNA mean = 0.1 ± 0.01; $P = .16$, two-tailed Student t test; Figure 5D).

Furthermore, we measured neuronal excitability using a multielectrode array (Figure 6A,B).[33] Hippocampal neuronal cultures were transduced at 1 DIV, and unit activity was recorded at 21 DIV (Figure 6A). We analyzed the mean bursting rate (MBR), which was significantly higher in neurons transduced with LGI1-shRNA compared to the neurons transduced with Scr-shRNA (Scr-shRNA MBR mean = 5.61 ± 0.68 bursts/min, LGI1-shRNA MBD mean = 8.92 ± 1.18 burst/min; $P = .04$, two-tailed Student t test; Figure 6C). Despite similar mean burst duration (MBD), spike frequency intraburst was higher in LGI1-shRNA–transduced cultures than that in Scr-shRNA–transduced cultures (Figure 6D,E; Scr-shRNA MBD mean = 135.4 ± 7.12 milliseconds, LGI1-shRNA MBD mean = 132 ± 6.5 milliseconds; $P = .73$, two-tailed Student t test; Scr-shRNA mean firing rate [MFR] median = 91.3 spikes/s, IQR = 19.42 spikes/s; LGI1-shRNA MFR median = 107.1 spikes/s, IQR = 32.41 spikes/s; $P = .03$, two-tailed Mann-Whitney test). Further analysis showed that there was a tendency for the average MFR of Scr-shRNA–transduced cultures to be less than that of LGI1-shRNA–transduced cultures, although this did not reach significance (Scr-shRNA MFR median = 1.06 spikes/s, IQR = 1.05; LGI1-shRNA MFR median = 1.79 spikes/s, IQR = 2.01; $P = .07$, two-tailed Mann-Whitney tests; Scr-shRNA, n = 18 wells; LGI1-shRNA, 22 wells).

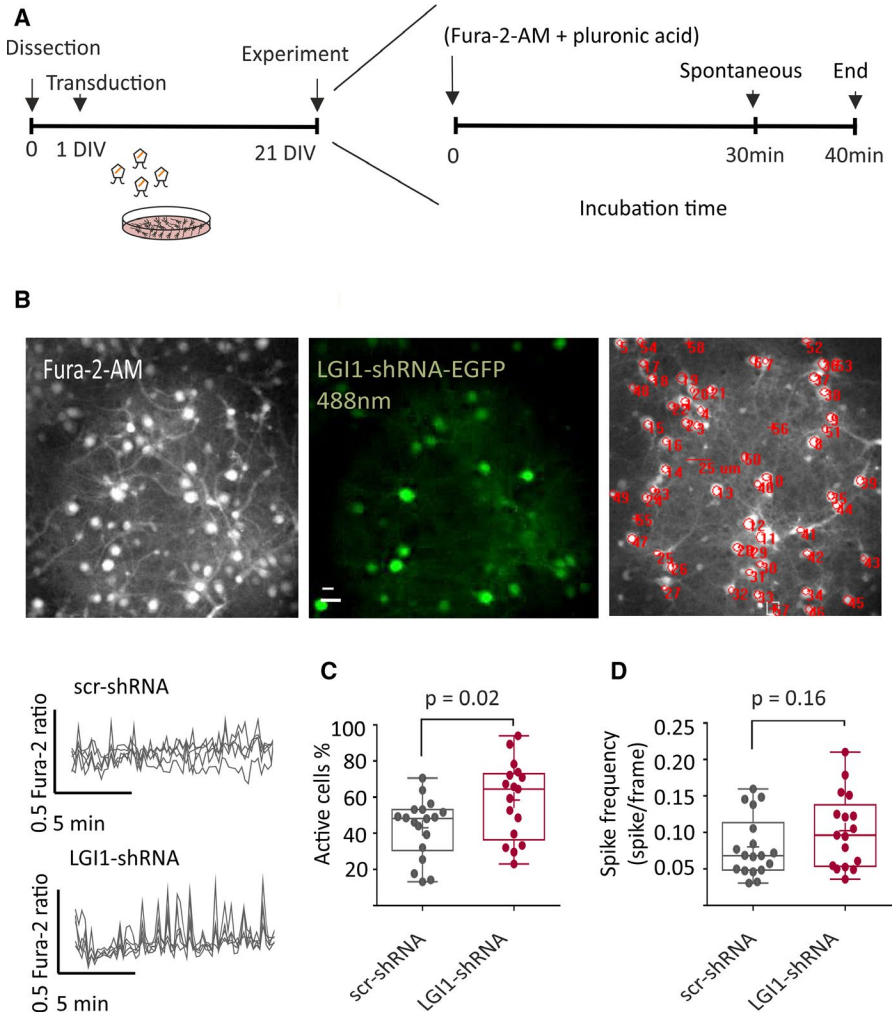

FIGURE 5 LGI1-shRNA affects basal calcium concentration in primary cultures. A, Protocol timeline for the live calcium imaging experiment. DIV, day in vitro. B, The first picture shows a representative field of view of neuronal cultures treated with Fura-2-AM and pluronic acid (510-nm emission filter; original magnification, ×20). The second picture shows the 488-nm enhanced green fluorescent protein (EGFP)-positive cells (scale bar = 25 μm). The third picture is a representative snapshot of cells capturing Fura-2-AM within their cellular membrane. Red circles are manually selected regions of interest of their soma. Representative calcium traces are shown at the bottom left of the panel. C, Percentage of active cells in basal conditions. D, Spike frequency for Scr-shRNA and LGI1-shRNA treatment in basal activity (Scr-shRNA, n = 18; LGI1-shRNA, n = 17 from four independent preparations) [Correction added on 18 November 2020, after first online publication: Figure 5 image has been updated.]

3.5 | LGI1 downregulation does not affect cellular viability and number of excitatory synapses in vitro

Because LGI1 has been reported to be important for glutamatergic synapse development, we investigated the number of active excitatory synapses of in vitro hippocampal neurons.[34] Colocalization of vesicular glutamate transporter 1, a presynaptic marker of excitatory synapses, and homer1, a postsynaptic marker of active excitatory synapses, did not reveal any significant difference between neurons transduced with Scr-shRNA or LGI1-shRNA (Mander coefficient: Scr-shRNA, 0.6 ± 0.04; LGI1-shRNA, 0.62 ± 0.03; $P = .58$, two-tailed t test; Scr-shRNA, n = 11; LGI1-shRNA, n = 12; Figure S6A,B).

Neuronal death in cultures and brain slices is an important mechanism of homeostasis.[35] To check whether removal of LGI1 in cultures affects cell viability which could lead to the difference in calcium signaling and burst activity, we used a mortality cell assay with propidium iodide staining.[36] The mortality rate between LGI1-shRNA–transduced and Scr-shRNA–transduced cultures were comparable (Scr-shRNA mortality rate median = 51.38%, range = 44.9% [38%-82.9%], IQR = 10.68%; LGI1-shRNA mortality rate median = 48.25%, range = 25.3% [42.1%-6.4%], IQR = 12.38%; $P = .9$, Mann-Whitney two-tailed test; Figure S6C,D).

4 | DISCUSSION

Extracellular matrix proteins have recently emerged as key contributors to the maintenance and regulation of physiological processes.[37] Here, we investigated the role of LGI1, a transsynaptic secreted protein, part of the synaptic extracellular matrix, after its subacute depletion in neuronal networks.

In this work, we established an RNA-silencing method to downregulate LGI1 subacutely in both ex vivo hippocampal slices and neuronal culture. We demonstrated that subacute removal of LGI1 in MF-CA3 hippocampal circuits was able to alter local network and neuronal excitability. Pharmacological experiments with a blocker of Kv1 ion channels indicated that modification of Kv1 was the likely basis of the observed

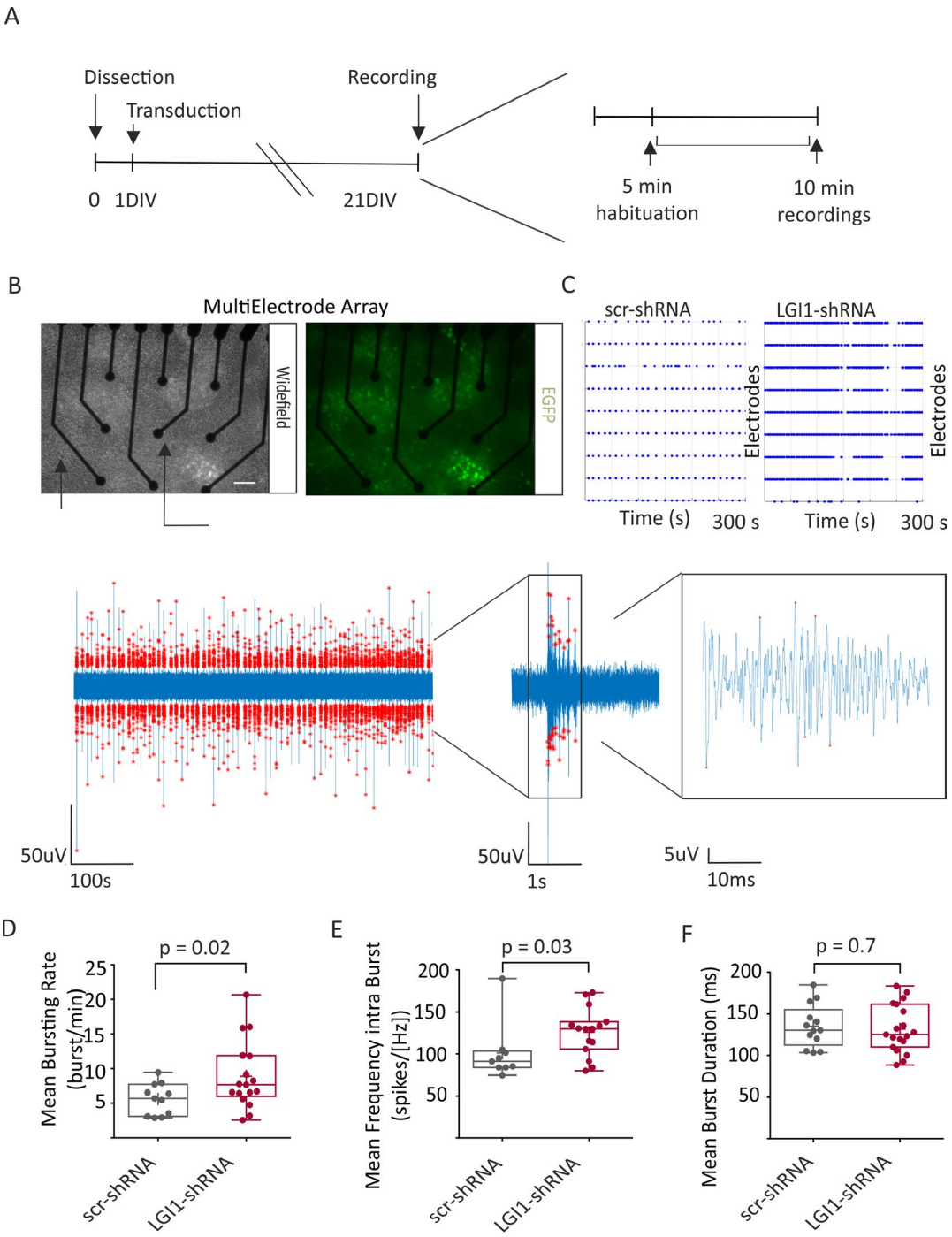

FIGURE 6 Subacute knockdown of LGI1 affects network burst activity. A, Timeline of the experiments. DIV, day in vitro. B, Representative picture of a single well of a multielectrode array. The nine recording electrodes are in black, over a field of neuronal networks. Each electrode is of 30-µm diameter. The picture shows 21-day-old multielectrode arrays (left: wide field; right, in green: expression of enhanced green fluorescent protein [EGFP] in transduced cultures; bottom: merged picture; scale bar = 60 µm). C, Raster plot (spikes) of the neuronal activity detected by single electrodes in LGI1-shRNA and Scr-shRNA. D, Mean bursting rate (Scr-shRNA, n = 11 wells; LGI1-shRNA, 17 wells; $P = .04$, Student t test). E, Mean firing intraburst (Scr-shRNA, n = 11 wells; LGI1-shRNA, 17 wells; $P = .03$, Mann-Whitney test). F, Mean burst duration (Scr-shRNA, n = 11 wells; LGI1-shRNA, 17 wells; $P = .73$, Student t test). Data were plotted and averaged from five independent preparations

increased facilitation observed in MF-CA3 circuits. Moreover, we demonstrated that a 40% downregulation of LGI1 in primary neuronal cultures was sufficient to alter the basal calcium levels and network bursts, without affecting neuronal survival.

So far, investigation of LGI1 in the physiology of neuronal circuits has been explored with the use of transgenic animals or with acute applications of autoantibodies against LGI1 in primary cultures and slices.[38] These models demonstrated

that LGI1 plays a crucial role during embryonic development and modulates both pre- and postsynaptic transmission. Several discrepancies in the effects described from multiple groups could be ascribed to the different genetic background of the animal models used, the differing ages of the brain samples examined, and the diverse anatomical zones investigated.[1,19,39] Moreover, some LGI1 mutations cause a loss of functional monomer, whereas others have a dominant-negative effect, hence triggering epilepsy probably via two different mechanisms of action. Finally, experiments with autoantibodies from patients with LGI1 limbic encephalitis suggest that acute interference with LGI1 is sufficient to alter neuronal circuits and synaptic transmission, without a significant immune/inflammatory component.[14,15,40]

To separate the contribution of developmental issues and of the inflammatory response from the removal of LGI1 protein in neuronal circuits, we designed and validated a subacute method based on small interfering RNA for downregulation of LGI1, avoiding the use of mutants or of polyclonal IgGs from encephalitic patients. To our knowledge, this approach has not been used before in the investigation of the role of LGI1, but it can establish the effects of subacute LGI1 downregulation. We achieved ~40% KD of the endogenous LGI1 protein.[8,41,42] This KD level is close to a heterozygous state, helping to dissect realistic physiological consequences of LGI1 molecular alterations on neuronal circuits.

Despite evidence that LGI1 has a pivotal role in glutamatergic neurons, LGI1 staining is also present in interneurons and glia.[38] Therefore, in this study, we used a broad promoter (mU6) for silencing RNA activity that is able to induce a constant production of the shRNA in any cell type.

Measurements of short-term plasticity using ex vivo hippocampal slices previously injected with LGI1-shRNA showed greater facilitation of MF to CA3 transmission. This result is crucial to attribute functional alterations of excitatory transmission to subacute downregulation of LGI1, without the contribution of developmental circuit abnormalities or temporal lobe inflammation. These results are in line with previous work that measured higher spontaneous burst activity and interictal-like depolarization by extracellular field recordings in CA1 of *Lgi1*-KO.[15,19,30] Also, the acute application of VGKC-IgGs in the DG-CA3 pathway confirmed that acute interference with LGI1 provoked higher neurotransmitter release from the granule cells and increased postsynaptic excitability.[40] The effects that we observed on low-frequency facilitation at the MF-CA3 synapse suggest that activation of presynaptic Kv1.1 during low-frequency facilitation restricts the degree of facilitation. Absence of LGI1, however, results in Kv1.1 becoming a fast-inactivating, slow-deinactivating channel.[9] Under these conditions, repetitive stimulation results in a progressive inactivation of Kv1.1, which further contributes to low-frequency facilitation (Figure S5).[27] Loss or a reduction in LGI1 will enhance DG to CA3 neurotransmission and could thus act as an initiator of seizures and could facilitate propagation in the hippocampus, as previously hypothesized.[10] That an extracellular structural protein influences network excitability and glutamate release has been debated for many years. The first evidence that LGI1 indirectly has a role in cellular excitability arrived when it was first co-purified in the macromolecular structure formed by Kv1.1 presynaptic potassium channel and postsynaptic AMPA receptors. Since then, it has been suggested that LGI1 influences Kv1.1 channels by two mechanisms: a prolongation of its opening state by interference with the Kvβ1 regulatory subunit, and an influence on the number of Kv1.1 channels recruited to the presynapse. Inside-out patches in oocytes showed that mutations or removal of LGI1 proteins lead to a shortening of the opening time of Kv1.1 channels. This has been attributed to the ability of a single kvβ1 unit to block the whole Kv1.1 tetramer. Instead, scr-shRNA and LGI1-shRNA are required to interfere with kvβ1, so that a small decrease in LGI1 relative to kvβ1 causes a much greater effect than expected.[9] Ex vivo LGI1 reduction affected short-term plasticity of MF to CA3 synapses, resulting in a significant overfacilitation during repetitive stimulation. This is probably caused by early inactivation of Kv1.1 channels, because blocking Kv1 with α-DTX occluded this effect. Therefore, it is also possible that subacute reduction of LGI1 proteins causes alteration of neurotransmission by functional modification of Kv1.1 kinetics. We cannot, however, completely exclude a postsynaptic contribution from LGI1 reduction due to its effect on AMPA receptors too.[1] Application of α-DTX in both Scr-shRNA and LV-shRNA caused a small increase in the amplitude of the response (in slope). At the same time, we also measured a trend to a decrease in the fiber volley slope, implying fewer axons recruited by the stimulation. Together, these results indicate that the release probability for any single fiber may be increased, suggesting that Kv1.1 could be contributing to the initial release probability. Our findings can therefore be explained by the reduction of LGI1 promoting inactivation of Kv1.1 during the windup, resulting in a greater release probability. In the future, it would be interesting to confirm whether different types of short-term plasticity, such as occurs with high-frequency stimulation, are also affected by LGI1 subacute downregulation.[27] The lack of effect of LGI1 KD on paired pulse stimulation with short intervals together with the likely predominant effect of LGI1 KD on Kv1.1 inactivation kinetics suggest that the effects on high-frequency stimulation will be less evident.

Patch-clamp experiments of cells near to $EGFP^+$ neurons in LGI1-shRNA slices revealed that reduction of LGI1 affects the physiological properties of neurons close to transduced LGI1-shRNA–positive cells. Rescue experiments in previous work supported that LGI1 probably has paracrine effects on neighboring neurons, hence local and

circumscribed reduction may affect other neurons too.[28] Our data show that subacute reduction of LGI1 reduces AP threshold.[10] This was also observed with acute incubation of VGKC-IgG by another group.[40] LGI1 subacute reduction also decreases the interspike interval, but does not affect the AP half-width. This is probably due to the expression pattern of Kv1.1, which is more abundant at the axon initial segment and axons.[43]

We asked whether these changes in excitability that we observed translated into an effect on network excitability. We employed calcium measurements and MEA devices to show, for the first time, that subacute LGI1 reduction results in an alteration in neuronal network excitability. The measurement of calcium waves is of critical importance, because they are strongly correlated with epileptic-like bursts and APs in vitro.[32] These initial studies indicated that broad heterozygous-like LGI1 downregulation was sufficient to alter basal calcium levels and burst activity of in vitro neuronal networks. These results corroborate previous findings from studies of LGI1 mutants in cell lines[44,45] and extend the present knowledge about LGI1-IgGs. Interestingly, only one previous study investigated calcium waves in neuronal cultures, showing the opposite effect to what we showed here. This might be attributable to long-term (24-72 hours) IgG incubations leading to neuronal toxicity in that study.[36] To conclude, the results reported here demonstrate that a subacute decrease of LGI1 can result in alterations of the DG-CA3 pathway excitability and increases in network hyperexcitability in vitro. Our findings support new pathophysiological mechanisms by which alteration of LGI1 affects brain excitability and opens up new clinical intervention routes through, for example, the use of small molecules that inhibit Kv1.1 inactivation.[46]

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CONFLICT OF INTEREST

None of the authors has any conflict of interest to disclose.

ETHICAL PUBLICATION STATEMENT

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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