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LGI1-associated epilepsy through altered ADAM23-dependent neuronal morphology

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Abstract

Most epilepsy genes encode ion channels, but the LGI1 gene responsible for Autosomal Dominant Partial Epilepsy with Auditory Features produces a secreted protein. LGI1 is suggested to regulate PSD-95 via ADAM22. However, no unbiased screen of LGI1 action has been conducted. Here, we searched for brain genes supporting high affinity LGI-1 binding. ADAM23 was the only LGI1 interactor identified. The related proteins, ADAM22 and ADAM11, but not ADAM12, bind LGI1. Neither ADAM23 nor ADAM11, nor some forms of ADAM22, contain PDZ-interacting sequences, suggesting PSD-95-independent mechanisms in ADPEAF. Because ADAMs modulate integrins, we examined LGI1 effect on neurite outgrowth. LGI1 increases outgrowth from wild type but not ADAM23-/- neurons. Furthermore, CA1 pyramidal neurons of ADAM23-/- hippocampi have reduced dendritic arborization. ADAM23-/- mice exhibit spontaneous seizures, while ADAM23+/- mice have decreased seizure thresholds. Thus, LGI1 binding to ADAM23 is necessary to correctly pattern neuronal morphology and altered anatomical patterning contributes to ADPEAF.

Introduction

The vast majority of Mendelian-inherited epilepsy genes encode ion channels (for review see (Gutierrez-Delicado and Serratosa, 2004)), leading to a central hypothesis that genetically altered channel function lowers seizure threshold. In contrast, two human epilepsy genes do not encode ion channels. The MASS1 (also termed Very Large G-protein-coupled Receptor, VLGR1 (Nakayama et al., 2002) gene shares the epitempin repeat (EPTP) or epilepsy-associated repeat (EAR) (Scheel et al., 2002) with the second non-channel gene associated with epilepsy, LGI1. Mutations of LGI1 are found in many families with Autosomal Dominant Partial Epilepsy with Auditory Features (ADPEAF) (Kalachikov et al., 2002; Morante-Redolat et al., 2002; Fertig et al., 2003; Michelucci et al., 2003; Bisulli et al., 2004). A range of amino acid substitutions, small insertions, deletions and nonsense mutations have been identified in different families with ADPEAF (Kalachikov et al., 2002; Morante-Redolat et al., 2002; Fertig et al., 2003; Michelucci et al., 2003; Bisulli et al., 2004; Ottman et al., 2004; Rosanoff and Ottman, 2008; Nobile et al., 2009). The common clinical symptom is of a seizure aura with auditory features or aphasia. Distinct ADPEAF phenotypes have not been observed with different LGI1 mutations (Nobile et al., 2009). The LGI1 protein contains two EPTP repeats

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in the carboxyl half of the protein, and three Leucine Rich Repeats (LRR) near its amino terminus.

It has been shown that LGI1 is widely expressed in neurons of the adult mouse brain and levels in peripheral tissues are low (Gu et al., 2002). However, the cell biology of LGI1 function and the pathophysiology of ADPEAF have been less clear. Several groups initially predicted that the LGI1 protein is a transmembrane polypeptide because its primary structure contains a signal sequence and a second region with moderately hydrophobic character that might constitute a transmembrane segment (Kalachikov et al., 2002; Morante-Redolat et al., 2002). It is now clear that LGI1 is secreted from transfected cells and that disease-associated mutations impair secretion (Senechal et al., 2005; Fukata et al., 2006; Sirerol-Piquer et al., 2006).

One hypothesis for LGI1 function derives from proteomic analysis of immunopreciptitates of brain voltage-gated potassium channels Kv1.1. LGI1 is enriched in Kv1.1 complexes (Schulte et al., 2006). Moreover, LGI1 protein expression was reported to reduce Kv1.1 inactivation cell autonomously via the intracellular β subunit of the channel. ADPEAF was postulated to occur through a cytoplasmic alteration in Kv1.1 function and excitability (Schulte et al., 2006) although this hypothesis does not fit with the later recognition that LGI1 is secreted.

A second hypothesis for LGI1 is based on the presence of LGI in a different immunoprecipitate, one for PSD-95 (Fukata et al., 2006). Secreted LGI1 interaction with intracellular PSD-95 is not direct, but is mediated through a transmembrane protein present in the PSD-95 immunoprecipitate, ADAM22 (a disintegrin and metalloprotease-22). One splice form of ADAM22 contains a carboxyl terminal motif that binds to a PDZ domain of PSD-95. Secreted LGI1 was shown to bind the extracellular domain of ADAM22, creating a link from LGI1 to PSD-95 and GluR channels. Both of these hypotheses link LGI1 to ion channel dysfunction in causing epilepsy.

Here, we investigated LGI1 function through an unbiased genome-wide search for cell surface binding sites. Our identification of ADAM23 validates the notion that the ADAM23/22/11 family is the primary site of LGI1 action. Although one splice form of ADAM22 binds to PSD-95 (Fukata et al., 2006; Godde et al., 2007), ADAM23 does not contain PDZ binding motifs. Therefore, we explored other mechanisms of action for LGI1/ADAM23 function. We find that LGI1 enhances neurite outgrowth in an ADAM23-dependent mechanism in vitro and in vivo. Moreover, mice lacking ADAM23 exhibit seizure activity and mice heterozygous for ADAM23 mutation have lowered seizure thresholds. We postulate that LGI1 mutation results in reduced ADAM23 function and an altered brain circuitry that underlies the propensity to epilepsy in ADPEAF individuals.

Methods

LGI1 expression vectors and fusion proteins

The LGI1-AP fusion protein was produced from a cDNA vector created by ligating the cDNA sequence encoding the full length wild type or mutant LGI1 without the stop codon into the pAPtag-5 vector (GenHunter, Nashville, TN). The resulting protein contains LGI1, AP, myc and His tags in order from amino to carboxyl terminus and migrates as a 140kDa species by immunoblot (IB). The T953G mutation of LGI1 was introduced into the full-length human LGI1 cDNA sequence by site-directed mutagenesis, resulting in an F318C substitution in the first EPTP domain. This mutation was previously reported in a large Italian-American ADPEAF family (Fertig et al., 2003).

Using Fugene-6 (Roche) LGI1 expression plasmids were transfected into the human embryonic kidney 293T (HEK293T) cells and conditioned media was collected after 14 days. The

conditioned medium was adjusted to 450 mM NaCl and 5 mM imidazole, pH 7.0 and applied to Ni-containing resin (Invitrogen). After washing with 50 volumes of 150 mM NaCl and 5 mM imidazole, bound LGI1-AP-Myc-His was eluted with 150 mM NaCl plus 50 mM imidazole. The AP activity of the eluate was determined using PNPP as substrate (Sigma) and protein size determined by immunoblot.

We created a stably transfected HEK293A cell line, expressing the same LGI1-AP fusion protein. This line was created by transfection of HEK293A cells as described above for HEK293T cells and subsequent selection of expressing cells by application of G418 (Gibco) at 10 μg/ml of media. To isolate clonal populations, cells were transferred to 96-well plate at 1 cell/well while maintaining selection medium. Several expanded clones yielded concentrations of 30-80 nM recombinant protein in conditioned medium. Clone 4H7 was utilized for subsequent LGI1-AP production.

LGI1-AP binding

COS-7 cells were transfected using Fugene-6 (Roche) in serum free OPTI-MEM (Gibco). After 8 h, the medium was changed to DMEM with 10% FBS (Fetal bovine serum, Gibco) and penicillin/streptomycin (Gibco). After 24 hrs cells were transferred to 24 or 48 well plates and 24 h later washed twice with HBH (Hanks Balanced Salt Solution (HBSS) with 0.5 mg/ml Bovine Serum Albumin and 1 mM NaHepes, pH 7.4). AP ligand was applied for 2 h at room temperature. Then cells were washed 3 times with ice cold HBH and fixed with 4% formaldehyde with 20% sucrose for 30 min. Endogenous AP activity was inactivated at 65°C for 6-8 h bound LGI1 was detected by incubation with NBT/BCIP (Sigma, (Fournier et al., 2001)). Human cDNA expression vectors for ADAM23, ADAM22, ADAM11, and ADAM12 (Origene) were used in AP binding experiments.

Expression cloning

COS-7 cells were transfected with pools of 5,000 non-redundant arrayed mouse adult brain cDNA (OriGene) as described (Fournier et al., 2001; Rajagopalan et al., 2004; Lauren et al., 2009). After 36-48 h, cells were incubated with 1-50 nM LGI1-AP for 2 h, washed, fixed, heat inactivated and bound LGI-AP was detected as described above. Individual clones supporting LGI1 binding were isolated from library pools by sub-selection and DNA sequence was determined at the William M. Keck Biotechnology Center of Yale University.

Neuronal cultures

Dorsal root ganglia were collected from E7 chick into dissection medium (HBSS, 10 mM Hepes, 33 mM Glucose, 12 mM $MgSO_4$, penicillin/streptomycin), digested in dissection medium with 2 mg/ml of Trypsin (Sigma) at 37°C for 15 minutes and triturated in plating medium (Neurobasal with B27 supplementation (Gibco) plus 2 mM L-Glutamine and penicillin/streptomycin (Gibco)). Cells from one ganglia were plated in one well of a 96-well laminin-coated (Invitrogen) plate.

Hippocampal and cortical cultures were prepared by dissecting hippocampi or cortices from individual P1 ADAM23 mouse pups or from entire litters of E18 rat pups in dissection medium (HybernateE without Calcium (BrainBits)) on ice. Tissue was dissociated in dissection medium with 1 mg/ml papain (Sigma) and 1 mg/ml DNase (Sigma) at 37°C for 15 ml. Then dissociation medium was aspirated and plating medium (Neurobasal-A (Gibco) with B-27, Na Pyruvate, Glutamax, Gentamycin 1:1000 (Gibco)) was added, cultures were dissociated by pipette trituration and plated at 15,000 cells/well in 96-well PDL-coated plates (BIOCOAT) pretreated with 10 μg/ml laminin for 1 h.

Neurite outgrowth assay

Dissociated neurons were incubated for 24 hrs with buffer control, AP control or LGI1-AP and then fixed, and stained with β-III tubulin (Promega) and Hoescht (Sigma, B-2261). Neuronal outgrowth was imaged and measured in an automated fashion using ImagExpress imaging system (Molecular Devices).

ADAM23 mice

ADAM23 gene targeted mice were originally created as part of a large scale secretory trap project (Leighton et al., 2001). Heterozygous ADAM23 mice were generously donated by Dr. Thomas Jessell of Columbia University. ADAM23 -/- mice were generated from ADAM23 +/- crosses. Mouse genotype was determined by PCR targeting the secretory trap. The location of the secretory trap was determined by PCR with series of forward and reverse primers for every 1500 base pairs of the ADAM23 mRNA. Once the location was established, forward and reverse primers corresponding to the 3′ end of the secretory trap and one reverse primer for the native ADAM23 mRNA sequence immediately after the trap were used to determine the genotype.

Brain histochemistry

Adult mice were anesthetized and perfused transcardially with 100 ml of 4% paraformaldehyde (PFA). The brain was removed and post-fixed in PFA for 24 h before cryoprotection with 20% sucrose and freezing. From P9-14 mice, the brain was fixed directly in 4% PFA at 4°C for 48 h, cryoprotected and frozen. Sections of 25 μm thickness were obtained with a cryostat and collected by air drying on Superfrost Plus glass slides. The expression of β-galactosidase was detected using X-gal as substrates and the expression of placental AP was detected using NBT/BCIP as described (Fournier et al., 2001).

Golgi stain

Brains of P10 ADAM23+/+, +/- and -/- mice were collected, weighed and fixed in 4% PFA for 48 h at 4°C, then stained by the Golgi method (Glaser and Van der Loos, 1981; Gibb and Kolb, 1998) for 21 days. Golgi stain was prepared as follows: Three solutions were made: (A) 5% potassium dichromate (Sigma P2588) in dH_2O, (B) 5% mercuric chloride (Sigma M1136) in dH_2O, (C) 5% potassium chromate (Sigma P0454) in dH_2O. Five volumes of A were mixed with 5 volumes of B. Four volumes of C were diluted in 10 volumes of dH_2O. The AB mixture was combined slowly with the C dilution. The stain was kept in a dark glass bottle for 5 days to allow precipitate to settle. The liquid part of the solution was used for staining the brain. After staining, brains were embedded in 10% (w/v) gelatin and post-fixed in PFA for 48 h at 4°C. Coronal sections of 40 μm were cut on a vibrating microtome (Leica, VT1000S) and washed in PBS. Sections were developed with KODAK developer for 30 minutes in the dark, washed with PBS and coverslipped on Superfrost glass slides (Fisher Scientific) with aqueous mounting medium (Biomeda). Z-stack pictures of individual neurons in CA1 region on the hippocampus were taken with ImagerZ1 on AX10 Zeiss microscope at 63× magnification. Images were analyzed with ImageJ software. First, camera lucida drawings of individual neurons were created from collapsed Z-stacks. Then concentric circles were drawn around the cell body at 5 μm intervals and number of basal dendrite intersections with each circle was counted. Then, the area covered by dendritic arborization was measured by connecting the points on the outermost dendrites and their origins on the cell body. The investigator was unaware of the mouse genotype during the entire process of image acquisition and quantification.

Behavioral analysis

ADAM23-/-, ADAM23+/- or ADAM23+/+ mice were placed in an isolated cage and videotaped for 60 min with or without stimulation every 10 min. Stimulation was provided by mild tactile pressure to the tail. Seizures were defined behaviorally by the abrupt onset of either tonic or clonic head motion with evidence of unresponsiveness.

Electroencephalography

A differential EEG signal was recorded from two stainless steel intracranial electrodes manufactured by Plastics One (Roanoke, VA) positioned at coordinates anteroposterior (AP), +0.0; mediolateral (ML), -0.5; dorsoventral (DV), −0.3 mm and at AP, −4.0; ML, -0.5; DV, -0.3 mm. A steel alligator clip attached to the neck muscles served as ground. The signal was amplified using a Model 12 acquisition system from Grass Instruments (W. Warwick, RI) with a gain of 200 and bandpass filtered at 1.0 to 100 Hz. Data were recorded for 30 min using Spike2 software (Cambridge Electronic Design; Cambridge, England), with an occasional mild tactile stimulation to the tail using forceps for arousal.

Seizure threshold and kindling

Seizure threshold was measured in adult (6 month old) ADAM23+/- and ADAM23+/+ mice. Mice were injected subcutaneously (sc) with 15-60 mg/kg Pentylenetetrazole (PTZ) (Sigma) and observed. Reaction to the injection was measured according to the following scale: 0, no response; 1, ear and facial twitching; 2, myoclonic body jerks; 3, clonic forelimb convulsions; 4, generalized clonic convulsions, turn over into side position; 5, generalized clonic–tonic convulsions. The highest dose that did not produce a response – 15 mg/kg – was considered subconvulsive.

Short term kindling was induced in adult mice by repeated sc injections of subconvulsive doses of PTZ every 30 min until a level 4 response was observed, at which point the total dose was recorded and the mouse euthanized. The investigator was blinded to the mouse genotype throughout the experimental procedure.

Results

Secreted LGI-1 binds to neuronal membranes

To create a tagged version of LGI1 suitable for characterization of cell surface binding sites relevant for epilepsy, the protein was expressed as a fusion protein with Alkaline Phosphatase (AP). Similar to native LGI1 (Senechal et al., 2005; Fukata et al., 2006; Sirerol-Piquer et al., 2006), the LGI1-AP fusion protein is secreted from transfected HEK293T cells (Fig. 1A). It has been reported that disease-causing mutations prevent LGI1 secretion (Senechal et al., 2005). Similarly, a disease-causing T953G mutation (Fertig et al., 2003) incorporated into the LGI1-AP fusion protein prevents secretion (Fig. 1A).

Secreted wild-type LGI1 is postulated to act as a ligand (Senechal et al., 2005; Fukata et al., 2006; Sirerol-Piquer et al., 2006), binding to neurons to alter function. We applied conditioned media from the LGI1-AP transfected cells to dissociated E7 chick DRG (dorsal root ganglia) neurons and E18 rat hippocampal neurons. LGI1-AP binds to both types of neurons in a dose-dependent manner (Fig. 1B). The apparent Kd is 30 nM.

ADAM23 is a high affinity LGI1 Binding Site

We utilized an unbiased expression cloning strategy to search the mouse genome for high affinity LGI1 binding sites. Although the LGI1-AP fusion protein binds to neurons, binding to non-transfected COS-7 is negligible (Fig. 2A). Forty-eight pools of 5,000 independent clones

from an arrayed adult mouse cDNA expression library were transfected into COS-7 cells. A single pool supported detectable binding of 20 nM LGI1-AP, and this pool was fractionated by sub-selection and repeated binding assays, until a single clone was isolated. Sequence analysis revealed that this cDNA encoded the entire open reading frame of ADAM23, a transmembrane protein possessing disintegrin and metalloprotease domains. ADAM23-expressing COS-7 cells exhibit saturable LGI1-AP binding with an apparent Kd of 15 nM (Fig. 2C, D). The affinity of this interaction is consistent with mediation of the neuronal binding of LGI1 (Fig. 1).

LGI1 interaction with ADAM22 has been reported (Fukata et al., 2006; Sagane et al., 2008). ADAM23, ADAM22 and ADAM11 constitute a subfamily of ADAM proteins enriched in brain and containing an inactive metalloprotease domain (Primakoff and Myles, 2000). To examine the specificity of LGI1 binding, we expressed ADAM23, ADAM22, ADAM11 or the more distantly related ADAM12 in COS7 cells to compare binding of LGI1 to this family's members. LGI1-AP binds with a Kd of 15 nM to cells expressing ADAM23, ADAM22 or ADAM11 (Fig. 2B, C). However, it does not bind to cells expressing ADAM12 or to vector control cells (Fig. 2B, C). These findings confirm and extend previous analysis LGI1 binding to ADAM proteins (Fukata et al., 2006; Sagane et al., 2008).

LGI1 stimulates neurite outgrowth through ADAM23

Since ADAM23, ADAM11 and some splice forms of ADAM22 (Godde et al., 2007) do not contain PDZ domain binding sites they are predicted not to interact with PSD-95. We explored other potential mechanisms of LGI1-ADAM23 action relevant to epilepsy. ADAM23 is known to interact with avβ3 integrins (Cal et al., 2000), and these adhesion molecules are expressed widely in the brain and are capable of modulating neurite outgrowth and cell adhesion (Cal et al., 2000; Hu and Strittmatter, 2008). Therefore, we considered whether LGI1 might regulate outgrowth. Addition of LGI1-AP to either DRG or hippocampal neurons resulted in a significant increase in outgrowth over 24 hours with a dose response relationship in the nM concentration range (Fig. 3).

Distribution of ADAM23 expression in brain

To consider the in vivo relevance of LGI1 binding to ADAM23 for neurite outgrowth and for epilepsy, we characterized mice lacking ADAM23 expression. In a secretory trap genomic project (Leighton et al., 2001), ADAM23 was one of the trapped genes. In this paradigm, the genetic disruption is accompanied by the expression of *lacZ* and heat-stable placental Alkaline Phosphatase from the endogenous promotor. The *lacZ*-derived β-galactosidase is targeted to the nucleus to reveal cell body position and the AP is targeted to axons by virtue of its GPI membrane anchorage. These markers allow a survey of ADAM23 expression pattern with simple histochemical reporters in heterozygous mice. If ADAM23 is to function as a receptor for secreted LGI1, then it must be expressed in the central nervous system where LGI1 binds to neurons, regulates outgrowth and prevents the development of epilepsy. ADAM23 +/- mice are viable and fertile and exhibit no overt phenotype. β-Galactosidase expressed from the ADAM23 locus is detected at multiple levels of the mature central nervous system in these mice (Fig. 4). Expression levels are not noticeably different in auditory cortex as compared to other areas. High expression is present in cerebral cortex pyramidal cells and in the CA1 and CA3 pyramidal cells of the hippocampal formation. The expression of the reporter gene is low in the dentate gyrus. The ADAM23 locus is also highly active in cerebellar Purkinje cells.

The axonal projection territories of ADAM23 expressing neurons can be assessed by AP reaction product. Not surprisingly, the wide distribution of ADAM23 locus expressing-positive cell bodies creates a wide distribution of presumptive ADAM23 positive axons. The molecular

layer of the hippocampus and the cerebellum are intensely stained and processes are positive throughout multiple layers of cerebral cortex (data not shown).

Normal cell positioning in ADAM23 null mice

ADAM23 -/- mice are born at close to Mendelian ratios from heterozygous crosses but do not survive past postnatal day 15 (P15). By P10, the homozygous mice exhibit obvious ataxia and fail to gain weight, as described earlier (Mitchell et al., 2001). Since a number of epilepsies are associated with cortical malformations and since disintegrin/integrin function might modify cell migration, we examined the distribution of presumptive ADAM23 expressing cells in the ADAM23 null mice (Fig. 5). Histological examination of β-galactosidase at the onset of symptoms reveals no shift in the position of ADAM23-expressing cells in ADAM23 null mice (Fig. 5). Thus, ADAM 23 is not required for normal neuronal cell migration.

ADAM23 is required for LGI1 binding and regulation of neurite outgrowth

The LGI1-AP ligand stimulates hippocampal neurite outgrowth at low concentrations. If ADAM23 is a principal receptor for LGI1, then this effect is expected to be reduced or absent in neurons cultured from ADAM23 -/- mice. Hippocampal and cortical cultures were established from P1 mice lacking ADAM23 and their littermates. The ability of LGI1 to stimulate neurite outgrowth from either cell type is significantly reduced in the absence of ADAM23 (Fig. 6). This is consistent with ADAM23 functioning as the primary receptor, but not the sole receptor, mediating axonal outgrowth effects of LGI1.

Altered neuronal morphology in ADAM23 null mice

The ability of LGI1 binding to stimulate neuronal outgrowth in tissue culture might be reflected in altered axonal and/or dendritic morphology in vivo. To consider abnormalities in axonal trajectories, the pattern of AP staining in ADAM23 -/- mice was compared to heterozygous littermates at P12. Because so many axonal populations are positive for AP in these mice, the sensitivity for detecting errors in axonal targeting is low. At a population level, all major ADAM23-tagged tracts appear normally developed in the absence of the protein (data not shown).

To assess dendritic morphology at higher resolution, we utilized Golgi impregnation (Fig. 7A). Though ADAM23 is widely expressed, we focused basal dendrite morphology of CA1 pyramidal neurons because this population is easily identified and there is relatively less variability than for some other neurons. The branching of basal dendrites of CA1 pyramidal neurons was characterized by Scholl analysis of Z-stack images. Compared to ADM23+/+ or +/- mice, ADAM23-/- mice had a significant decrease in the number of grid intersections from 55 μm from the cell body and no intersections greater than 110 μm from the cell soma (Fig. 7C). The reduced branching of ADAM23-/- basal dendrites cannot be attributed to changes in gross anatomy of the brain, since brain weight was identical to wild type at P10, and because general patterns of AP and LacZ staining were indistinguishable between ADAM23 -/- and +/- mice. The reduced branching of ADAM23 -/- pyramidal cells is associated with a lesser brain area enclosed within the perimeter of an individual neuron's dendritic field neuron in collapsed Z-stacks from a 40 μm-thick section (Fig. 7B). Since similar dendritic reductions are observed in P3 null mice (Fig. 8), before the onset of seizures (see below), they are not secondary to epilepsy. Thus, the lack of ADAM23 leads to less LGI1-stimulated outgrowth in vitro, and less dendritic arborization in vivo. Thus, the lack of ADAM23 protein leads to less LGI1-stimulated outgrowth in vitro, and less dendritic arborization in vivo.

Seizure phenotype of ADAM23 -/- mice

The presence of neonatal ataxia and death in ADAM23 -/- mice has been documented (Mitchell et al., 2001). To examine to a potential role for ADAM23 as a receptor mediating LGI1-associated epilepsy, we observed the mice for seizure activity in the neonatal period. Videotape recording documented numerous instances of tonic and tonic/clonic seizure activity in the ADAM-23 -/- mice but none in ADAM23+/- or wild type littermate mice (Fig. 9A). Seizures typically were characterized by 10-60 seconds of tonic or tonic/clonic posturing and unresponsiveness to tactile stimuli. A period of 1-5 minutes of reduced activity followed most seizures, consistent with post-ictal state. Between seizures, episodes of ataxic behavior were apparent. The frequency of convulsive episodes increased as a function of age, such that few were recorded at P7 and a maximum incidence was reached by P11 before the mice became moribund (Fig. 9B). To provide further evidence of seizure activity, electroencephalography was obtained in P10 ADAM23-/- mice via intracranial electrodes. One example of a tactile stimulation leading to a discharge lasting 6 seconds of 15 Hz low voltage fast activity typical of tonic seizures is presented in Fig. 9C.

Human LGI mutations produce ADPEAF as a dominant trait, so we assessed mice heterozygous for the ADAM23 loss of function allele. ADAM23+/- mice do not display any behavioral nor anatomical abnormalities. They survive to adulthood, breed and behave as wild type mice do. We tested whether the lack of one ADAM23 allele lowers the threshold for seizure induction by pentylenetetrazole (PTZ). Six month old mice were injected with a range of PTZ doses and assessed for behavioral changes consistent with seizure activity. A significantly greater fraction of ADAM23+/- mice (n=27) responded to 30 mg/kg injections of PTZ, than observed for ADAM23+/+ mice (n=18; $p<0.05$; Fig. 9D). Only one ADAM23 +/- mouse responded to injections from 10 to 20mg/kg. A dose of 15mg/kg was defined as subconvulsive. We then repeatedly injected adult mice with subconvulsive doses of PTZ at 30 minute intervals and observed for a response of 4 (see scale definition in Methods) or higher. The combined dose of injected PTZ at which each individual mouse was considered kindled, was significantly higher for ADAM23+/- mice ($p<0.05$) than ADAM23+/+ mice.

Discussion

The major conclusion of this study is that ADPEAF pathophysiology includes aberrant LGI1 regulation of neuronal morphology through ADAM23 family proteins. This hypothesis is supported by the observations that ADAM23 was the only binding site for LGI1 identified in an unbiased genome-wide screen, that LGI1 increases neurite outgrowth in vitro, that this regulation requires ADAM23 expression, that ADAM23 null mice have aberrant dendrite morphology, that ADAM23 null mice exhibit seizures and that reduced ADAM23 expression lowers seizure threshold. Our model for ADPEAF based on altered anatomy and neuronal connectivity is distinct from epilepsy models stemming from aberrant cell positioning or from modified ion channel properties.

Previous models for LGI1 loss of function in ADPEAF have focused on altered excitability through ion channel dysfunction, without a consideration of neuronal morphology. Schulte and colleagues described LGI1 participation in Kv1.1 potassium channel function (Schulte et al., 2006). In particular, they demonstrated LGI1 blocks the inactivation gating function of cytoplasmic Kvβ1 subunit. Given the now well-documented secretion of LGI1, any regulation of Kvβ1 must occur via a transmembrane protein. Since both ADAM22 and LGI1 coprecipitated with Kv1.1, ADAM22 may in retrospect provide an explanation. Fukata and colleagues identified both LGI1 and ADAM22 in a PSD-95 immunoprecipitation complex (Fukata et al., 2006). The ADAM22 interaction with PSD95 relies on the carboxyl-terminal 4 aa of ADAM22 binding to the PDZ domain of PSD95. They showed that secreted LGI1 can act through transmembrane ADAM22 to enhance AMPA-type glutamate receptor-mediated

synaptic transmission in hippocampal slices. However, neither ADAM23 nor ADAM11, nor certain splice forms of ADAM22, contain PDZ-binding C-termini. While LGI1 and a subset of ADAM22 may regulate ion channel in certain situations, this is unlikely to be an action of LGI1 binding to ADAM23. Consistent with the existence of other mechanisms in ADPEAF, genetic analysis of ADAM22 and Kv1.1 have not identified any human epilepsy-linked variants in these genes (Chabrol et al., 2007; Diani et al., 2008). While we cannot rule out the contribution of channel mechanisms in ADPEAF, the data here support the hypothesis that an ADAM23-mediated alteration in neuronal morphology contributes to this epilepsy. We show that LGI1 acts via ADAM23 to alter neurite outgrowth in tissue culture models prior to synapse formation, so at least this manifestation of LGI/ADAM interaction precedes neural network formation and synaptic channel modulation. However, we cannot exclude the possibility that subsequent and/or parallel changes in ion channel localization occur.

The expression of LGI1 and ADAM23 are widespread in the brain, so multiple aspects of neuronal connectivity may be altered by the ability of LGI1 binding to promote neurite outgrowth through ADAM23. In this study, we show that both cortical and hippocampal cultures exhibit LGI1-ADAM23 stimulated neurite outgrowth. We document a role for ADAM23 in the arborization of CA1 basal dendrites in vivo. There are likely to be many other aspects of neuronal connectivity that are dependent on LGI1-ADAM23 interaction. The anatomical site(s) where altered connectivity leads to epilepsy is(are) not clear. In this regard, it is notable that the clinical characteristics of ADPEAF include a predominance of partial complex seizures with a temporal focus and a predilection for auditory aura (Kalachikov et al., 2002; Morante-Redolat et al., 2002; Ottman et al., 2004; Nobile et al., 2009). ADAM23 expression is not specific to auditory pathways.

The epilepsy of ADAM23-/- mice is severe and it is likely that status epilepticus is reached in the terminal state. The heterozygous mice show reduced seizure thresholds, but no evidence of frank epilepsy. Since LGI1-associated ADPEAF is inherited in a dominant fashion, it may reflect partial loss of function of the LGI1 ligand at all three receptor proteins, ADAM23, ADAM22 and ADAM11. This hypothesis suggests that ADPEAF might best be modeled in ADAM11/22/23 triple heterozygous mice. Nonetheless, it is clear from the data here that ADAM23 alone can account for a majority of LGI1-stimulate outgrowth in culture, and can promote dendrite growth in vivo and can prevent seizures. Moreover, ADAM23 +/- mice exhibit lower seizure threshold. Thus, it will be of interest to assess the presence of ADAM23 mutations in non-LGI1 families with ADPEAF. While LGI1 mutations are specific for ADPEAF, it is possible that ADAM22 or ADAM23 may play roles in other developmental forms of epilepsies.

Together these data demonstrate that LGI1 can act through ADAM23 to pattern connectivity between neurons in vivo. ADPEAF pathophysiology is likely to extend beyond ion channel biophysics to include altered anatomy.

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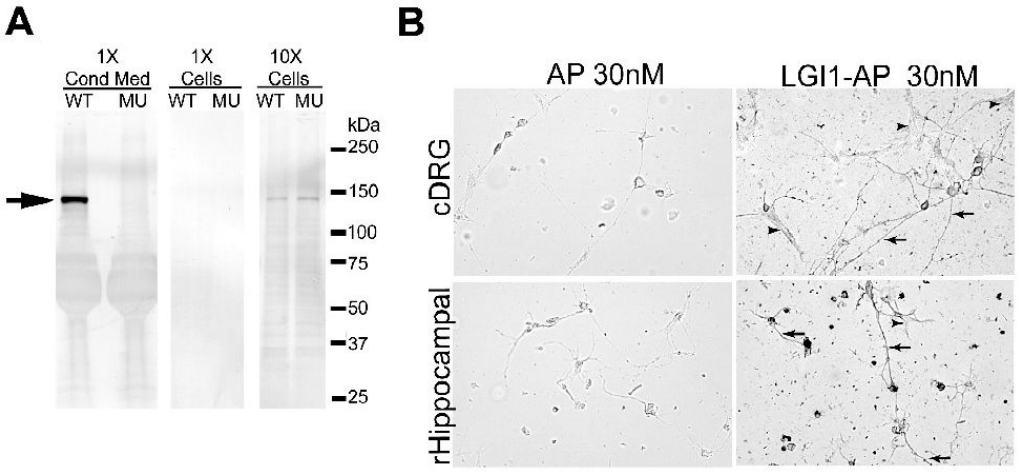

Figure 1. The secreted ADPEAF protein LGI1 binds to neurons

(A) The LGI1-AP fusion protein (WT) is secreted from transfected HEK293T cells. The AP-LGI1-F318C mutant (MU) is detectable in the cell lysate but is not secreted into the medium. Anti-AP immunoblot.

(B) LGI1-AP fusion protein, but not AP, binds to mouse E18 DRG and hippocampal neurons. Bound AP is detected by NBT/BCIP reaction.

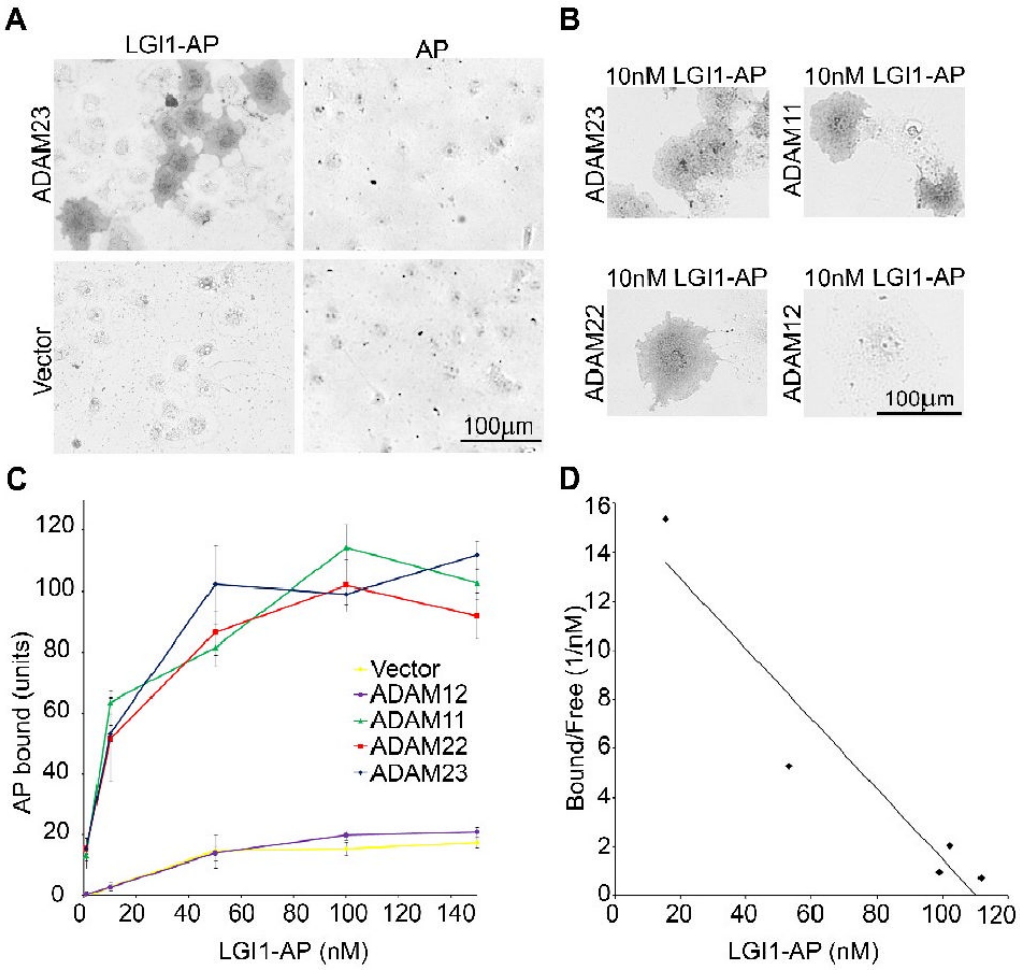

Figure 2. Expression cloning identifies ADAM23 as a high affinity LGI-1 binding site

(A) COS-7 cells were transfected with empty vector or ADAM23 expression vector and then exposed to 30 nM AP or LGI1-AP for 1 hr. Bound exogenous heat-stable AP was visualized as a dark enzyme reaction product after washing, fixing and heat inactivating endogenous AP. Scale bar is 100 µm.

(B) LGI1-AP binding to COS-7 cells transfected with ADAM23, ADAM22, ADAM11, ADAM12 and vector. Scale bar is 100 µm.

(C) The amount of LGI1-AP bound to ADAM23, ADAM22, ADAM11, ADAM12 and vector-expressing COS-7 cells is plotted as a function of ligand concentration. Data are mean ± sem from 3 independent determinations.

(D) The data from C are replotted by the Scatchard method. The apparent ADAM23 Kd is 10 nM.

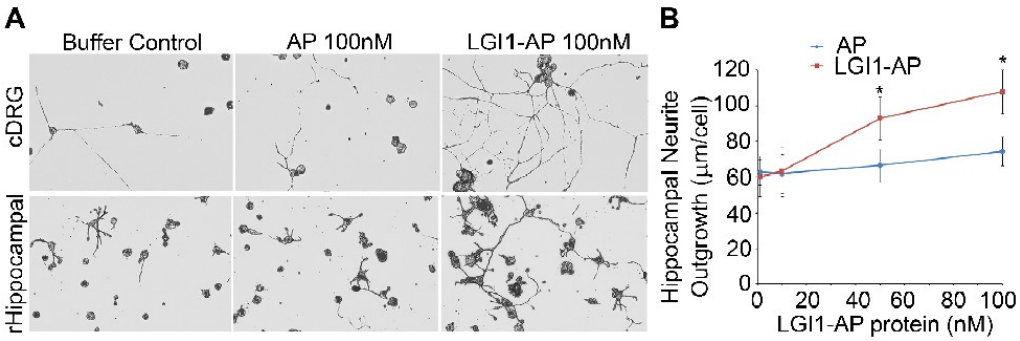

Figure 3. Extracellular LGI1 stimulates neurite outgrowth

(A) Exposure of chick E7 DRG or rat E18 hippocampal neurons to 100 nM LGI1-AP increases neurite outgrowth.

(B) Quantitation of hippocampal neurite outgrowth as a function of AP or LGI1-AP concentration. Data are mean ± sem from 3 independent experiments. *, P<0.05 versus AP, Student's two-tailed *t* test.

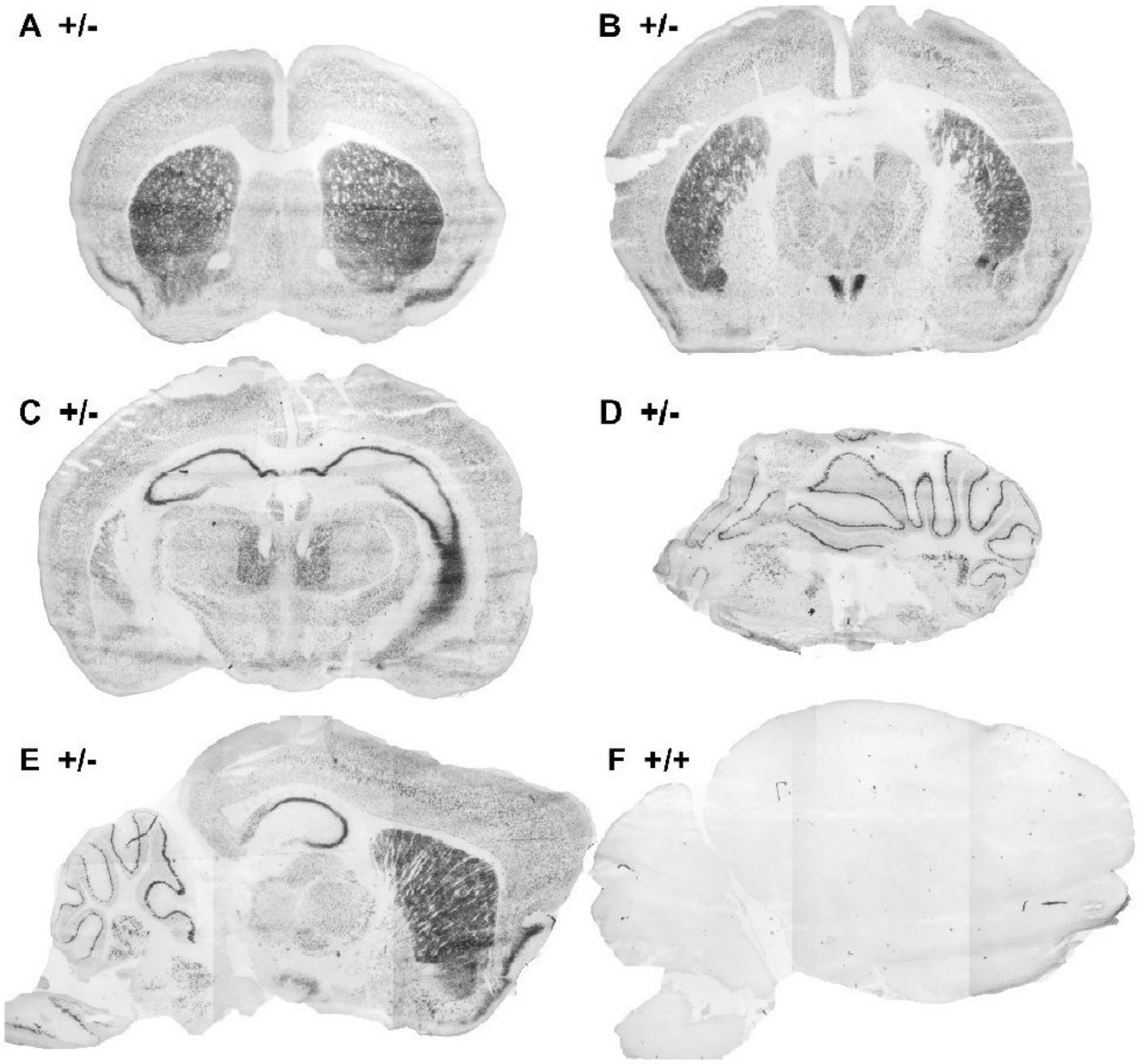

Figure 4. Distribution of ADAM23 expression in the CNS

(A-D) β-Galactosidase staining of coronal brain sections from adult ADAM23 +/- mice reveals the distribution of cells expressing ADAM23. Note the widespread expression in pyramidal cells of both cerebral cortex (A-C) and hippocampus (B-C), in Purkinjie cell of the cerebellum (D) and in numerous diencephalic and brain stem nuclei (A-D). There is an absence of ADAM23 expression in the dentate gyrus of the hippocampus (B-C).

(E) Parasagittal section of adult ADAM23 +/- mice stained for β-galactosidase activity.

(F) There is no β-galactosidase reaction product in WT mouse brain sections.

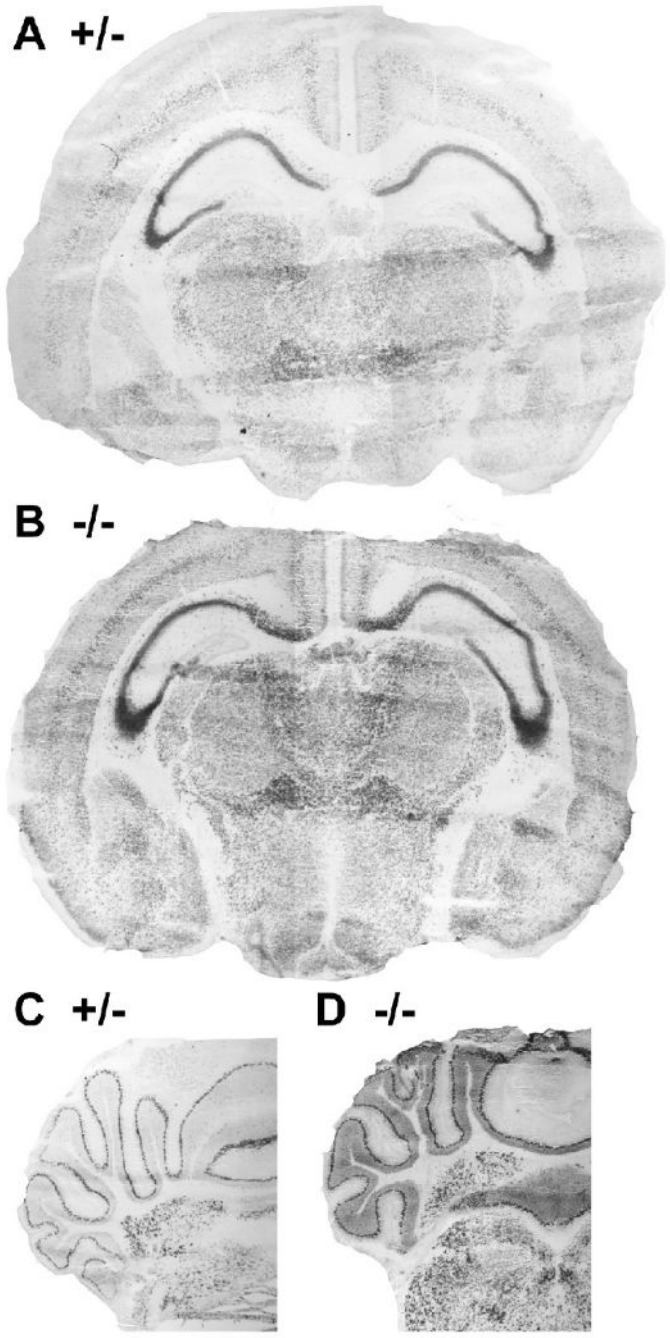

Figure 5. Position of cells expressing ADAM23 does not require the protein

(A-D) Coronal sections of P12 mouse brain from an ADAM23 +/- (A, C) or ADAM23 -/- (B, D) mouse were processed for β-galactosidase. There is no evidence for cell migration abnormalities in the cerebral cortex or hippocampus in mice lacking ADAM23.

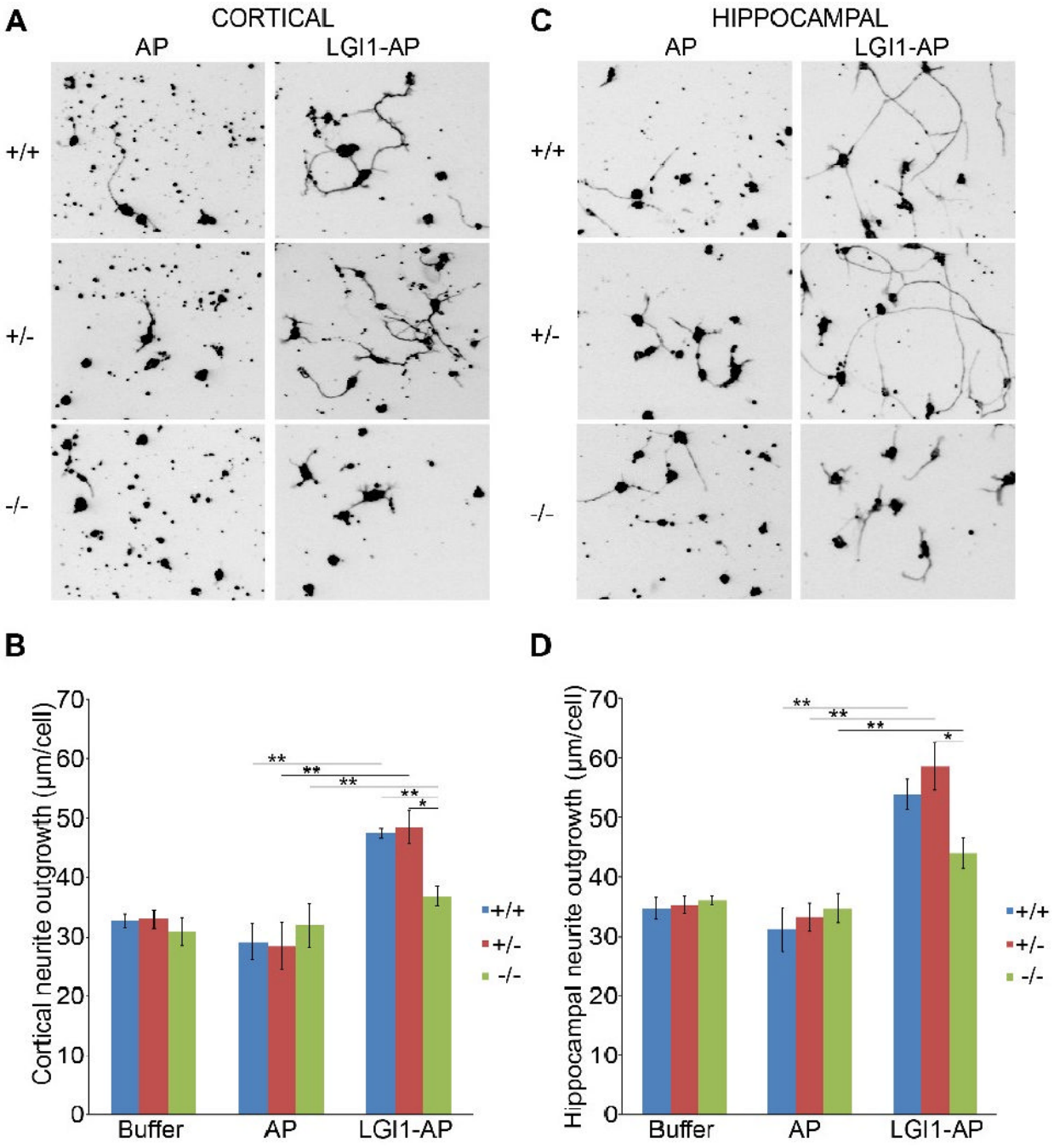

Figure 6. Reduced ability of LGI1 to stimulate hippocampal and cortical outgrowth from ADAM23-/- neurons

Cortical (A) and hippocampal (C) cultures from ADAM23+/+, ADAM23+/- and ADAM23-/- P1 mice exposed to purified AP or LGI1-AP protein at 100 nM ligand for 24 hours before fixation and staining of neuronal processes with anti- βIII-tubulin (dark in this inverted fluorescence image). Images were obtained at 10× magnification. Average outgrowth from P1 mouse cortical (B) and hippocampal (D) neurons was measured in µm/cell for ADAM23+/+ (n=6 separate cultures, each from a different mouse), ADAM23+/- (n=6) and ADAM23-/- (n=7) after culture in buffer, 100 nM AP or 100 nM LGI1. Data are mean ± sem. ANOVA: *

$p<0.05$, ** $p<0.01$. Cell number varied by less than 15% between different conditions in any one experiment.

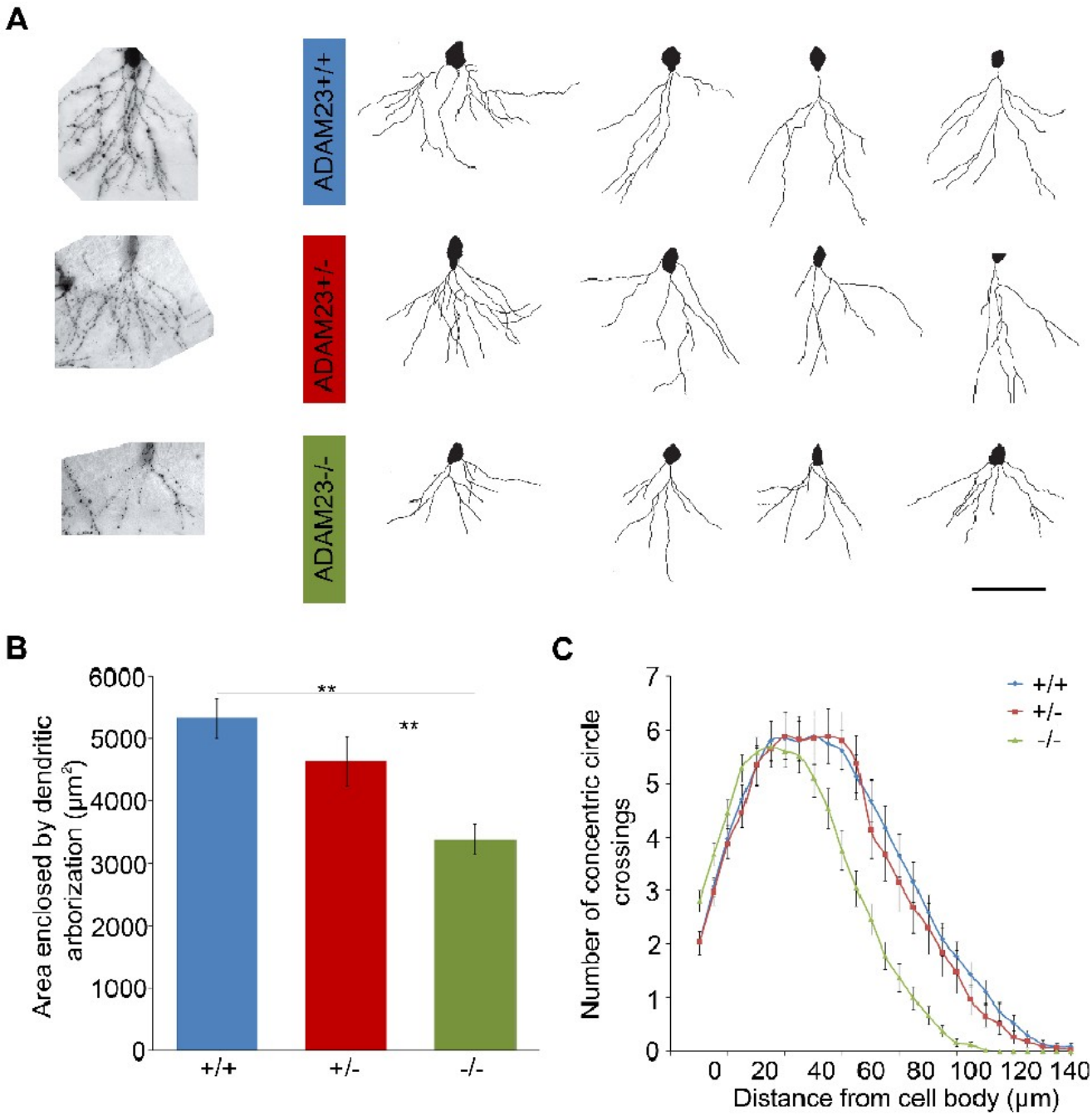

Figure 7. CA1 dendritic arborization and outgrowth in ADAM23+/+, AADAM23 +/- and ADAM23-/- mice

(A) At left, micrographs illustrate Golgi strains of CA1 pyramidal from mice of the indicated genotypes. At right, camera Lucida drawings of collapsed Z-stack of all basal dendrites in coronal 40 μm sections of CA1 pyramidal neurons from ADAM23+/+, ADAM23+/- and ADAM23 -/- P10 mice. Scale bar is 50 μm.

(B) Average area enclosed by the basal dendritic arborization of ADAM23+/+ (n=43 cells from 6 mice), ADAM23+/- (n=33 from 6 mice), ADAM23-/- (n=47 from 6 mice) mice. Data are mean ± sem. Student's two-tailed *t*-test: * $p<0.05$, ** $p<0.01$.

(C) Scholl analysis of the average number of basal dendrites crossing concentric circles at the specified distances from the cell soma. For ADAM23+/+, n=43 from 6 mice; ADAM23+/-, n=33 from 6 mice; and ADAM23-/-, n=47 from 6 mice. Data are mean ± sem. Repeated measures ANOVA: * $p<0.05$, ** $p<0.01$.

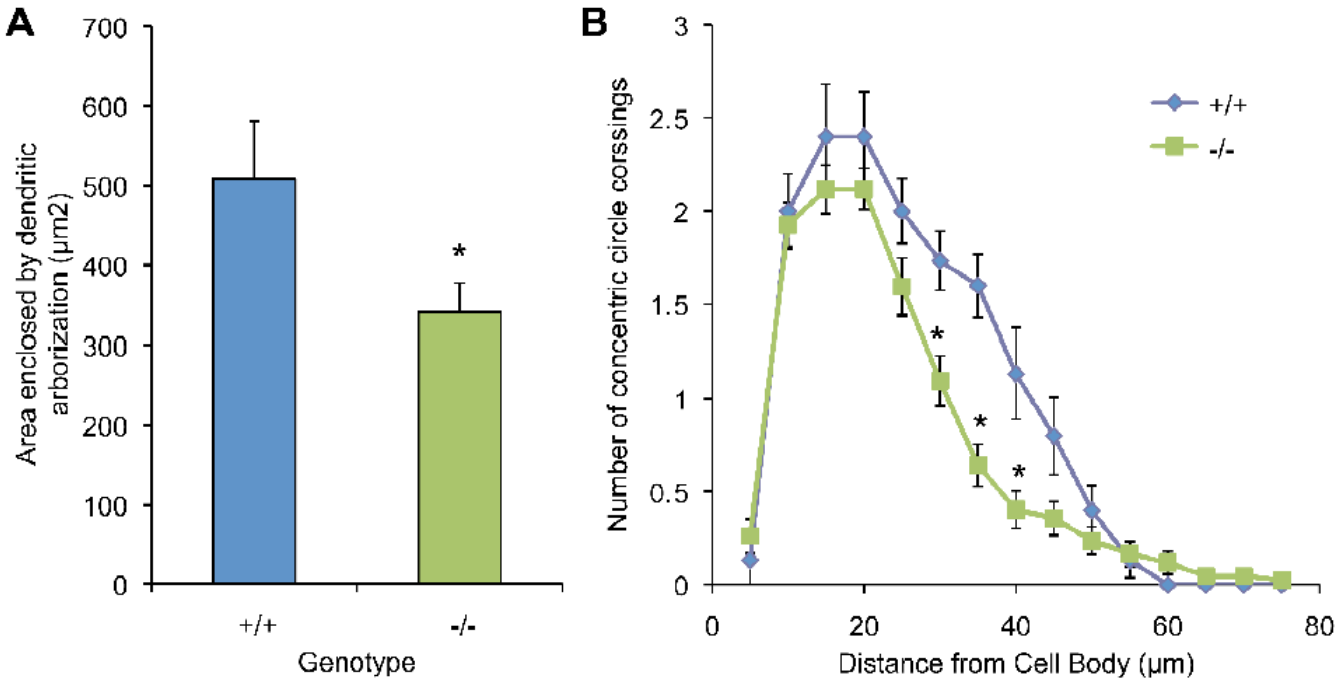

Figure 8. Reduced CA1 dendritic arbor in postnatal day 2 ADAM23-/- mice

(A) CA1 pyramidal from P2 mice of the indicated genotypes were impregnated by the Golgi method and basal dendrites of CA1 pyramidal neurons were assessed. Average area enclosed by the basal dendritic arborization of ADAM23+/+ (n=15 cells from 2 mice), ADAM23-/- (n=42 from 3 mice) mice. Data are mean ± sem. Student's two-tailed *t*-test: * $p<0.05$.

(C) Scholl analysis of the average number of basal dendrites crossing concentric circles at the specified distances from the cell soma. For ADAM23+/+, n=15 from 2 mice and ADAM23-/-, n=42 from 3 mice. Data are mean ± sem. Repeated measures ANOVA: * $p<0.05$, ** $p<0.01$.

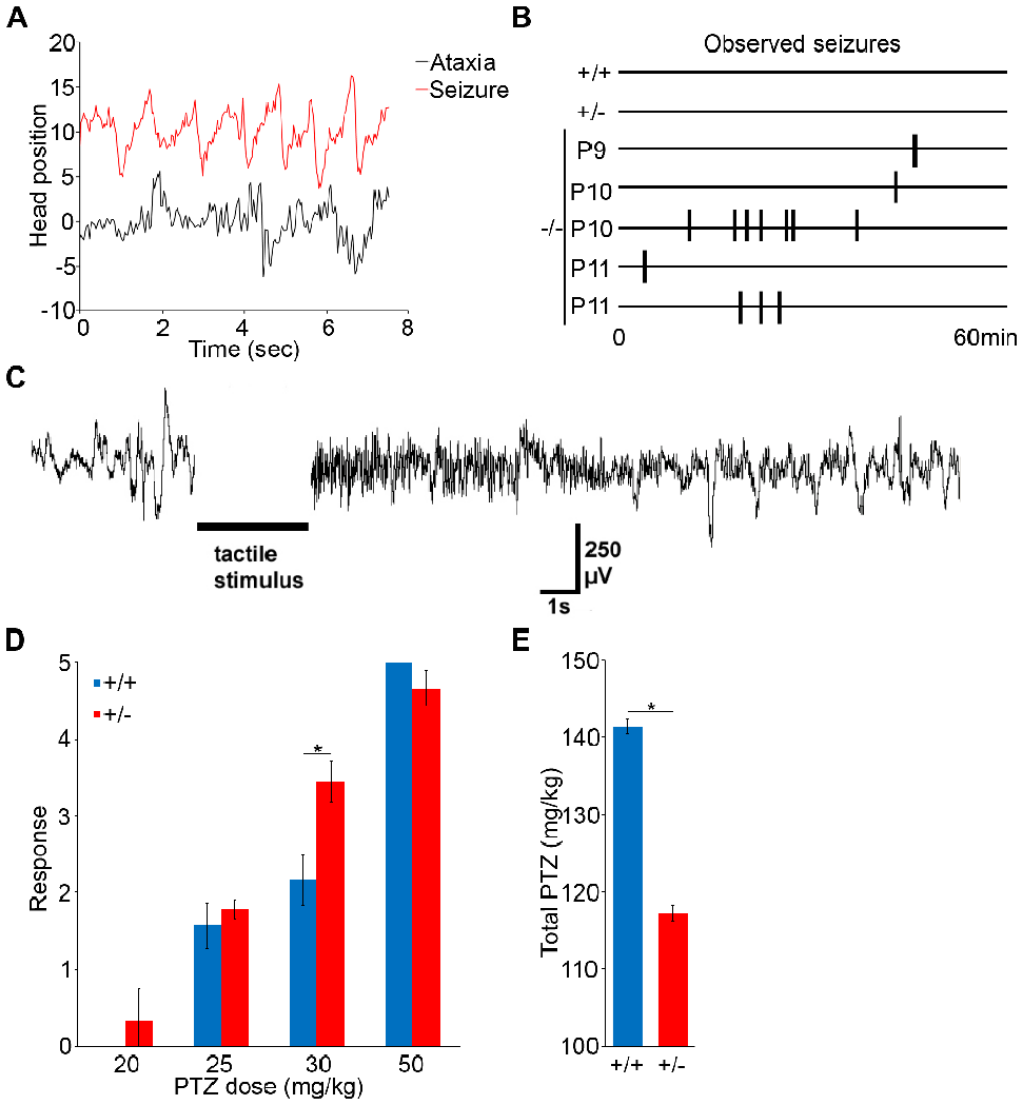

Figure 9. Epileptic events in ADAM23-/- mice and reduced seizure threshold in ADAM23+/- mice

(A) Head position plotted as a function of time for P10 ADAM23-/- mouse. Top trace (--) represents repetitive clonic movements of a seizure and bottom trace (--) represents irregular ataxic movements in the same mouse.

(B) The frequency of convulsive episodes illustrated in (A) reported for a 60 min observation period (horizontal lines) for ADAM23 +/+, ADAM23+/- and ADAM23-/- P10 mice. Each occurrence is represented as a vertical line.

(C) EEG recording from a P10 Adam23-/- mouse illustrates an example of tactile stimulation leading to tonic seizure.

(D) Response of 6 month old ADAM23+/+ and +/- mice to single sc injection of PTZ (mg/kg). Data are mean ± sem, student t-test * $p<0.05$, ** $p<0.01$. ADAM23+/+ 20mg/kg n=3, 25mg/kg n=14, 30mg/kg n=18, 50mg/kg n=3. ADAM23+/- 20mg/kg n=3, 25mg/kg n=19, 30mg/kg n=27, 50mg/kg n=6.

(E) Short term kindling of 6 month old ADAM23+/+ and ADAM23+/- mice to repeated (every 30min.) sub convulsive (15mg/kg) sc injection of PTZ (mg/kg). Data are mean ± sem, student t-test * $p<0.05$, ** $p<0.01$, ADAM23+/+ n=40, ADAM23+/- n=50.