

## Glutamatergic neuron-targeted loss of *LGI1* epilepsy gene results in seizures

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Leucin-rich, glioma inactivated 1 (*LGI1*) is a secreted protein linked to human seizures of both genetic and autoimmune aetiology. Mutations in the *LGI1* gene are responsible for autosomal dominant temporal lobe epilepsy with auditory features, whereas *LGI1* autoantibodies are involved in limbic encephalitis, an acquired epileptic disorder associated with cognitive impairment. We and others previously reported that *Lgi1*-deficient mice have early-onset spontaneous seizures leading to premature death at 2–3 weeks of age. Yet, where and when *Lgi1* deficiency causes epilepsy remains unknown. To address these questions, we generated *Lgi1* conditional knockout (cKO) mice using a set of universal Cre-driver mouse lines. Selective deletion of *Lgi1* was achieved in glutamatergic pyramidal neurons during embryonic (*Emx1-Lgi1cKO*) or late postnatal (*CaMKII $\alpha$ -Lgi1cKO*) developmental stages, or in gamma amino butyric acidergic (GABAergic) parvalbumin interneurons (*PV-Lgi1cKO*). *Emx1-Lgi1cKO* mice displayed early-onset and lethal seizures, whereas *CaMKII $\alpha$ -Lgi1cKO* mice presented late-onset occasional seizures associated with variable reduced lifespan. In contrast, neither spontaneous seizures nor increased seizure susceptibility to convulsant were observed when *Lgi1* was deleted in parvalbumin interneurons. Together, these data showed that *LGI1* depletion restricted to pyramidal cells is sufficient to generate seizures, whereas seizure thresholds were unchanged after depletion in gamma amino butyric acidergic parvalbumin interneurons. We suggest that *LGI1* secreted from excitatory neurons, but not parvalbumin inhibitory neurons, makes a major contribution to the pathogenesis of *LGI1*-related epilepsies. Our data further indicate that *LGI1* is required from embryogenesis to adulthood to achieve proper circuit functioning.

**Keywords:** epilepsy; *LGI1*; ADEAF; genetics; conditional knockout

**Abbreviations:** ADEAF = autosomal dominant epilepsy with auditory features; cKO = conditional knockout

## Introduction

Most genes mutated in monogenic epilepsies encode ion channel subunits, suggesting that familial epilepsies are effectively classed as channelopathies (Reid *et al.*, 2009). One exception is the leucine-rich glioma-inactivated 1 (*LGI1*) gene, encoding a secreted protein. Mutations in *LGI1* have been reported in families with autosomal dominant epilepsy with auditory features (ADEF) (Kalachikov *et al.*, 2002; Morante-Redolat *et al.*, 2002). ADEF is a well-defined inherited condition consisting of adolescence/early adulthood-onset lateral temporal seizures. The clinical distinctive feature of this syndrome is the presence of prominent auditory component, either as an aura or as a triggering factor of seizures (Michelucci *et al.*, 2009). One-third of the 39 published ADEF-causing mutations result in a premature stop codon, which causes haploinsufficiency. Numerous missense mutations prevent LGI1 secretion from transfected cells (Senechal *et al.*, 2005; Sirerol-Piquer *et al.*, 2006; Chabrol *et al.*, 2007; Striano *et al.*, 2008; de Bellescize *et al.*, 2009; Nobile *et al.*, 2009; Di Bonaventura *et al.*, 2011; Leonardi *et al.*, 2011; Fanciulli *et al.*, 2012; Sadleir *et al.*, 2013; Lee *et al.*, 2014). Other pathogenic mutations may impair LGI1 interactions with protein partners rather than suppressing protein secretion (Striano *et al.*, 2011). Overexpression of two different truncating LGI1 mutant proteins in mammalian cells (Schulte *et al.*, 2006) and in transgenic mice (Zhou *et al.*, 2009) point to dominant negative effects. In addition to its role in inherited epilepsies, LGI1 is involved in a subset of patients with acquired autoimmune limbic encephalitis, a neurological disorder of adulthood (Irani *et al.*, 2010; Lai *et al.*, 2010). Patients with autoantibodies directed against LGI1 protein suffer from psychiatric symptoms, including memory loss and confusion, and from epilepsy. Seizures are described as: (i) characteristic faciobrachial dystonic seizures with brief, repeated unilateral motor manifestation; or (ii) mesial temporal lobe seizures in the case of limbic involvement (Irani *et al.*, 2013).

Mechanisms of LGI1-related epilepsies remain unclear (Kegel *et al.*, 2013). Four main functions have been proposed for LGI1 in the CNS: (i) inhibition of the inactivation of the presynaptic voltage-gated potassium channel Kv1.1 (Schulte *et al.*, 2006); (ii) potentiation of AMPA receptor-mediated synaptic transmission in the hippocampus through interaction with ADAM22/23 ('a disintegrin and metalloprotease' domain) transmembrane proteins (Fukata *et al.*, 2006, 2010; Ohkawa *et al.*, 2013); (iii) enhancement of neuronal growth on myelin-based inhibitory substrates by interactions with Nogo receptor 1 (Thomas *et al.*, 2010); and (iv) postnatal developmental maturation of glutamatergic transmission, in hippocampal dentate gyrus through pre- and postsynaptic functional maturation, pruning of spines and dendritic branches, and in thalamus through axonal pruning (Zhou *et al.*, 2009, 2012). Remarkably, all animal models of *Lgi1*-deficiency display spontaneous seizures: *Lgi1*<sup>-/-</sup> knockout (KO) mice (Chabrol *et al.*, 2010; Fukata *et al.*, 2010; Yu *et al.*, 2010), *Lgi1*-mutant rats (Baulac *et al.*, 2012) and *lgi1a* zebrafish knock-down (Teng *et al.*, 2010). Homozygous *Lgi1*<sup>-/-</sup> mice have spontaneous seizures with onset at postnatal Day 10 and all pups die at

2–3 weeks of age, while heterozygous *Lgi1*<sup>+/-</sup> mice are more susceptible to sound-induced (Chabrol *et al.*, 2010) or pentylene-tetrazole-induced seizures (Fukata *et al.*, 2010). Two studies based on records of hippocampal miniature excitatory postsynaptic currents (mEPSCs) in *Lgi1*<sup>-/-</sup> brain slices suggested effects of LGI1 on glutamatergic synapses, though with opposite conclusions: Yu *et al.* (2010) showed an increased frequency of mEPSCs in *Lgi1*<sup>-/-</sup> mice, whereas Fukata *et al.* (2010) found a reduced amplitude of mEPSCs mediated by AMPA receptors. A specific role for LGI1 in glutamatergic circuits is intriguing as *Lgi1* mRNA and protein are expressed in both glutamatergic and gamma amino butyric acid-ergic (GABAergic) neurons, as well as glial cells, of multiple brain regions (Kalachikov *et al.*, 2002; Senechal *et al.*, 2005; Head *et al.*, 2007; Silva *et al.*, 2011; Ohkawa *et al.*, 2013).

This study was based on conditional knockout (cKO) mice permitting a brain region-, cell type-, and time-restricted deletion of *Lgi1*. In this way, we first aimed to determine whether spontaneous seizures of *Lgi1*<sup>-/-</sup> mice could be reproduced by a selective deficiency of *Lgi1* in cortical glutamatergic neurons. Secondly, we aimed to ask whether loss of *Lgi1*, beyond the early neurodevelopmental period, can also trigger seizures later in life. Independent *Lgi1* cKO mice strains were generated using a set of universal Cre-driver mouse lines (Emx1-Cre and CaMKII $\alpha$ -Cre) targeting cortical neuronal populations: (i) Emx1-Cre targets forebrain neural progenitor cells, the precursors of neocortical and hippocampal glutamatergic neurons at embryonic stage E10.5 (Gorski *et al.*, 2002); and (ii) CaMKII $\alpha$ -Cre targets neocortical and hippocampal glutamatergic neurons at late-onset postnatal stage (from 5 weeks) (Zeng *et al.*, 2001). We also investigated whether GABAergic cortical interneurons might be involved in seizure generation using PV-Cre mice, which targets parvalbumin (PV) interneurons from the embryonic stage (Hippenmeyer *et al.*, 2005).

## Materials and methods

### Generation of *Lgi1* conditional knockout mice

Three universal Cre-driver mouse lines were selected to excise the floxed allele of *Lgi1*: Emx1-Cre (JAX # 005628), allowing embryonic glutamatergic forebrain-specific deletion of *Lgi1* (Gorski *et al.*, 2002); CaMKII $\alpha$ -Cre (also reported as CW2-Cre, generated in Tonegawa's laboratory, RIKEN-MIT Neuroscience Research Center, Massachusetts Institute of Technology, Cambridge, MA, USA), allowing postnatal glutamatergic forebrain-specific deletion of *Lgi1* (Zeng *et al.*, 2001; Anderson *et al.*, 2005) and PV-Cre (JAX #008069), allowing embryonic deletion of *Lgi1* in parvalbumin interneurons (Hippenmeyer *et al.*, 2005). *Lgi1* floxed (*Lgi1*<sup>fl/fl</sup>) mice and heterozygous KO mice (*Lgi1*<sup>+/-</sup>) were previously generated (Chabrol *et al.*, 2010). All mouse lines were maintained on the C57BL/6J genetic background. Mice were housed in groups of four to six littermates/cage with food and water *ad libitum* and kept in a 21  $\pm$  1°C and 12-h light/dark cycle under specific pathogen-free conditions at the ICM animal core facility and at the Nouvelle Animalerie Commune (Pitié-Salpêtrière, Paris). The Animal Ethics Committee approved all experiments. Animals were treated according to the guidelines of the European Community

(authorization number 75-1622). All efforts were made to minimize the number of animals used in the study and their suffering.

## Genotyping

All mice were genotyped by PCR at the genotyping and sequencing ICM platform. Genomic DNA was extracted from tail biopsies of mice. *Lgi1* wild-type, floxed and null alleles were analysed by PCR amplification using the following primer pairs: 5'-ACATTCCTTAGTGCCCTGTTT-3'/5'-CCTCTTAGCCACTGAGGCATCT-3' (wild-type and floxed alleles) and 5'-ATTCCTTAGTGCCCTGTTTTA-3'/5'-TGTCTGATTCAATGCTGCTTAGA-3' (null allele). A 120-bp band was observed for the wild-type allele, a 160-bp band for the floxed allele, and a 200-bp band for the null allele. Genotyping regularly detected germinal knockout mice in the offspring of Emx1-Cre breedings due to expression of Cre recombinase in germline cells. Germinal knockout mice were systematically excluded from the study. Primers and PCR conditions recommended by the Jackson Laboratory were used to screen for the presence of each Cre transgene. Age-matched littermates of the genotypes of interest were used for all subsequent analyses.

## Western blot

Conditional knockout mice and their littermate controls were decapitated and their whole brains were rapidly removed. Cortex, hippocampus and cerebellum were dissected and lysed in 2.5 M urea, 2.5% SDS, 50 mM Tris, 30 mM NaCl buffer. Total protein concentrations were determined by the BCA Protein Assay Kit (Pierce). Twenty micrograms of protein for each sample was separated on 10% Bis-Tris polyacrylamide gels (Novex, Invitrogen). Western blot analyses were performed using the following antibodies: rabbit polyclonal anti-LGI1 antibody (1/500, ab30868, Abcam) and rabbit polyclonal anti- $\alpha$ -actin antibody (1/1000, A2066, Sigma-Aldrich). Quantification of LGI1 expression was done using MultiGauge densitometry software and normalized with actin. Data are reported as mean  $\pm$  standard error of the mean (SEM).

## Histochemistry

Conditional knockout mice and their littermate controls were intraperitoneally injected with a lethal dose of sodium pentobarbital, and then perfused with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Brains were removed, post-fixed in the same fixative for 24 h at 4°C and then paraffin embedded. Paraffin sections of 7- $\mu$ m thickness were cut. For all experiments, at least two littermate mice of each genotype were processed simultaneously. Antibodies used were rabbit polyclonal anti-LGI1 antibodies (ab30868, ab69491, ab67319; Abcam) and goat polyclonal anti-LGI1 antibodies (sc-9581, sc-9583; Santa Cruz). Nissl staining was used to reveal neuronal cytoarchitecture.

## Mouse lifespan

Mice were housed in groups of four to six same-sex littermates under specific pathogen-free conditions. They were observed and weighed daily, but were otherwise left undisturbed until they died. Survival was assessed in male and female mice. Kaplan–Meier survival curves were made using known birth and death dates. Data are reported as mean  $\pm$  SEM. Mice that died spontaneously were not used for other experiments.

## Seizure analysis by SeizureScan

SeizureScan software is designed to automatically identify seizures of rodents in a home cage, providing a non-invasive tool to screen for epileptic phenotype (CleverSys Inc). Mice were placed individually in their home cage and habituated for 30 min before recording. Recurrent video recordings of Emx1-*Lgi1*cKO ( $n = 4$ ), CaMKII $\alpha$ -*Lgi1*cKO ( $n = 5$ ) and PV-*Lgi1*cKO ( $n = 6$ ) mice and their control littermates ( $n = 11$ ) were analysed by SeizureScan software. In collaboration with CleverSys, settings fitted to juvenile or adult animals were developed and used for automatic detection of behavioural seizures. A systematic manual control of the results was performed. Data are reported as mean  $\pm$  SEM.

## Animal surgical procedure

This work was performed at Rennes University and followed the European Community Council Directive of 24 November 1986 (86/609/EEC). Conditional knockout mice and their littermate controls were anaesthetized by an intraperitoneal injection of a chloral hydrate solution in NaCl (4%, 10 ml/kg), and then placed in a stereotaxic frame in a flat skull position. Bipolar depth electrodes (homemade polyester insulated stainless steel electrodes) were implanted in both hippocampi (coordinates from bregma: anteroposterior =  $-2.0$  mm, mediolateral =  $\pm 1.5$  mm, dorsoventral =  $-1.9$  mm) and monopolar surface electrodes over both motor cortex (M1). A reference electrode was placed over the cerebellum.

## Intracranial video-EEG recordings

EEG recordings were obtained from Emx1-*Lgi1*cKO ( $n = 2$ ), CaMKII $\alpha$ -*Lgi1*cKO ( $n = 7$ ), PV-*Lgi1*cKO ( $n = 2$ ) and littermate controls ( $n = 12$ ) mice using a video-EEG system (Deltamed, 2048 Hz). Recording sessions always started at the same time of the day to minimize diurnal variation. Mice were placed in a transparent cage, positioned in a Faraday cage, first habituated for 30 min and then recorded. EEG from Emx1-*Lgi1*cKO mice were recorded immediately after complete recovery from surgery, and EEG records were obtained for 4–6 h/day over 2 days, until animal death. EEG from CaMKII $\alpha$ -*Lgi1*cKO mice were recorded during  $>9$  months on a weekly basis. EEG from PV-*Lgi1*cKO mice were recorded over a period of up to 2.5 months on a weekly basis. To verify location of depth electrodes after the experiment, brains were removed and sliced (20- $\mu$ m thickness) with a cryostat. Data are reported as mean  $\pm$  SEM.

## Pentylentetrazole administration

Three groups of 2-month-old mice were injected with a 53 mg/kg dose of pentylentetrazole (Sigma-Aldrich): PV-*Lgi1*cKO mice ( $n = 11$ ), PV-*Lgi1*<sup>+/-</sup> mice ( $n = 18$ ) and PV-*Lgi1*<sup>+/+</sup> ( $n = 20$ ). Pentylentetrazole was dissolved in saline solution. Behavioural response to pentylentetrazole was followed 30 min after the injection to assess severity of seizures visually scored to four stages as previously reported (Harai et al., 2012). Mice were observed by two investigators blinded to their genotype. Distribution of maximal score of seizure severity was assessed using the  $\chi^2$  test. Latency between pentylentetrazole injection and the occurrence of the first generalized seizure was assessed using the *t*-test. At the end of the experiments, all animals were euthanized. Data are reported as mean  $\pm$  SEM.

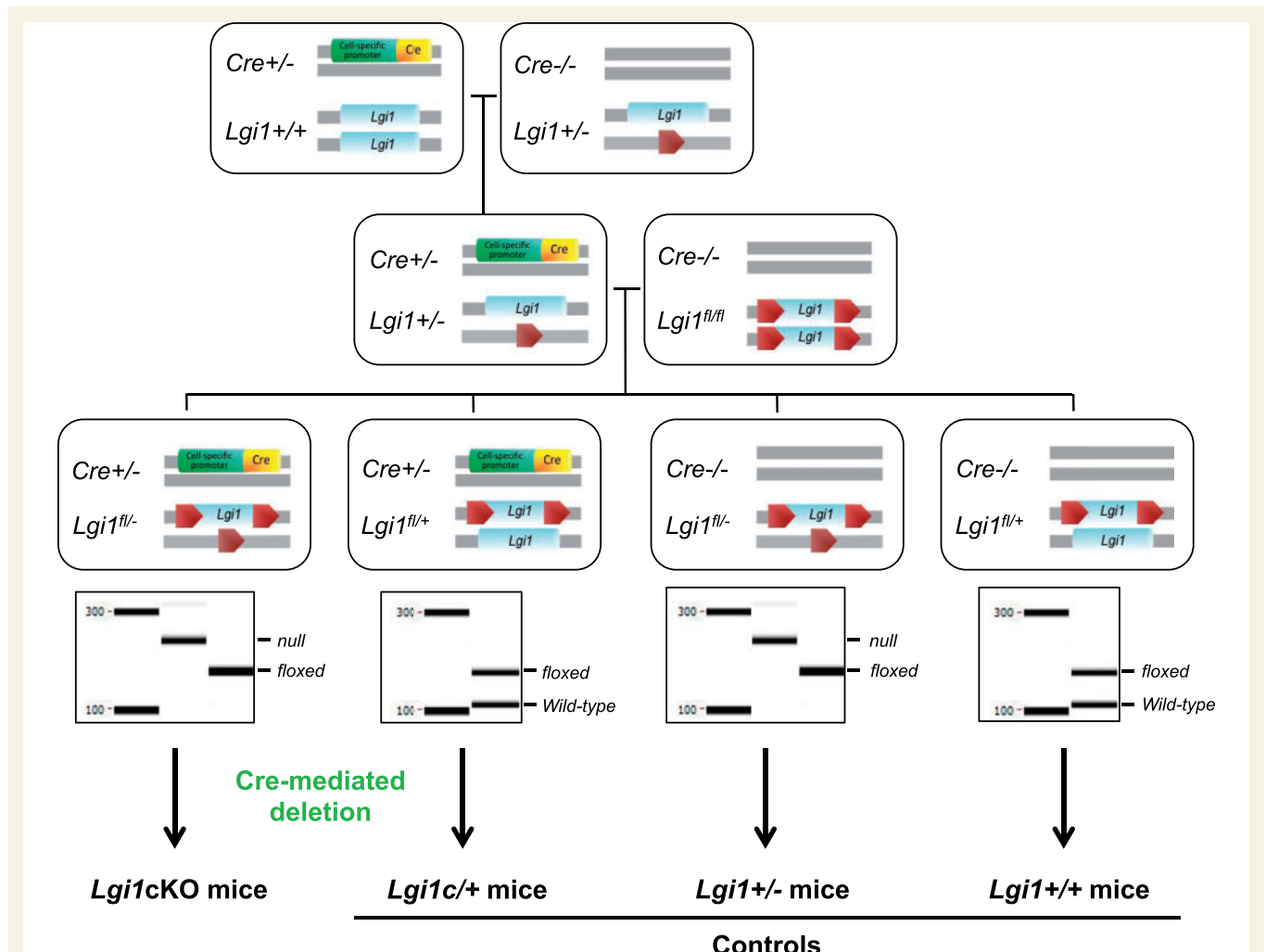
## Results

### Conditional deletion of *Lgi1* in forebrain pyramidal neurons

We examined the effects of conditional *Lgi1* deletion in forebrain glutamatergic pyramidal cells at two different developmental stages by using two standard Cre transgenic mouse lines. We used previously generated homozygous *Lgi1*-floxed (*Lgi1<sup>fl/fl</sup>*) and heterozygous *Lgi1* knockout mice (*Lgi1<sup>+/-</sup>*) (Chabrol *et al.*, 2010). *Lgi1<sup>fl/fl</sup>* and *Lgi1<sup>+/-</sup>* mice are fertile, viable and display neither gross structural or histological brain abnormalities nor spontaneous seizures (data not shown). Figure 1 shows the breeding cascade performed to generate distinct mouse lines on a *Lgi1<sup>fl/-</sup>* heterozygous knockout background: *Lgi1<sup>fl/-</sup>*; Emx1-Cre<sup>+/-</sup> designated as Emx1-*Lgi1*cKO, and *Lgi1<sup>fl/-</sup>*; CaMKII $\alpha$ -Cre<sup>+/-</sup> designated

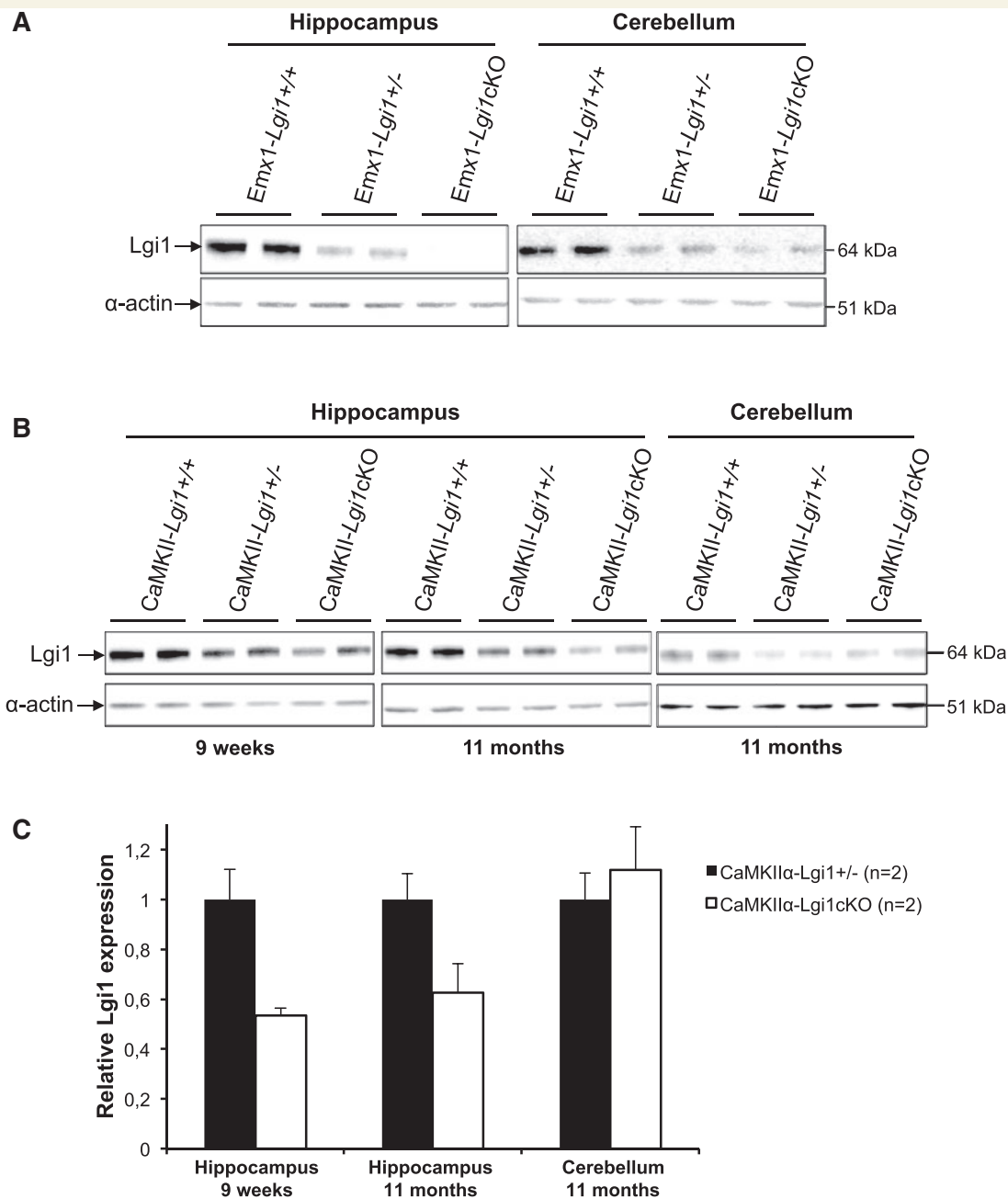
as CaMKII $\alpha$ -*Lgi1*cKO (Fig. 1). Littermates carrying the alternative genotypes (*Lgi1<sup>fl/+</sup>*; Cre<sup>+/-</sup> designated as *Lgi1<sup>cl/+</sup>*), (*Lgi1<sup>fl/-</sup>*; Cre<sup>-/-</sup> designated as *Lgi1<sup>+/-</sup>*) and (*Lgi1<sup>fl/+</sup>*; Cre<sup>-/-</sup> designated as *Lgi1<sup>+/+</sup>*) were used as controls. In Emx1-Cre (males and females) derived mouse strain, expected genotypes were not recovered in Mendelian ratios due to recurrent ectopic recombination of the floxed *Lgi1* allele. At birth, all conditional knockout and littermate control mice were viable with no gross anatomical abnormalities.

LG11 protein expression from conditional knockout mice and their respective littermate controls was assessed by Western blot of hippocampus, neocortex (data not shown) and cerebellum lysates. Immunoblots showed the intensity of the LG11 64 kDa band in the hippocampus of Emx1-*Lgi1*cKO mice ( $n = 2$ ) was greatly reduced compared to controls, whereas it was still expressed in the cerebellum (Fig. 2A). In adult CaMKII $\alpha$ -*Lgi1*cKO mice, immunoblots of hippocampus lysates revealed LG11 expression was



**Figure 1** Breeding schemes of the generation of *Lgi1*cKO mice. Two sequential crosses were made: 1/ breeding Emx1-Cre, CaMKII $\alpha$ -Cre or PV-Cre mice with *Lgi1<sup>+/-</sup>* mice resulted in *Lgi1<sup>+/-</sup>*; Emx1-Cre<sup>+/-</sup>; *Lgi1<sup>+/-</sup>*; CaMKII $\alpha$ -Cre<sup>+/-</sup> and *Lgi1<sup>+/-</sup>*; PV-Cre<sup>+/-</sup> animals, 2/ these mice were then crossed with *Lgi1<sup>fl/fl</sup>* mice to generate experimental genotypes. Results representative of PCR genotyping from *Lgi1<sup>+/+</sup>* and *Lgi1<sup>cl/+</sup>* mice (wild-type *Lgi1*: 120-bp band, floxed *Lgi1*: 160-bp band), *Lgi1<sup>+/-</sup>* and *Lgi1*cKO mice (floxed *Lgi1*: 160-bp band, null *Lgi1*: 200-bp band) are shown.





**Figure 2** Specific deletion of *Lgi1* in forebrain glutamatergic neurons. Representative Western blot cropped around LGI1 and actin bands showing (A) a severely reduced LGI1 expression in the hippocampus but not cerebellum of two *Emx1-Lgi1*cKO mice aged postnatal Day 18, (B) a reduction of LGI1 expression in the hippocampus of two *CaMKIIα-Lgi1*cKO mice aged 9 weeks and 11 months. LGI1 level was not reduced in the cerebellum of *CaMKIIα-Lgi1*cKO mice. (C) Quantification of LGI1 protein levels by densitometry of Western blots and normalized with actin for loading. Bar graph with error bars indicating SEM shows mean values of LGI1 band intensity normalized to actin.

reduced to  $47 \pm 3\%$  at 9 weeks ( $n=2$ ) and  $37 \pm 12\%$  at 11 months ( $n=2$ ) after birth compared to *CaMKIIα-Lgi1*<sup>+/-</sup> mice (Fig. 2B and C). However, we noted that the expression of LGI1 varied between animals of the same age, which may reflect a variable efficiency of the deletion. As expected, LGI1 expression was similar in the cerebellum of *CaMKIIα-Lgi1*cKO and *CaMKIIα-Lgi1*<sup>+/-</sup> mice up to 11 months of age (Fig. 2B and C). These data

demonstrate a successful recombination of the floxed *Lgi1* allele in the hippocampus of *Emx1-Lgi1*cKO and *CaMKIIα-Lgi1*cKO mice resulting in a specific deletion. Because five commercial LGI1 antibodies (ab30868, ab69491, ab67319, sc-9581 and sc-9583) gave non-specific staining by immunohistochemistry of brain tissue from germinal *Lgi1*<sup>-/-</sup>, we could not determine the neuronal populations of the Cre-mediated deletion (Supplementary Fig. 1).

Nevertheless, Emx1-Cre and CaMKII $\alpha$ -Cre mouse strains have been extensively used and proven to be cell-type specific (Tsien *et al.*, 1996; Madisen *et al.*, 2010; Liang *et al.*, 2012) (Allen Brain Institute, mouse brain connectivity section: <http://connectivity.brain-map.org>).

### Embryonic loss of LGI1 in forebrain glutamatergic neurons is sufficient to generate epileptic seizures

First, we examined gross brain morphology by Nissl staining of coronal and sagittal brain sections of Emx1-*Lgi1*cKO mice at postnatal Day 19. No major abnormalities of cortical and hippocampal organization and lamination were detected, suggesting that *Lgi1* deletion in embryonic forebrain cells does not affect neuronal migration (Supplementary Fig. 2A–D).

We assessed seizure activity by using two complementary methods: (i) a non-invasive video-based approach that automatically recognizes seizure behavioural manifestations (SeizureScan software) which was used to detect the occurrence of seizures and their frequency; and (ii) intracranial video-EEG monitoring to assess electrographic features of the seizures. First, we performed video monitoring coupled to SeizureScan analysis in Emx1-*Lgi1*cKO mice ( $n = 4$ ) from the age of postnatal Day 13 up to a maximum of postnatal Day 22, until animals death. We detected recurrent spontaneous behavioural seizures in 100% of Emx1-*Lgi1*cKO mice. Age at first seizure ranged from postnatal Days 16 to 21 (Table 1). Typically, seizures consisted of stereotyped behavioural sequences of repeated forelimb clonic jerks and a hypertonic neck and tail, generally followed by loss of postural equilibrium and clonic jerks of the head and all limbs (Supplementary Video 1). In some cases, seizures continued with myoclonic jerks and hyperkinetic running or hypertonic posture of the trunk, limbs and tail. During the postictal period, mice were immobile for several seconds. Secondly, video-EEG recordings were monitored to characterize the electrographic epileptic activity. Multiple depth electrodes were implanted in two Emx1-*Lgi1*cKO mice (at postnatal Days 21 and 26). EEG recordings were limited to 4–6h/day as a maximum as pups were not weaned, and were obtained during 2 days, until animal death. During spontaneous seizures, ictal electrographic activities were apparent in both hippocampal and cortical electrodes (Fig. 3A).

Typically, a high-amplitude spike and wave (5–6 mV/1–2 Hz) generally initiated a seizure in the hippocampus, followed by low voltage fast activities (70–100 Hz), as illustrated in the time-frequency representation (Fig. 3A). Spike discharges and bursts of polyspikes of increasing amplitude in hippocampus and cortex finally ended the seizures, which were followed by a flattening of the EEG activity for  $43.3 \pm 15$  s. The majority of seizures were recorded earlier in the hippocampus than in the cortex (Fig. 3). However, in one seizure, epileptic activities were detected in the cortex before the hippocampus (Supplementary Fig. 3).

Six Emx1-*Lgi1*cKO mice were investigated for the epileptic phenotype with 18 total seizures recorded. SeizureScan and EEG records provided concordant data that are summarized in Table 1. All mice had seizures, indicating a complete penetrance of the epileptic phenotype. They had frequent, recurrent and severe spontaneous seizures with an age at onset comprised between postnatal Days 16 and 21. The mean seizure frequency was  $0.76 \pm 0.23$ /h and the mean duration was  $31.7 \pm 2.9$  s. Emx1-*Lgi1*cKO mice died within 3 days after seizure onset, presumably due to cardiac or respiratory arrest during the postictal period accompanied by a flat EEG. We also found several mice ( $n = 4$ ) dead in their cages, in a hypertonic posture. Age-matched controls never showed spontaneous behavioural or electrographic seizures ( $n = 7$  by SeizureScan,  $n = 4$  by EEG) (Fig. 3B).

Lifespan study revealed that all Emx1-*Lgi1*cKO mice died prematurely, within the first month of age. The majority of mice (15/17) died before postnatal Day 23 but two mice survived up to the age of postnatal Days 28 and 32. The average lifetime was  $20 \pm 0.9$  days according to Kaplan-Meier survival curves (Fig. 3C). Control littermates always survived beyond this period.

### Postnatal loss of LGI1 in forebrain glutamatergic neurons leads to occasional epileptic seizures

We examined the consequences of LGI1 depletion induced postnatally in forebrain pyramidal neurons by using the CaMKII $\alpha$ -Cre transgenic mouse line. CaMKII $\alpha$ -*Lgi1*cKO mice were viable with no overt physiological or behavioural phenotypes. No gross morphological abnormalities were evident in Nissl stained whole-brain sections from 9-month-old CaMKII $\alpha$ -*Lgi1*cKO mice (Supplementary Fig. 2E–H).

We asked whether spontaneous seizures emerged in CaMKII $\alpha$ -*Lgi1*cKO mice using non-invasive video records of behaviour coupled to SeizureScan analysis. Video monitoring was performed

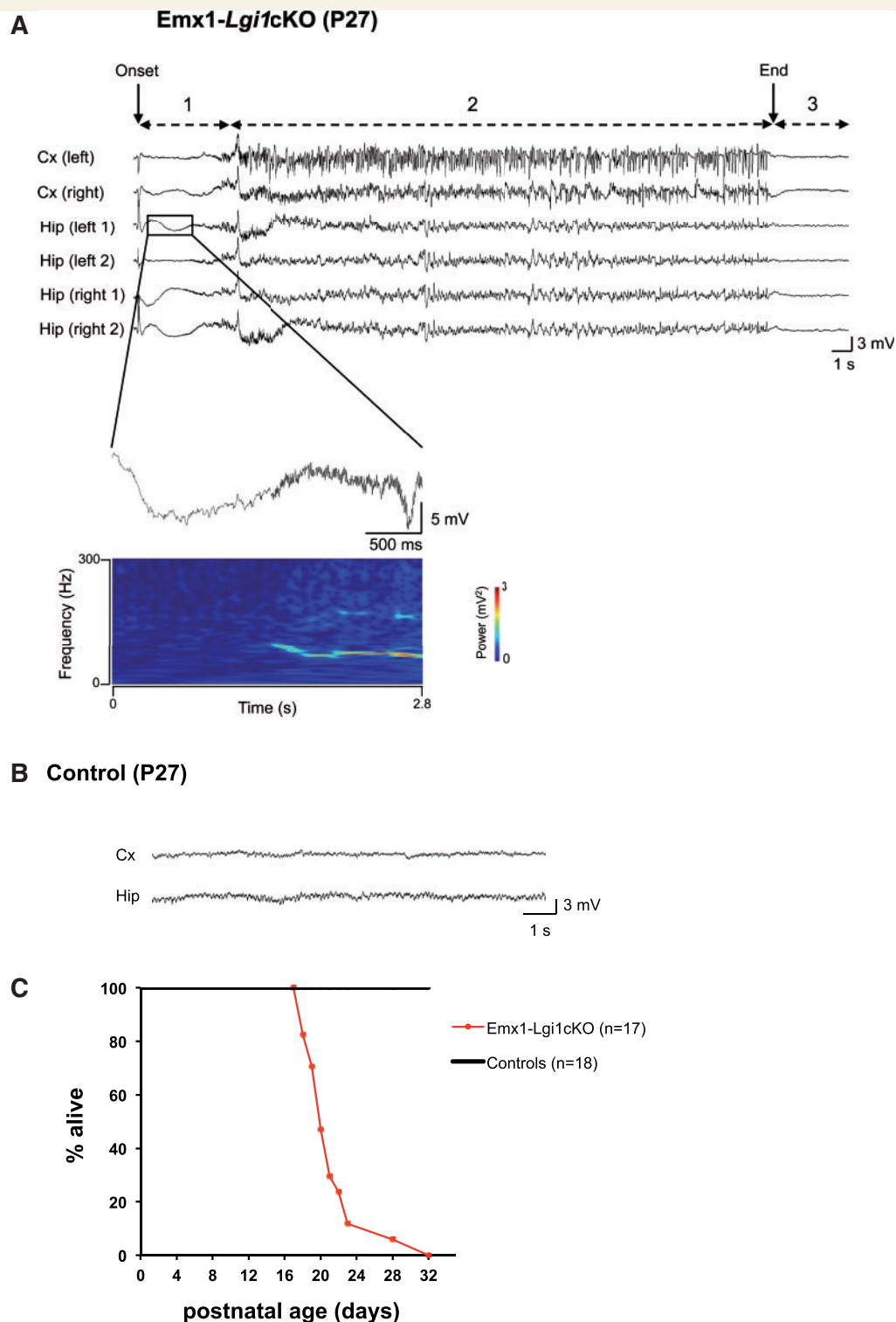
**Table 1 Summary of SeizureScan and video-EEG findings and characteristics of spontaneous seizures**

Genotype	Number of mice with seizures (total $n$ )		% of mice with seizures*	Number of seizures recorded*	Age range of recordings (days)*	Age range at seizure onset (days)	Frequency range of seizures (/h)*	Mean seizure duration (s)**
	EEG	SeizureScan						
Emx1- <i>Lgi1</i> cKO	2 (2)	4 (4)	100	18	13–27	16–21***	0.34–1.79	$31.7 \pm 2.9$
CaMKII $\alpha$ - <i>Lgi1</i> cKO	4 (7)	3 (5)	58	41	46–330	94–221*	0.07–1.4	$67.7 \pm 8.3$
PV- <i>Lgi1</i> cKO	0 (2)	0 (6)	0	0	30–160	NA	NA	NA
Controls	0 (12)	0 (11)	0	0	13–248	NA	NA	NA

\*Data combined from SeizureScan and video-EEG.

\*\*Data from video-EEG.

\*\*\*Data from SeizureScan.



**Figure 3** Spontaneous epileptic seizures and premature death in *Emx1-Lgi1cKO* mice. (A) *Top*: Cortical and hippocampal EEG records show a spontaneous seizure from an *Emx1-Lgi1cKO* mouse aged postnatal Day 27. Monopolar electrodes were implanted in both cortices (EEG traces: Cx left and right) and bipolar electrodes were implanted in both hippocampi (EEG traces: Hip left 1/2, Hip right 1/2). The seizure started with a high-amplitude spike and wave (arrow indicates onset of seizure) and terminated abruptly with pronounced postictal flattening (arrow indicates end of seizure). Behavioural modifications were correlated with EEG changes: 1 = behavioural arrest; 2 = repeated clonic jerks of all limbs and hypertonic neck; 3 = postictal immobility. *Enlarged panel*: Expanded hippocampal EEG trace showing low voltage fast activities, corresponding to the frequencies with the highest powers in the spectrogram. The time frequency representation was obtained with a wavelet transform of the EEG signal. (B) Epileptic activity was not generated by age-matched controls ( $n = 4$ ). Representative EEG traces from one cortical and one hippocampal electrode in age-matched control mouse. (C) Kaplan-Meier survival curves of *Emx1-Lgi1cKO* mice ( $n = 17$ ) and littermate controls ( $n = 18$ ) from birth until postnatal Day 32 showing early premature death. Half of *Emx1-Lgi1cKO* mice died before postnatal Day 20.

twice a week (up to 24 continuous hours) from adult CaMKII $\alpha$ -*Lgi1*cKO ( $n = 5$ ) over a period of 5.5 months (2.5- to 8-month-old mice). We detected spontaneous behavioural seizures in three of five CaMKII $\alpha$ -*Lgi1*cKO mice with an age at onset of 3.5 to 5 months (postnatal Days 110–150). Seizures occurred both during sleep (12 seizures) or waking (10 seizures) periods. Typically, behavioural manifestations of seizures started with a hypertonia of the neck and tail, followed by repeated clonic jerks, restricted to forelimbs, sometimes unilaterally, and accompanied by loss of posture (Supplementary Video 2). A brief immobility was observed during the postictal period. In rare seizures, the behavioural manifestations were subtle, consisting only of hypertonia of the neck and brief forelimb and head clonic jerks. Epileptic activity was confirmed from intracranial video-EEG recordings. Intracranial electrodes were stereotactically implanted in adult CaMKII $\alpha$ -*Lgi1*cKO mice ( $n = 7$ ). Simultaneous video-EEG records of 4 h/week duration were acquired during a period of up to 9 months (mice aged 1.5- to 11-month-old). Occasional behavioural and electrographic seizures (19 seizures total) were recorded from the hippocampus and cortex of both hemispheres of four of seven CaMKII $\alpha$ -*Lgi1*cKO mice with an age at onset ranging from 3 to 7 months (postnatal Days 94–221) (Fig. 4A). Typically, seizure onset in the hippocampus was marked by high-amplitude hippocampal spikes or polyspikes (4–7 mV, 8–15 Hz), resembling interictal activity and followed by low voltage fast activities (50–70 Hz). Then, a rhythmic activity of progressively increasing amplitude appeared, which secondarily spread to involve the cortex (Fig. 4A). Electrographic seizures terminated with a decrease in spike frequency and amplitude, and then a postictal EEG flattening period of  $7.5 \pm 3.2$  s. EEG epileptic activity was never detected in three littermate controls (Fig. 4B). In addition to seizures, hippocampal interictal spikes were common in records from epileptic CaMKII $\alpha$ -*Lgi1*cKO mice, but never detected in control animals. The number of spikes ranged from  $\sim 100$  to  $\sim 1200$ /h during ictal periods whereas it was  $\sim 50$ /h in records without seizures. We also detected interictal-like spikes in video-EEG records from one CaMKII $\alpha$ -*Lgi1*cKO mouse where no seizure was recorded ( $\sim 200$  spikes/h at age 7 months) (Fig. 4C).

Twelve CaMKII $\alpha$ -*Lgi1*cKO mice were investigated by both SeizureScan and video-EEG over 2000 hours, and seizures (total of 41) detected in half of them (Table 1). While electrographic manifestations of seizures and their duration (mean  $67.7 \pm 8.3$  s) were similar between animals, their frequency (mean =  $0.36 \pm 0.1$ /h, range: 0.07–1.4/h), age at onset (3 to 7 months) and timing of occurrence (from 3 to 7–8 months), assessed by both EEG and SeizureScan varied greatly, possibly due to a variable timing of *Lgi1* deletion mediated by CaMKII $\alpha$ -Cre mouse. Overall, seizures of both Emx1-*Lgi1*cKO and CaMKII $\alpha$ -*Lgi1*cKO mice began with a similar behavioural sequence but were clearly milder in CaMKII $\alpha$ -*Lgi1*cKO mice in which hyperkinetic running or hypertonic postures were never observed and postictal flat EEG period was shorter.

Beside the epileptic phenotype, CaMKII $\alpha$ -*Lgi1*cKO mice had a shorter lifespan than littermate controls: during the time of the experiment (up to 7 months), 60% of CaMKII $\alpha$ -*Lgi1*cKO mice died at various ages (mean lifetime of  $193 \pm 13.8$  days) (Fig. 4D). Death may have been caused by respiratory or cardiac

arrest occurring after a seizure during the postictal period of EEG flattening. However, in contrast to Emx1-*Lgi1*cKO mice, some CaMKII $\alpha$ -*Lgi1*cKO mice survived up to 90 days after seizures onset. All littermate controls survived throughout the period.

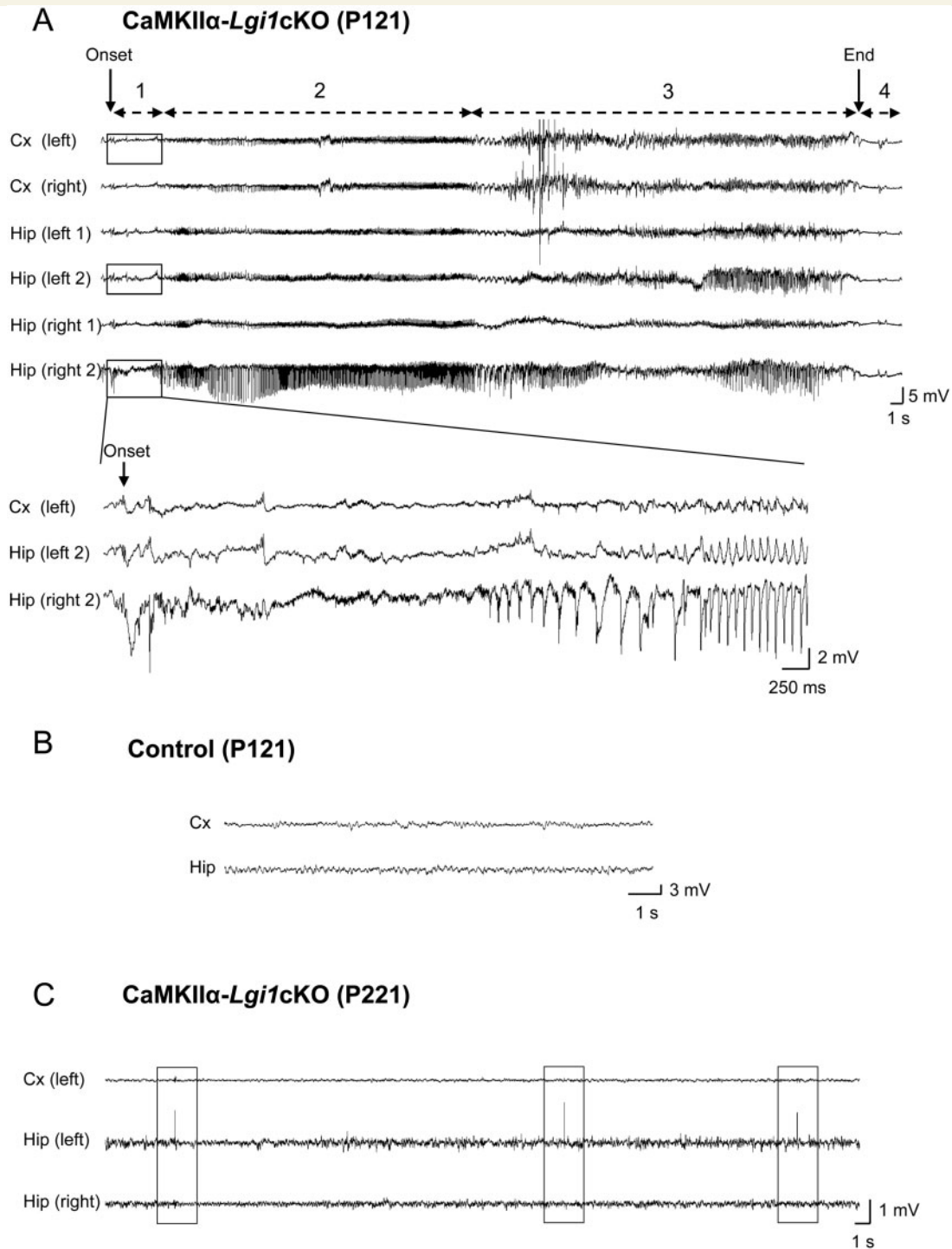
## Parvalbumin interneurons have no major role in the generation of seizures

The expression of LGI1 protein in inhibitory interneurons has recently been confirmed using human serum containing LGI1 autoantibodies, which overcome the poor specificity of commercial antibodies (Ohkawa *et al.*, 2013). Moreover, LGI1 has been suggested to play an important role in the development of cortical interneurons (Friocourt and Parnavelas, 2011). To determine a possible involvement of inhibitory neurons in the epileptic phenotype of *Lgi1*-deficient mice, we made a genetic deletion of *Lgi1* from parvalbumin GABAergic interneurons using a PV-Cre mouse line (Hippenmeyer *et al.*, 2005), which has been shown to be specific to parvalbumin interneurons using a reporter mouse strain (Yi *et al.*, 2014). Animals were generated from two sequential crosses to produce PV-*Lgi1*cKO mice (*Lgi1*<sup>fl/fl</sup>; PV-Cre<sup>+/-</sup>) and littermate controls (Fig. 1). There was no difference in body weight or gross brain morphology (Supplementary Fig. 2I–L) between PV-*Lgi1*cKO mice and their littermate controls. Moreover, over 8 months of experiments, lifespan was preserved in PV-*Lgi1*cKO mice.

We next sought for spontaneous seizures in PV-*Lgi1*cKO mice by SeizureScan and video-EEG monitoring (Table 1). SeizureScan recordings were acquired for 16 h/week (light and dark monitoring) from PV-*Lgi1*cKO mice aged 1 to 5 months ( $n = 6$ ). Video-EEG recordings were collected over 4 h/week in two PV-*Lgi1*cKO mice aged 2 to 4.5 months. Neither observation, nor SeizureScan and EEG data revealed spontaneous seizures or interictal spikes in PV-*Lgi1*cKO mice over a total period of  $> 1000$  h of recordings.

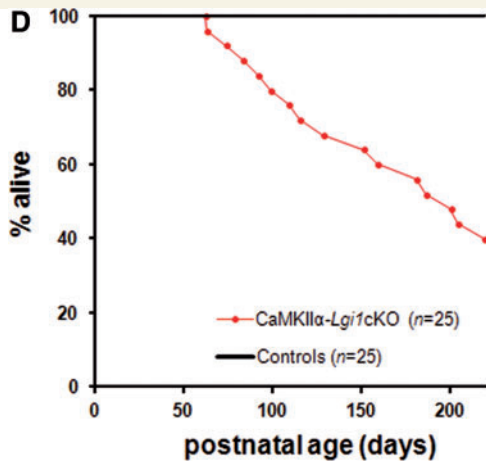
Next, we searched for a more subtle hyperexcitable phenotype by investigating the susceptibility of PV-*Lgi1*cKO mice to GABA<sub>A</sub> receptor antagonist pentylenetetrazole-induced seizures. We compared the severity of seizures induced by a 53 mg/kg intraperitoneal injection of pentylenetetrazole in 2-month-old PV-*Lgi1*cKO, PV-*Lgi1*<sup>+/-</sup> and PV-*Lgi1*<sup>+/+</sup> mice. As expected with this pentylenetetrazole dose, all mice exhibited at least myoclonic body jerks and forelimb clonic jerks, corresponding to score 1 according to Harai *et al.* (2012). There were no significant differences in the global distribution of seizure severity scores between PV-*Lgi1*cKO and PV-*Lgi1*<sup>+/-</sup> mice (PV-*Lgi1*<sup>+/-</sup>: score 1: 17.6%, score 2: 70.6%, score 3: 0%, score 4: 11.8%; PV-*Lgi1*cKO: score 1: 9.1%, score 2: 81.8%, score 3: 0%, score 4: 9.1%;  $\chi^2$  test  $P$ -value = 0.78; Fig. 5). We did not observe significant differences in the latency between pentylenetetrazole injection and the occurrence of the first generalized seizure between PV-*Lgi1*cKO and PV-*Lgi1*<sup>+/-</sup> mice (PV-*Lgi1*<sup>+/-</sup>:  $246.3 \pm 34.3$  s; PV-*Lgi1*cKO:  $267.6 \pm 70.4$  s;  $t$ -test  $P$ -value = 0.77). However, in contrast to previous work (Fukata *et al.*, 2010), no significant difference in the severity of response to pentylenetetrazole-induced seizures was detected between *Lgi1*<sup>+/+</sup> and *Lgi1*<sup>+/-</sup> mice. Possibly the





**Figure 4** Late-onset epileptic phenotype and reduced lifespan in CaMKII $\alpha$ -Lgi1cKO mice. (A) *Top*: Cortical and hippocampal EEG records of a spontaneous seizure of a 4-month-old CaMKII $\alpha$ -Lgi1cKO mouse. Monopolar electrodes were implanted in both cortices (EEG traces: Cx left and right) and bipolar electrodes were implanted in both hippocampi (EEG traces: Hip left 1/2, Hip right 1/2). The seizure started with hippocampal polyspikes (Hip right 2, arrow indicates onset) and terminated with brief postictal flattening. Behavioural modifications were correlated with EEG changes: 1 = behavioural arrest; 2 = agitation, gyration; 3 = repeated clonic jerks of head and forelimbs with a hypertonic neck and tail; 4 = postictal immobility. *Enlarged panel*: Expanded EEG traces of the seizure onset show low voltage fast activities in the right hippocampus, followed by spike discharges of increasing amplitude, which begin earlier in the hippocampus than in the cortex. (B) Epileptic activities were never seen in age-matched controls ( $n = 3$ ), as illustrated by the representative EEG traces from a CaMKII $\alpha$ -Lgi1 $^{+/-}$  littermate control mouse. (C) Hippocampal 'interictal-like' spikes recorded in a CaMKII $\alpha$ -Lgi1cKO mouse. The mean frequency of spikes recorded at this age (postnatal Day 221) was  $\sim 200/h$ . Spikes are boxed.

(continued)



**Figure 4** Continued. (D) Kaplan-Meier survival curves of CaMKII $\alpha$ -Lgi1cKO mice ( $n = 25$ ) and littermate control mice ( $n = 25$ ) from postnatal Day 0 until Day 220. 60% of CaMKII $\alpha$ -Lgi1cKO mice died in the first seven postnatal months.

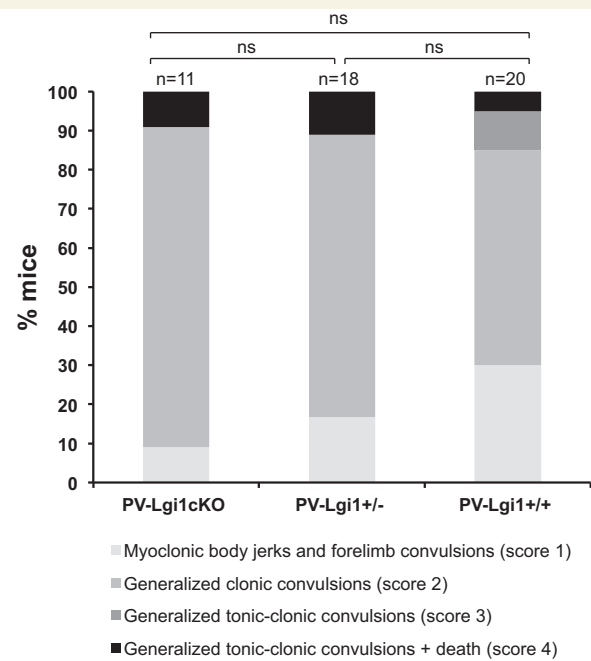
result depended on different experimental conditions (pentylene-tetrazole dose, age of mice, or genetic background).

## Discussion

LGI1 is a key protein for CNS function as its loss, either due to genetic haploinsufficiency or acquired antibody-mediated depletion, causes epilepsy. However, mechanisms of abnormal brain excitability are not clear. Here we used conditional knockout mice with restricted spatiotemporal and cell-type deletion of *Lgi1*. These animals let us define contributions of glutamatergic pyramidal neurons and GABAergic interneurons to seizure generation, and ask whether the role of LGI1 in seizure generation is purely neurodevelopmental or whether depletion in adult can also trigger seizures.

### Glutamatergic neuron-targeted loss of *Lgi1* causes a severe epileptic phenotype

Several studies suggested that LGI1 may regulate glutamatergic transmission (Fukata *et al.*, 2006, 2010; Zhou *et al.*, 2009, 2012; Yu *et al.*, 2010). We attempted to validate this hypothesis *in vivo*, using two conditional knockout mouse lines (Emx1-Lgi1cKO and CaMKII $\alpha$ -Lgi1cKO) to achieve a selective deletion of *Lgi1* in glutamatergic pyramidal neurons of the neocortex and hippocampus. Our results revealed that both conditional knockout mice presented spontaneous seizures. The epileptic phenotype of Emx1-Lgi1cKO mice, consisting of early-onset, frequent seizures associated with premature death, was reminiscent of that of germinal *Lgi1*<sup>-/-</sup> mice (Chabrol *et al.*, 2010). Behavioural manifestations and electrographic pattern of seizures of germinal *Lgi1*<sup>-/-</sup> and Emx1-Lgi1cKO mice were very similar, suggesting an identical sequential involvement of different brain areas. We noticed that ictal epileptic activities in the hippocampus of Emx1-Lgi1cKO mice preceded discharges from the motor cortex in most seizures,



**Figure 5** Loss of LGI1 in parvalbumin interneurons does not trigger seizures. Pentylene-tetrazole-injection induced seizures of similar severity in PV-Lgi1cKO mice compared to PV-Lgi1<sup>+/-</sup> mice. Quantification of maximal reaction to pentylene-tetrazole injection (53 mg/kg) in 2-month-old PV-Lgi1cKO ( $n = 11$ ), PV-Lgi1<sup>+/-</sup> ( $n = 18$ ) and PV-Lgi1<sup>+/+</sup> ( $n = 20$ ) mice: score 1, myoclonic body jerks and forelimb convulsions; score 2, generalized clonic convulsions; score 3, generalized tonic-clonic convulsions; score 4, generalized tonic-clonic convulsions followed by death. ns = non-significant.

suggesting seizures in mice originate in mesial temporal structures. Although some patients with ADEAF describe psychic ('déjà-vu') and autonomous (epigastric sensations) symptoms, characteristic of mesial temporal lobe auras (Morante-Redolat *et al.*, 2002; Winawer *et al.*, 2002; Ottman *et al.*, 2004), the human syndrome rather implicates lateral temporal lobe. We envisage two explanations for the discrepancy: (i) seizures might originate in lateral temporal structures, but have passed undetected with no electrodes in these areas; (ii) different structures might be involved in mice and in humans. In contrast, restricting deletion of *Lgi1* to GABAergic parvalbumin interneurons did not trigger spontaneous seizures or confer increased susceptibility to pentylene-tetrazole-induced seizures. Parvalbumin-positive cells account for about half of cortical interneurons (Wonders and Anderson, 2006). They innervate perisomatic regions on pyramidal cells of hippocampus and neocortex and are therefore suited to control the output of these principal cells (Freund and Katona, 2007). Recent evidence points to a direct role for parvalbumin interneurons in the pathogenesis of some genetic epilepsies, in particular Dravet syndrome, a severe epileptic encephalopathy caused by *de novo* mutations in the *SCN1A* gene encoding the voltage-gated sodium alpha 1 subunit channel (Ogiwara *et al.*, 2007; Dutton *et al.*, 2012; Rossignol *et al.*, 2013). Although these results do not exclude a contribution of GABAergic interneurons to the genesis of seizures in *Lgi1*-deficient mice, they do suggest it may be relatively weak. Instead,

similarities between the epileptic phenotype of *Emx1-Lgi1*cKO mice and that of the germinal *Lgi1*<sup>-/-</sup>, and the lack of seizures in PV-*Lgi1*cKO mice suggest that glutamatergic neurons are the main contributors to the pathogenesis of LGI1-related epilepsy. This study also demonstrates that deletion of *Lgi1* in the forebrain alone suffices to generate spontaneous seizures in mice.

### LGI1 displays an essential role in brain during the whole life

Postnatal deletion of *Lgi1* in *CaMKII $\alpha$ -Lgi1*cKO mice caused a milder phenotype, with infrequent and late-onset electroclinical spontaneous seizures associated with a variable reduction in lifespan. Long-term EEG recordings will help better characterize the dynamics of the seizure emergence in these mice. Nevertheless, our data imply that LGI1 depletion induces seizures after main synaptic and neuronal developmental processes have matured properly. Our findings are reminiscent of the human acquired autoimmune encephalitis with adult onset, supporting a direct link between LGI1 loss of function due to LGI1 autoantibodies and limbic encephalitis (Ohkawa *et al.*, 2013). We therefore conclude that LGI1 is critical from perinatal through late postnatal development. However, our results clearly indicate that the phenotype of *Emx1-Lgi1*cKO mice is more severe than that of *CaMKII $\alpha$ -Lgi1*cKO mice in terms of behavioural manifestations, seizure frequency (0.76 versus 0.36 seizures/h), duration of postictal period and EEG flattening (43.3 versus 7.5 s), and latency between seizure onset and death (3 versus 90 days). This may result from an enhanced vulnerability of immature brain to seizures (Ben-Ari and Holmes, 2006). Yet, the more severe phenotype associated with the earlier LGI1 deletion is also consistent with the postnatal neurodevelopmental role of LGI1 in transgenic mice expressing a truncating mutant form of LGI1 that was shown to act as a dominant-negative (Zhou *et al.*, 2009, 2012). However, in this conditional loss-of-function mouse model, seizures also arose during adult life, indicating LGI1 function is not limited to early postnatal development processes. LGI1, like reelin, a secreted protein of the extracellular matrix, could serve different functions during brain development and adulthood. During embryonic development, reelin contributes to a correct lamination of cortical and hippocampal regions, while at postnatal and adult stages, it modulates synaptic plasticity and dendritic growth (Campo *et al.*, 2009). The importance of LGI1 in adulthood is also consistent with a role of LGI1 helping circuits respond to seizures by recruiting K<sub>v</sub>4.2 potassium voltage-gated ion channel to the membrane as a seizure dampening mechanism (Smith *et al.*, 2012).

### Novel insight into the function of LGI1

Various intracellular and extracellular functions have been attributed to LGI1. It remains unclear whether secreted and/or cytoplasmic LGI1 protein is involved in the circuit hyperexcitability that triggers seizures. The identity of neurons that synthesize and secrete LGI1 and the binding sites for secreted LGI1 also remain to be fully characterized. Single-CA1 neuron genetic invalidation by stereotaxic injection of siRNA against *Lgi1* could help clarify potential cell-autonomous effects. Our results show that a lack of LGI1 synthesis by glutamatergic neurons can trigger seizures even

while secretion from interneurons and glial cells was presumably maintained. This lack of compensation from different cell types might support a cytoplasmic function for LGI1, or possibly suggests a local, rather paracrine action. These findings may limit the search for the effective pro-epileptic site of mutant *LGI1* to pre- or extra-synaptic regions at glutamatergic synapses.

Our data emphasize that cortical excitatory neurons, rather than inhibitory interneurons, contribute to the pathogenesis of LGI1-related epilepsy. We also show that LGI1 is an essential protein to maintain normal brain function throughout life. Loss of LGI1 during late postnatal period leads to seizures pointing to a role in adult regulation of neuronal excitability as well as a function in early developmental processes.

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## Supplementary material

Supplementary material is available at *Brain* online.

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