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CUX2 deficiency causes facilitation of excitatory synaptic transmission onto hippocampus and increased seizure susceptibility to kainate

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CUX2 **gene encodes a transcription factor that controls neuronal proliferation, dendrite branching and synapse formation, locating at the epilepsy-associated chromosomal region 12q24 that we previously identified by a genome-wide association study (GWAS) in Japanese population. A *CUX2* recurrent de novo variant p.E590K has been described in patients with rare epileptic encephalopathies and the gene is a candidate for the locus, however the mutation may not be enough to generate the genome-wide significance in the GWAS and whether *CUX2* variants appear in other types of epilepsies and physiopathological mechanisms are remained to be investigated. Here in this study, we conducted targeted sequencings of *CUX2*, a paralog *CUX1* and its short isoform *CASP* harboring a unique C-terminus on 271 Japanese patients with a variety of epilepsies, and found that multiple *CUX2* missense variants, other than the p.E590K, and some *CASP* variants including a deletion, predominantly appeared in patients with temporal lobe epilepsy (TLE). The CUX2 variants showed abnormal localization in human cell culture analysis. While wild-type CUX2 enhances dendritic arborization in fly neurons, the effect was compromised by some of the variants. *Cux2*- and *Casp*-specific knockout mice both showed high susceptibility to kainate, increased excitatory cell number in the entorhinal cortex, and significant enhancement in glutamatergic synaptic transmission to the hippocampus. CASP and CUX2 proteins physiologically bound to each other and co-expressed in excitatory neurons in brain regions including the entorhinal cortex. These results suggest that**

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***CUX2* and *CASP* variants contribute to the TLE pathology through a facilitation of excitatory synaptic transmission from entorhinal cortex to hippocampus.**

Cux2 gene encodes a homeobox transcription factor CUX2 that is predominantly expressed in progenitor cells of the subventricular zone in mouse embryos and pyramidal neurons of the upper neocortical layers (II–IV) in adult mice[1]. CUX2 is also expressed in Reelin-positive neurons distributed throughout the layers II–IV in postnatal day 0 (P0) mice[2]. CUX2 controls neuronal proliferation, dendrite branching, spine morphology and synapse formation[3,4]. We recently reported a genome-wide association study (GWAS) on 1825 Japanese patients with variable epilepsies which identified an associated region at chromosome 12q24.11–12q24.13 harboring 24 transcripts including *CUX2* gene[5]. In the region, *CUX2* is only gene which has been reported to be relevant for epilepsy; a recurrent de novo variant (c.1768G>A, p.E590K) has been identified in patients with rare epileptic encephalopathies (EEs)[6,7]. *CUX2* is therefore one of the most promising candidate genes in this 12q24 epilepsy-associated region, but the mutation reported in rare EEs may not be enough to explain the association detected in the Japanese GWAS study.

To investigate whether *CUX2* and its paralogues' mutations are involved in other types of epilepsies, here we performed targeted sequencing of *CUX2*, its paralog *CUX1,* and *CASP* which is a short isoform of *CUX1* with a unique C-terminus, in Japanese patients with variable epilepsies including genetic generalized and structural/metabolic epilepsies, and identified their variants predominantly in patients with temporal lobe epilepsy (TLE), the most common but intractable form of epilepsy[8]. Analyses in human cultured cell and transgenic fly showed that the variants have loss-of-function effects. CUX2 and CASP deficiencies in mice increased their seizure susceptibilities to a convulsant, kainate, which has long been used to generate TLE animal models[9]. Histological and electrophysiological analyses revealed increases of excitatory neuron numbers in entorhinal cortex and those in excitatory input to hippocampus in both mice, proposing a circuit mechanism for the pathology of TLE.

Materials and methods

Subjects. Genomic DNAs from 271 Japanese patients with a variety of epilepsies (Table S1) and 311 healthy Japanese volunteers recruited by cooperating hospitals were used for the targeted sequencing analyses for *CUX2*, *CUX1* and *CASP*. For the frequency calculation of c.3847G>A (p.E1283K) variant in *CUX2* gene, additional DNA samples from independent 69 Japanese patients with TLE from 2 additional independent facilities were further recruited (Table S1). The patients' DNAs analyzed in our GWAS[5] were not used in the present study, because their epilepsy subtype information [TLE, etc.] were not available for those materials.

Targeted sequencing. Genomic DNAs were extracted from peripheral venous blood samples using QIAamp DNA Blood Midi Kit (Qiagen). Genomic DNA samples were amplified with the illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare). We designed PCR primers to amplify all coding regions of *CUX2* (NM_015267), *CUX1* (NM_001202543 and first exon of NM_181552), unique C-terminus region of *CASP* (NM_001913), and amplified genomic DNA by PCR using the PrimeSTAR HS DNA Polymerase (TaKaRa) or KOD-plus Ver. 2 (TOYOBO). The PCR products were purified using ExoSAP-IT PCR product Cleanup (Thermo Fisher Scientific) and analyzed by direct sequencing using the ABI PRISM 3730xl Genetic Analyzer. All novel variants identified in amplified DNA by GenomiPhi were verified by direct-sequencing of patients' genomic DNAs. Primer sequences and PCR conditions are available upon request.

Quantification of mRNA Described in the Supplemental Methods.

Domain search Domain searches in CUX2, CUX1, and CASP amino acid sequences were performed using the SMART database.

Expression constructs and mutagenesis Described in the Supplemental Methods.

Cell imaging Described in the Supplemental Methods.

Drosophila stocks and crosses Described in the Supplemental Methods.

TUNEL assay in flies Described in the Supplemental Methods.

Mice. *Cux2* knockout (KO) mouse was obtained from Texas A&M Institute for Genomic Medicine (TIGM) as cryopreserved sperm of heterozygous *Cux2* gene trap mouse (129/SvEv × C57BL/6 background) derived from the gene trapped clone OST440231. Live animals were produced by in vitro fertilization at the Research Resources Division (RRD) of the Institute of Physical and Chemical Research (RIKEN) Center for Brain Science. The heterozygous mice were maintained on the C57BL/6J (B6J) background, and the resultant heterozygous mice were interbred to obtain wild-type (WT), heterozygous, and homozygous mice. Genotyping was carried out as described previously[10].

Casp-specific KO mice were generated using the CRISPR/Cas system. Plasmid vector pX330-U6-Chimeric_BB-CBh-hSpCas9 was a gift from Dr. Feng Zhang (Addgene plasmid # 42230). A pair of oligo DNAs corresponding to *Casp*-gRNA (TTTCCATCATCTCCAGCCAA AGG) in exon 17 of *Casp* (NM_198602) was annealed and ligated into pX330-U6-Chimeric_BB-CBh-hSpCas9. For knock-out mouse production, Cas9 mRNAs and

Casp-gRNA were diluted to 10 ng/μL each. Further, B6J female mice and ICR mouse strains were used as embryo donors and foster mothers, respectively. Genomic DNA from founder mice was extracted, and PCR was performed using gene-specific primers (CRISPR_check_F: 5′-GGAGCTATTGTAGGACATCACAGA-3′ and CRISPR_check_R: 5′-CCCCAGTGTTCTTTACTTTGAGTT-3′). PCR products were purified using ExoSAP-IT PCR product Cleanup and analyzed by direct sequencing using the ABI PRISM 3730xl Genetic Analyzer. The heterozygous mutant mice (c.1514^1515ins.TT, p.S506fs) were crossbred with B6J mice, and the resultant heterozygous mice were interbred to obtain WT, heterozygous, and homozygous mutant mice. The frame-shift mutation was confirmed by sequence analysis of cDNA from mutant mouse brains.

Seizure susceptibility in mice Described in the Supplemental Methods.

CUX2 antibody generation

To generate a rabbit polyclonal antibody to CUX2, a fusion protein was prepared, in which the glutathione-S-transferase (GST) protein was fused to the a.a. residues 356 to 415 of mouse CUX2 which has been used in the previous study's antibody generation[11]. Rabbits were injected with 0.5 mg of purified GST fusion protein in Freund's complete adjuvant, boosted five times with 0.25 mg of protein, and serum collection at 1 week following the last boost. Polyclonal antibody was purified by affinity chromatography. The serum was passed through a GST affinity column ten times, and the flow-through was then applied to a GST-CUX2 (356–415 a.a.) affinity column to isolate antibodies.

Histological analyses Described in the Supplemental Methods.

In vitro electrophysiology Described in the Supplemental Methods.

Co-immunoprecipitation Described in the Supplemental Methods.

Statistical analysis. In the in vitro and in vivo experiments, data are presented as Box-and-whisker plots or means ± s.e.m. The boxes show median, 25th and 75th percentiles, and whiskers represent minimum and maximum values. *P*-value for p.E1283K was calculated using the Cochrane–Armitage trend test. One-way or two-way ANOVA, Tukey's multiple comparison test, Chi-square test, or Kolmogorov–Smirnov test were used to assess the data as mentioned in the figure legends. Statistical significance was defined as $P < 0.05$.

Study approval. *Human study.* The experimental protocols were approved by the Ethical Committee of RIKEN and by the participating hospitals and universities. All human study experimental procedures were performed in accordance with the guidelines of the Ethical Committee of the RIKEN and with the Declaration of Helsinki. Written informed consents were obtained from all individuals and/or their families in compliance with the relevant Japanese regulations.

Animal study. All animal experimental protocols were approved by the Animal Experiment Committee of RIKEN. All animal breeding and experimental procedures were performed in accordance with the ARRIVE guidelines and the guidelines of the Animal Experiment Committee of the RIKEN.

Results

***CUX2* variants predominantly appeared in Japanese TLE patients.** We carried out a targeted sequencing of *CUX2* in 271 Japanese patients with variable epilepsies consisting of 116 genetic generalized epilepsies and 155 structural/metabolic ones (Table S1). Structural/metabolic epilepsy samples contained 68 TLEs, which were further divided to 57 mesial TLE (mTLE) and 11 lateral TLE (lTLE). We identified five *CUX2* heterozygous missense variants in nine unrelated patients (Fig. 1A, Table 1 and Supplemental Note). Notably, eight of the nine patients with *CUX2* variants had TLE (one lTLE and seven mTLE patients). All of patients carrying *CUX2* missense variants belonged to the subgroup of structural/metabolic epilepsy. None of patients with genetic generalized epilepsies showed *CUX2* variants except for silent variants. All of the mTLE patients showed hippocampal sclerosis. Three (p.R34W, p.P454L, and p.W958R) out of the five variants were absent or rare (< 0.5%) in the in-house Japanese control individuals (in-house controls) and databases and were also predicted to be damaging (Table 1). The p.E1283K variant, a frequent variant predicted to be less damaging, appeared in Japanese TLE patients at a significantly high ratio [$P = 5.93 \times 10^{-3}$, OR = 6.94, 95% CI = 1.39–34.61 calculated in 137 (above-mentioned 68 + additional independent 69; Table S1) TLE patients vs 311 in-house controls] and therefore we hypothesized it may also be a genetic contributor for TLE. The p.D337N variant appeared in one case with TLE and controls with a similar allele frequency.

Loss-of-function effects of *CUX2* variants. To investigate the functional impacts of *CUX2* variants appeared in patients with epilepsy (Fig. 1A, Table 1), we transfected HeLa.S3 cells with expression constructs of wild-type (WT) and the five variants (p.R34W, p.D337N, p.P454L, p.W958R, and p.E1283K). We calculated two parameters of abnormality, "leakage to cytoplasm" and "abnormal puncta" (Fig. 1B,C and Fig. S1A). Although some but not all variants showed abnormalities in each parameter, the combined data reached statistical significance except for p.R34W.

CUX2 is an ortholog of *Drosophila melanogaster cut,* which promotes dendritic arbor morphological complexity[12]. We generated transgenic fly lines to express human WT CUX2 or variants (p.R34W, p.D337N,

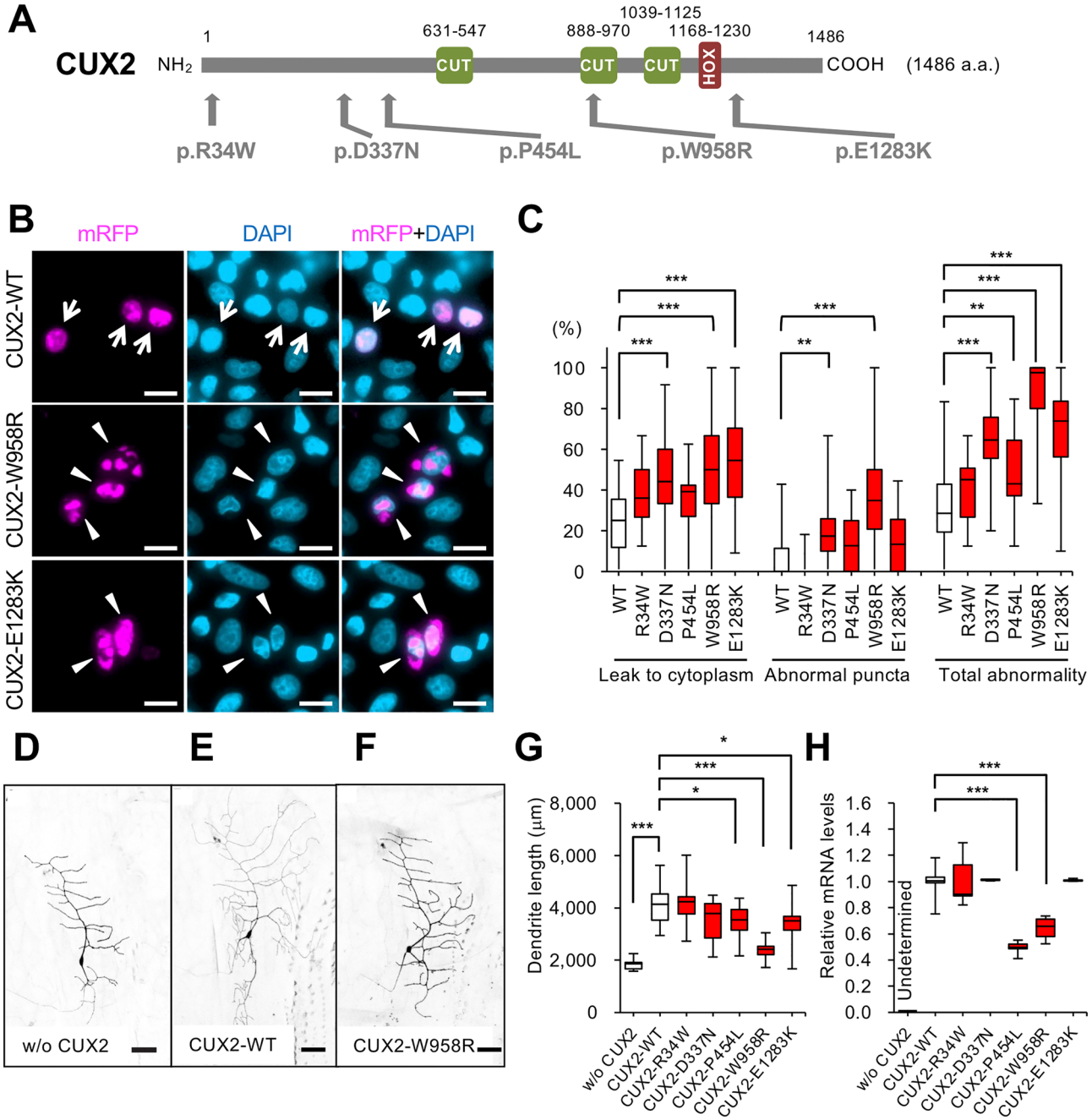


Figure 1. Loss-of-function effects of TLE variants in *CUX2*. (**A**) CUX2 protein structure (NP_056082) with variants appeared in patients with epilepsy. (**B**) Abnormal subcellular localization of CUX2 variant proteins. CUX2-WT protein (arrows) was limited to, but well distributed within, nuclei stained with DAPI (cyan), whereas variants showed abnormal aggregates in nuclei (W958R) or leaked-out to the cytoplasm (E1283K) (arrowheads). Scale bars = 20 μm. (**C**) Ratio of abnormally localized CUX2 proteins (> 200 cells counted). n = WT: 545, R34W: 282, D337N: 363, P454L: 239, W958R: 565, and E1283K: 323 cells. (**D**–**H**) CUX2 WT accelerated arborization of fly neurons and TLE variants lowered its activity and expression. Representative images of neurons without CUX2 (**D**), WT control (**E**), and W958R (**F**). Scale bars = 50 μm. (**G**) Shortened dendrite length in transgenic fly with mutants (n = 11–25 neurons per genotypes) and (**H**) lowered expression of mutants (n = 6). One-way ANOVA Tukey's multiple comparison test (**C**,**G**,**H**). *P < 0.05, **P < 0.01, ***P < 0.001.

p.P454L, p.W958R, and p.E1283K) and analyzed their dendritic arbor morphology in *Drosophila* larvae (Fig. 1D–G and Fig. S1B–D). Similar to its *Drosophila* orthologue[12], ectopic expression of CUX2 WT strongly increased dendritic arbor complexity (branch number and length). However, activities to drive arbor complexity in the variants were significantly decreased, except for p.R34W and p.D337N. RT-qPCR assays in the adult transgenic flies revealed that expression levels of the *CUX2* variants, p.P454L and p.W958R, were significantly lower (Fig. 1H). All *CUX2* constructs were inserted into the same genomic site, therefore the lower expression

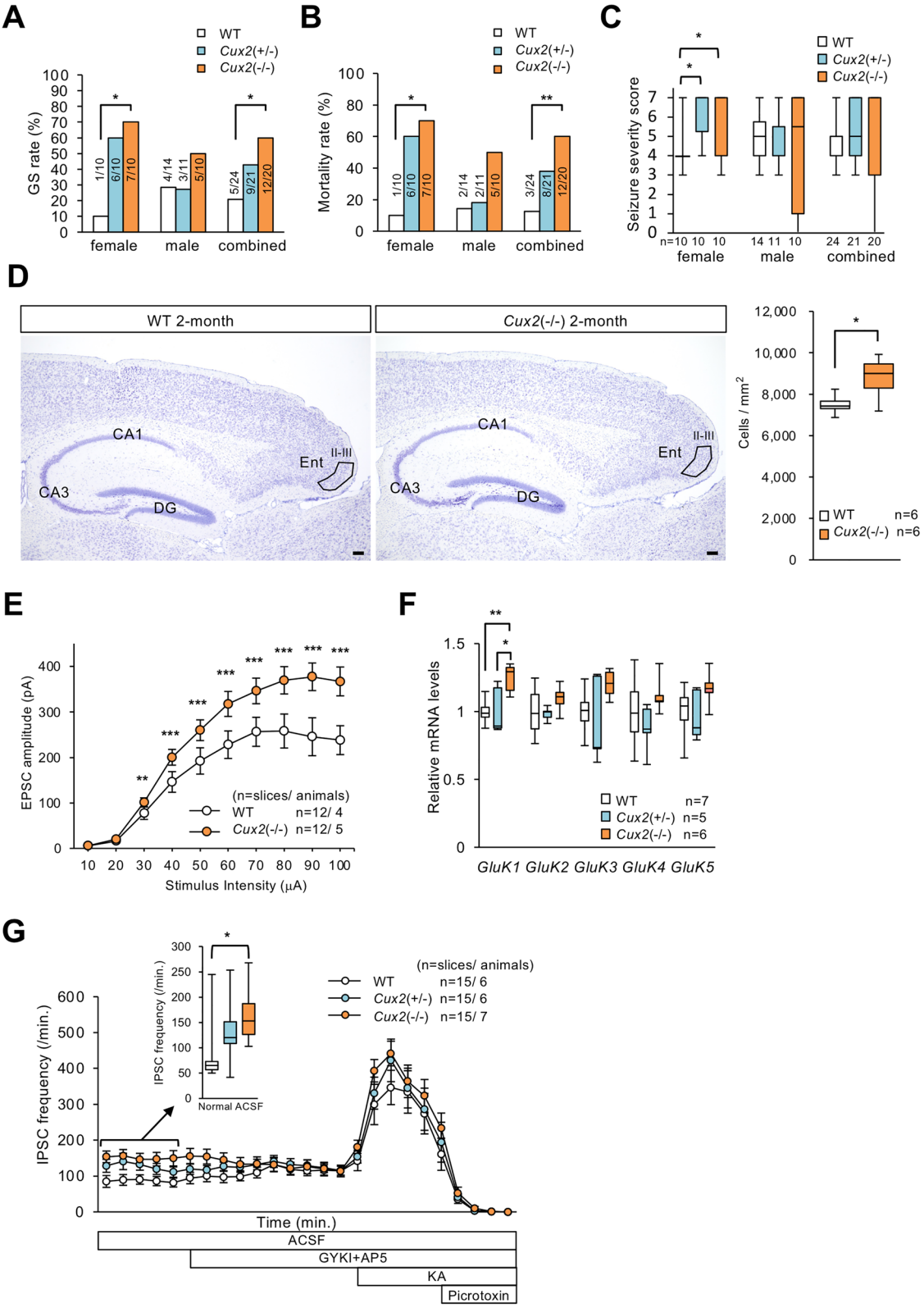
Patient ID	Gene	Nucleotide changes	Amino acid substitutions	SNP ID	Onset age (year)	Evaluation age (year)	Sex	Diagnosis	Variant allele count in		*P*-value	Variant allele count in					Mutation taster	PolyPhen-2	PROVEAN	SIFT	M-CAP
									Case	JP		J-HGVD	EVS	1kGP	ExAC	gnomAD					
SIZ-220	*CUX2*	c.100C>T	p.R34W	rs199531850	10	27	M	**mTLE**	1/542	0/622	0.284	1/1900	1/11,778	1/5008	21/119,874	62/273,306	++	–	++	++	–
SIZ-016		c.1009G>A	p.D337N	rs201601231	16	24	M	**mTLE**	1/542	5/622	0.140	4/2192	1/12,344	2/5008	18/117,772	34/279,160	++	++	–	–	–
SIZ-296		c.1361C>T	p.P454L	rs768144991	2	15	M	Doose syndrome	1/542	0/622	0.284	5/2184	NR	NR	1/24,448	8/167,304	++	+	++	–	+
SIZ-014		c.2872T>C	p.W958R	NA	3	42	M	**mTLE**	1/542	0/622	0.284	NR	NR	NR	NR	NR	++	++	++	++	+
SIZ-004		c.3847G>A	p.E1283K	rs61745424	11	37	F	**mTLE**	5/542	2/622	0.185	9/2126	227/12,676	143/5008	3893/120,384	8188/280,456	–	–	–	–	NA
SIZ-022					8	29	F	**mTLE**													
SIZ-073					19	27	M	**mTLE**													
SIZ-079					13	27	M	**lTLE**													
SIZ-190					16	34	F	**mTLE**													
SIZ-784	*CUX1*	c.3161C>T	p.S1054L	rs146486358	16	32	M	JME suspected	2/542	NT	NT	9/1912	NR	13/5008	101/120,970	231/282,774	++	++	–	–	NA
SIZ-891					0	8	M	GEFS													
SIZ-575		c.3281C>T	p.A1094V	rs184337744	3	5	F	FLE	1/542	NT	NT	NR	1/13,006	1/5008	38/120,936	38/250,814	–	+	–	–	+
SIZ-669		c.3815G>A	p.R1272Q	NA	0	30	F	SGE	1/542	NT	NT	2/2152	NR	NR	NR	NR	++	++	–	++	+
SIZ-456		c.4172C>T	p.T1391I	NA	2	5	F	CAE	1/542	0/620	0.284	NR	NR	NR	NR	NR	–	–	–	–	+
	CASP	c.1390G>A	p.A464T	rs803064	*	*	*	*	247/542	NT	NT	991/2210	NR	2800/5008	68,923/121,236	159,104/282,310	–	–	–	–	–
SIZ-127		c.1433C>T	p.A478V	NA	14	31	F	JME	1/542	0/622	0.284	NR	NR	NR	NR	NR	++	+	–	–	+
SIZ-063		c.1524delG	p.R509fs	rs782400087	6	9	F	CAE	1/542	0/622	0.284	NR	NR	NR	1/121,412	1/249,562	++	NA	NA	NA	NA
SIZ-068		c.1687G>A	p.G563S	rs187131238	12	28	F	JME	2/542	1/622	0.484	3/2164	NR	2/5008	4/120,438	7/247,922	–	–	–	–	–
SIZ-060					18	26	M	**mTLE**													
SIZ-638		c.1868_1870delTCT	p.F623del	NA	15	48	M	**lTLE**	1/542	0/622	0.284	NR	NR	NR	NR	NR	++	NA	++	NA	NA

Table 1. *CUX2*, *CUX1*, and *CASP* gene nonsynonymous variants in patients with epilepsy. *mTLE* mesial temporal lobe epilepsy, *lTLE* lateral temporal lobe epilepsy, *JME* juvenile myoclonic epilepsy, *CAE* childhood absence epilepsy, *SGE* symptomatic generalized epilepsy, *FLE* frontal lobe epilepsy, *GEFS* generalized epilepsy with febrile seizure plus, *M* male, *F* female, *JP* in-house Japanese control individuals, *J-HGVD* Japanese Human Genetic Variation Database, *EVS* Exome Variant Server NHLBI GO Exome Sequencing Project, *1kGP* The 1000 Genomes Project, *ExAC* Exome Aggregation Consortium, *gnomAD* Genome Aggregation Database, ++ Disease causing, Probably damaging, Deleterious, or Damaging, + Possibly damaging, – Polymorphism, Benign, Tolerated, or Neutral, *NA* not available, *NT* not tested, *NR* not registered. The *CUX2* reference sequence (NM_015267) has an error at c.4414, and the correct nucleotide is C. *CUX2* nucleotide change c.4414G>C (p.V1472L) (rs6490073 in dbSNP, NCBI) was observed in all sequences in databases and in our subjects, suggesting that the *CUX2* reference sequence (NM_015267) has an error at this position (the correct nucleotide is c.4414C). *Informations are not described because of more than 100 individuals. Significant texts are in bold.

levels of transgenes are not likely to be due to position effects but most likely due to these variants because these are only the differences in the constructs used for the analyses of fly. TUNEL of adult fly brains showed that the alleles did not promote apoptotic cell death (Fig. S1E). Together, these observations suggest that the *CUX2* variants present in patients with epilepsy cause loss-of-function of the protein.

***Cux2*-deficient mice show increased susceptibility to kainate.** Because of the loss-of-function nature of epilepsy-associated *CUX2* variants, we next investigated *Cux2*-KO mice[10]. The body weight of 2-month-old mice was comparable among genotypes (Fig. S2A). In electrocorticogram analysis, the median of the poly spike and wave discharges frequency was slightly higher in the primary somatosensory cortex forelimb region of *Cux2*(−/−) than WT mice, however the difference did not reach statistical significance (data not shown). No obvious epileptic behaviors or changes in local field potential recordings in the hippocampus were noted in *Cux2*(+/−) or *Cux2*(−/−) mice. Although patients with TLE often have past histories of febrile seizures[13], *Cux2*-KO mice did not show any seizure susceptibility to increased body temperature (data not shown). Seizure susceptibility to pentylenetetrazole (PTZ), a GABA-A receptor antagonist, remained unchanged in *Cux2*-KO mice (Fig. S2B–F). Importantly, however, *Cux2*-KO mice had a high susceptibility to kainate, which is commonly used to generate TLE animal models[9], in frequencies of generalized convulsive seizures (GS) (Fig. 2A) and lethality (Fig. 2B). The latencies to onset of GS and death were also significantly decreased in *Cux2*(−/−) mice (Figure S2G,H). Seizure severity was also significantly higher in *Cux2*-KO female mice (Fig. 2C). These results support the notion that *CUX2* loss-of-function mutations cause TLE.

***Cux2*-deficient mice show increased cell number in entorhinal cortex and glutamatergic input to hippocampus.** *Cux2*(−/−) mice have been reported to show overgrowth of the neocortical upper layers[3]. In a Nissl staining, we also found a significantly increased cell number in entorhinal cortex layers II-III, which projects to hippocampal dentate granule cells and CA3 pyramidal cells (Fig. 2D). In the slice-patch recordings, we further found that perforant path-evoked excitatory postsynaptic currents (eEPSCs) in the dentate granule cells were significantly higher in *Cux2*(−/−) mice (Fig. 2E), indicating that glutamatergic synaptic transmission from the entorhinal cortex layers II–III onto the hippocampus was significantly facilitated in *Cux2*-KO mice.


A
WT
Cux2(+/-)
Cux2(-/-)
GS rate (%)
1/10
6/10
7/10
4/14
3/11
5/10
5/24
9/21
12/20
female
male
combined
B
Mortality rate (%)
1/10
6/10
7/10
2/14
2/11
5/10
3/24
8/21
12/20
C
Seizure severity score
n=10 10 10
14 11 10
24 21 20
D
WT 2-month
Cux2(-/-) 2-month
CA1
CA3
DG
Ent
II-III
Cells / mm2
WT n=6
Cux2(-/-) n=6
E
EPSC amplitude (pA)
Stimulus Intensity (μA)
(n=slices/ animals)
WT n=12/ 4
Cux2(-/-) n=12/ 5
F
Relative mRNA levels
GluK1 GluK2 GluK3 GluK4 GluK5
WT n=7
Cux2(+/-) n=5
Cux2(-/-) n=6
G
IPSC frequency (/min.)
Normal ACSF
(n=slices/ animals)
WT n=15/ 6
Cux2(+/-) n=15/ 6
Cux2(-/-) n=15/ 7
Time (min.)
ACSF
GYKI+AP5
KA
Picrotoxin

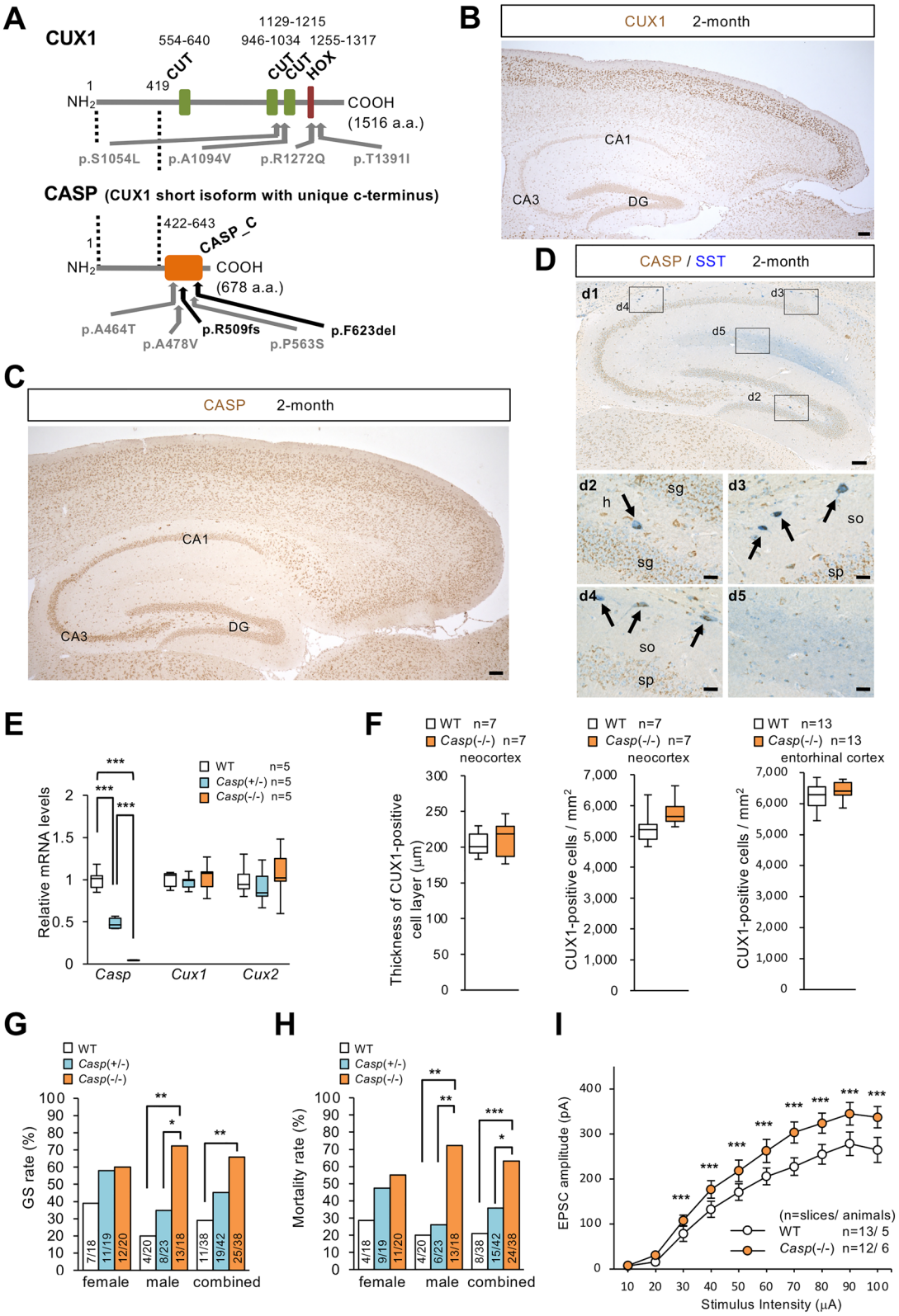
◂**Figure 2.** Increased kainate susceptibility, entorhinal cortical cell number, and excitatory input to hippocampal granule cells in *Cux2*-KO mice. (**A**–**C**) Seizure-related events in mice after intraperitoneal injection of kainate (KA). Ratio of animals exhibiting generalized convulsive seizure (GS) (**A**), mortality rate (**B**), and seizure severity scores (**C**) was significantly higher in *Cux2*(−/−) female and combined gender mice. (**D**) Number of entorhinal cortex layer II–III excitatory neurons was significantly increased in *Cux2*(−/−) mice (2-month-old). Scale bar = 100 μm. (**E**) Slice-patch analyses showed that perforant path-evoked EPSCs in dentate granule cells were significantly increased in *Cux2*(−/−) female. (**F**) RT-qPCR analyses revealed that *GluK1* mRNA was significantly increased in *Cux2*(−/−) mice. (**G**) Basal frequency of sIPSC in dentate granule cells of *Cux2*(−/−) female was significantly increased, and it was suppressed with subsequent applications of antagonists for AMPA receptor (GYKI) and NMDA receptor (AP5). Kainate (KA) increased the sIPSC frequency, which was then suppressed by the GABA-A receptor antagonist picrotoxin. *DG* dentate gyrus, *Ent* entorhinal area. Yates' correction after Pearson's Chi-square (**A**,**B**), one-way ANOVA Tukey's test (**C**,**F**,**G**), one-way ANOVA (**D**), or two-way ANOVA Tukey's test (**E**). n: mouse numbers. *P < 0.05, **P < 0.01, ***P < 0.001.

At a glance, hippocampal structures in *Cux2*-KO mice were comparable to 2- and 10-month-old WT mice (Fig. 2D, Fig. S3). We generated an anti-CUX2 antibody similarly to a previous study[11] and confirmed the presence of CUX2 immunosignals in WT and absence in *Cux2*(−/−) mice (Fig. S3A,B). CUX2 immunosignals were dense in the neocortical upper layers (II–IV) as previously reported[1] and also dense in the entorhinal cortex upper layers (II-III) (Fig. S3C). In WT hippocampus, we only observed CUX2 immunolabeling signals in inhibitory interneurons, specifically somatostatin (SST)-positive, reelin (RLN)-positive and parvalbumin (PV)-positive inhibitory, but not in excitatory neurons (Table S2, Fig. S4). We found that there were no significant differences in interneuron cell numbers between genotypes (Fig. S5A–D). Timm staining and immunohistochemistry for c-Fos (Fig. S5E,F), Doublecortin, phospho-Histone H3, Ki67, NeuN, GFAP, and ZnT-3 (data not shown) did not show differences in the hippocampus between genotypes. There are five subtypes of kainate receptors (KARs), GLUK1–GLUK5, in primates and rodents. We investigated KARs expression in the hippocampus of 2-month-old *Cux2*-KO female mice. RT-qPCR assays revealed that the expression of *GluK1* (formerly named *GluR5*) was significantly higher in *Cux2*(−/−) mice (Fig. 2F), which is presumably a homeostatic compensatory reaction to epileptic seizures (see "Discussion"). The baseline frequencies of spontaneous inhibitory postsynaptic currents (sIPSCs) in hippocampal dentate granule cells were significantly higher at 6–7 weeks old, and this difference was suppressed after bath-application of GYKI and AP5, which are AMPA and NMDA receptor antagonists, respectively (Fig. 2G). Frequency of sIPSC in dentate PV-positive interneurons was also increased in *Cux2*-KO mice (Fig. S6). These results suggest that, in *Cux2*(−/−) mice, the function of hippocampal inhibitory neurons remained intact, but the increased excitatory input from the entorhinal cortex to the hippocampus could facilitate firing activities of inhibitory neurons, which itself would also be a compensatory action to epileptic activities in mice. In CA3 pyramidal neurons of *Cux2*-KO mice, EPSCs were not significantly affected (Fig. S6), suggesting that the increased excitatory input in the upstream dentate granule cells may be neutralized by the increased inhibitory input in those cells.

Taken together, these results suggest that the increase in entorhinal cortex layers II–III cell numbers and the resultant facilitation of glutamatergic synaptic transmission from the entorhinal cortex layers II–III onto hippocampi are causal factors leading to the increased susceptibility to kainate of *Cux2*-KO mice. Increases in GLUK1 and facilitated firing of inhibitory neurons in the mouse hippocampus would rather be compensatory reactions.

CASP variants in TLE patients. *CUX1* is a paralog of *CUX2*, and *CASP* is an alternatively spliced short isoform of *CUX1* harboring a unique C-terminus[14] (Fig. 3A, Fig. S7). CUX1 and CUX2 proteins have four DNA binding domains (three CUT repeats and one homeodomain), but CASP lacks all of these domains and instead contains a transmembrane domain. We performed targeted sequencing analyses of *CUX1* and *CASP* in the 271 Japanese patients with epilepsy and identified nine nonsynonymous variants (Fig. 3A, Table 1, and Supplemental Note). Among those, one variant in *CUX1*, c.4172C>T (p.T1391I) and three variants in *CASP*, c.1433C>T (p.A478V), c.1524delG (p.R509fs), and c.1868_1870delTCT (p.F623del), were completely absent or very rare in in-house controls and databases (Table 1). No other truncation variants of the *CASP*-specific sequence were found in these databases, suggesting that *CUX1* and especially *CASP* variants contribute to epilepsy. Although epilepsies observed in patients with *CUX1* and *CASP* variants were rather heterogeneous, CASP-p.G563S and p.F623del variants appeared in mTLE and lTLE patients, respectively (Table 1). The mTLE patient SIZ-060 showed hippocampal sclerosis (Supplemental Note).

CASP and CUX2 proteins are co-expressed in excitatory neurons of entorhinal cortex upper layer and physiologically bind to each other. Immunohistochemistry with CUX1 antibodies in 2-month-old WT mice revealed CUX1 immunosignals in excitatory neurons at the neocortical upper layers (II-IV), as previously reported[3], and those at the entorhinal cortex upper layers (II-III) (Fig. 3B), similar to CUX2 (Fig. S3C). In contrast in the hippocampus, CUX1 was expressed in SST-positive, RLN-positive, and PV-positive interneurons, but not in excitatory neurons (Fig. 3B and Fig. S8), similar to CUX2 (Fig. S4). Using a CASP-specific antibody recognizing 400–650 a.a., we found that CASP was rather widely expressed in neurons of multiple brain regions, but still dense in the neocortical and entorhinal cortex upper layers, similar to CUX1 and CUX2 (Fig. 3C). In the hippocampus, CASP was dense in hilar and stratum-oriens SST-positive cells that expressed CUX2 (Fig. 3C,D), and more specifically, within the cytoplasm (Fig. S9), which is consistent with CASP expression in the Golgi apparatus[15].


A
CUX1
554-640
1129-1215
946-1034
1255-1317
CUT
CUT
CUT
HOX
1
419
NH2
COOH
(1516 a.a.)
p.S1054L
p.A1094V
p.R1272Q
p.T1391I
CASP (CUX1 short isoform with unique c-terminus)
422-643
CASP_C
1
NH2
COOH
(678 a.a.)
p.A464T
p.A478V
p.R509fs
p.P563S
p.F623del
B
CUX1 2-month
CA1
CA3
DG
C
CASP 2-month
CA1
CA3
DG
D
CASP / SST 2-month
d1
d2
d3
d4
d5
h
sg
so
sp
E
WT n=5
Casp(+/-) n=5
Casp(-/-) n=5
Relative mRNA levels
Casp
Cux1
Cux2
F
WT n=7
Casp(-/-) n=7
neocortex
Thickness of CUX1-positive cell layer (μm)
WT n=13
Casp(-/-) n=13
entorhinal cortex
CUX1-positive cells / mm2
G
WT
Casp(+/-)
Casp(-/-)
GS rate (%)
7/18
11/19
12/20
4/20
8/23
13/18
11/38
19/42
25/38
female
male
combined
H
Mortality rate (%)
4/18
9/19
11/20
4/20
6/23
13/18
8/38
15/42
24/38
I
EPSC amplitude (pA)
(n=slices/ animals)
WT n=13/ 5
Casp(-/-) n=12/ 6
Stimulus Intensity (μA)

◂**Figure 3.** *CASP* variants in epileptic patients, CASP distribution, and increases in kainate susceptibility and excitatory input to hippocampal granule cells in *Casp*-KO mice. (**A**) Locations of *CUX1* and *CASP* variants in patients with epilepsy (see Table 1). Dashed lines define the common region. (**B**) CUX1 immunosignals (brown) in neocortical and entorhinal cortex upper layer excitatory neurons and hippocampal interneurons. (**C**) CASP (brown) expressed more widely in neurons, and intensely expressed in neocortical and entorhinal cortex upper layer excitatory neurons. (**D**) In hippocampus, CASP (brown) was dense in SST-positive (blue) interneurons at hilus and stratum oriens (arrows). d2-d5; magnified images outlined in d1. Scale bars = 100 µm (**B**, **C** and d1), 20 µm (d2–d5). *so* stratum oriens, *sp* stratum pyramidale, *sg* stratum granulosum, *h* hilus. (**E**) RT-qPCR analyses revealed that *Casp* mRNA was decreased, while *Cux1* and *Cux2* mRNAs remained unchanged, in *Casp*-KO mice. (**F**) Thickness of the CUX1-positive neocortical layer (left), density of CUX1-positive cell in neocortex (middle), and CUX1-positive cell density in entorhinal cortex (right). CUX1-positive cell density tended increase at neocortex and entorhinal cortex in *Casp*(−/−) mice (2-month-old) but not statistically significant. (**G**,**H**) *Casp*-KO mice showed significantly higher susceptibility to kainate in seizure rate (**G**), mortality (**H**). (**I**) Perforant path-evoked EPSCs in dentate granule cells were significantly increased in *Casp*(−/−) male (6–7-week-old). One-way ANOVA Tukey's test (**E**), Yates' correction after Pearson's Chi-square (**G**,**H**), or two-way ANOVA Tukey's test (**I**). n: mouse numbers. *P < 0.05, **P < 0.01, ***P < 0.001.

A protein interaction between CUX1 and CASP has been previously reported[14]. Here we newly found that the CASP protein physically interacts with CUX2 (Figs. S10 and S11). All three tested CUX2 rare variants bound to CASP, and all three tested CASP rare variants bound to CUX2 (Figs. S10 and S11), suggesting that the variants did not affect protein binding between CASP and CUX2.

Casp-deficient mice also show increased cell number in entorhinal cortex and glutamatergic input to hippocampus. It has been reported that the number of cortical neurons was significantly increased in *Cux1*(−/−); *Cux2*(−/−) double-mutant mice, but this increase was no greater than that in the *Cux2*(−/−) single mutant; therefore, regulation of the upper layer neuronal number was assumed to be a unique function of CUX2 and not redundant with CUX1 activities[3]. Because of the low survivability of *Cux1*-KO mice[16] and our observation that the TLE variants appeared in the *CASP*-unique sequence but not in *CUX1* itself, we decided to investigate *Casp*-specific KO rather than *Cux1*-KO mice for analysis. We generated a *Casp*-specific KO mouse by targeting exon 17 at the unique C-terminus (Fig. S7C). *Casp*(+/−) and *Casp*(−/−) mutant mice were born at a Mendelian ratio, grew normally, and were fertile. RT-qPCR analyses revealed that the *Casp* mRNA became half and diminished in *Casp*(+/−) and *Casp*(−/−) mice, respectively, whereas *Cux1* and *Cux2* mRNA levels remained unchanged (Fig. 3E). CASP immunosignals well disappeared in *Casp*(−/−) mice (Fig. S9A), confirming the specificity of the CASP antibody. At a glance, there were no abnormal localizations and intensities of CUX1 and CUX2 proteins in *Casp*-KO mice (Fig. S9B,C). The median body weight was comparable among genotypes at 2 months of age (Fig. S9D). RT-qPCR assays of KARs mRNA in the hippocampi of 2-month-old *Casp*-KO mice did not show significant change in KARs expression levels (Fig. S9E).

In a Nissl staining of 2-month-old *Casp*-KO mice, no increase of neuron number was observed in the entorhinal cortex (Fig. S9F). However, immunohistochemical staining using the anti-CUX1 antibody as a marker of neurons at upper layers of the neocortical (II–IV) and the entorhinal cortex (II–III) showed a tendency of increase in both the neocortex and entorhinal cortex ($P = 7.57 \times 10^{-2}$ and $P = 4.50 \times 10^{-1}$, respectively) (Fig. 3F). Furthermore, *Casp*-KO mice also showed high susceptibility to kainate (Fig. 3G,H, Fig. S9G–I). After intraperitoneal application of kainate, a larger number of *Casp*-KO mice showed GS and lethality (Fig. 3G,H). Onset latencies of GS and death were significantly decreased in *Casp*-KO mice (Fig. S9H,I). Seizure severity in *Casp*(−/−) mice was also significantly higher (Fig. S9G). The differences in seizure susceptibility to kainate were seen mainly in male *Casp*-KO mice (Fig. S9), contrary to *Cux2*-KO mice in which the susceptibility is higher in female (Fig. S2). Notably again, perforant path-evoked EPSCs (eEPSCs) in the dentate granule cells were significantly higher in *Casp*-KO mice (Fig. 3I), which is similar to *Cux2*-KO mice (Fig. 2E).

All of these observations propose that facilitation of glutamatergic synaptic transmission from the entorhinal cortex onto hippocampal dentate granule cells is a common mechanism for TLE caused by *CUX2* and *CASP* variants.

Discussion

In this study, we performed targeted sequencing analyses of *CUX2, CUX1* and *CASP* on 271 Japanese patients with a variety of epilepsies, and found that *CUX2* missense variants predominantly appear in TLE patients, in that eight of 68 TLE patients (12%) had *CUX2* variants. Three variants (p.R34W, p.P454L, and p.W958R) are quite rare or even absent in various databases, and are consistently predicted to have a damaging effect, therefore these would be regarded as causal or large-effect susceptibility variants. Although the p.E1283K is a high-frequent, relatively common variant, its frequency in TLE patients is statistically higher compared with in-house controls and therefore would potentially be a genetic contributor for TLE. *CASP* variants also appeared in two TLE patients. All of these patients with *CUX* family variants showed symptoms of epileptic seizures, suggesting that the variants may contribute to the threshold for the triggering epileptic seizures through a facilitation of excitatory synaptic transmission from entorhinal cortex to hippocampus in epilepsies caused by *CUX* family variants. Our recent GWAS analysis of Japanese patients with variable epilepsies identified a region with genome-wide significance at chromosome 12q24 which harbors *CUX2*[5]. Although a recurrent de novo *CUX2* variant p.E590K has been described in patients with EEs[6,7], a previous whole exome sequencing study for Japanese patients with EEs[17] did not find the *CUX2* pathogenic variant. Therefore, the *CUX2* recurrent variant in EEs may not be enough to explain the genome-wide association with epilepsy at the 12q24 region in Japanese population. In our GWAS

study[5], sub-analyses for subtypes of epilepsies further revealed that a polymorphic marker at the 12q24 epilepsy-associated region showed genome-wide significant association with structural/metabolic epilepsy. Hippocampal sclerosis is the major entity for structural/metabolic epilepsy, and therefore the *CUX2* variants in patients with TLE would contribute to the association with epilepsy at 12q24 in Japanese population.

Human cell culture and fly dendritic arborization analyses revealed loss-of-function effects of the *CUX2* variants, which were found in TLE patients. *CASP* also showed variants in epilepsy patients including TLE at the unique C-terminus and we further found that the CASP physically binds to CUX2. Although all tested CASP variants did not affect the binding activity to CUX2, the CASP protein has been reported to play a role in intra-Golgi retrograde transport[18] and therefore the variants in CASP may still affect the subcellular transport or protein modification of CUX2.

Cux2- and *Casp*-KO mice did not show spontaneous seizures but showed significantly elevated seizure susceptibility to kainate, an agent which has been used to establish TLE animal models[9]. We previously reported a nonsense mutation of the *KCND2* gene encoding a voltage-gated potassium channel Kv4.2 in a patient with TLE[19]. Similar to *Cux2*- and *Casp*-KO mice, *Kcnd2*-KO mice did not show spontaneous epileptic seizures but showed increased susceptibility to kainate in seizure and mortality[20]. These observations suggest that increased seizure susceptibility to kainate correlates with the threshold for triggering epileptic seizures. However, other additional as-yet unknown modifying, genetic, or environmental factors may influence full expression of TLE symptoms including hippocampal sclerosis.

Cux2-KO mice showed a significant and *Casp*-KO mice showed a tendency of, increases in entorhinal cortex layer II–III excitatory neuron cell number. Although *Casp*-KO mice did not show any significant changes in *Cux2* mRNA expression levels and histological or cytological distributions of CUX2 protein, co-expression of CASP and CUX2 proteins in neurons including entorhinal cortex projection neurons, and the physiological interaction between CASP and CUX2 proteins still suggest that CASP deficiency may impair CUX2 function through an as-yet unknown mechanism, consequently leading to increased entorhinal cortex excitatory neuron cell number in *Casp*-KO mice. Furthermore, both *Cux2*- and *Casp*-KO mice revealed significant increases in perforant path-evoked EPSCs in dentate granule cells. These results suggest that facilitation of glutamatergic synaptic transmission from the entorhinal cortex onto hippocampal dentate granule cells is a causal basis for the significant increase in seizure susceptibilities to kainate in *Cux2*- and *Casp*-KO mice. In contrast, the observed changes in the hippocampus of *Cux2*-KO mice are assumed to be homeostatic compensatory reactions to the epileptic causal changes. In the hippocampus of WT mice, CUX2 immunolabelling was only observed in inhibitory interneurons such as SST-positive, RLN-positive, and PV-positive inhibitory, but not in excitatory neurons. In the *Cux2*-KO mice, although no changes were observed in interneuron cell numbers, a significant increase in sIPSC frequency was observed in dentate granule cells similar to patients with TLE[21], which would presumably be a compensatory reaction[22–24] to the increased excitatory input from the entorhinal cortex to the hippocampus. The increased GLUK1 expression in the hippocampus of *Cux2*-KO mice may also be suppressive for epileptic seizures because *GluK1* is expressed in inhibitory neurons[25], and GLUK1 expression has been assumed to be protective at least for kainate-induced epilepsy[26]. Taken together, the changes within the hippocampus of *Cux2*-KO mice may be homeostatic compensatory responses rather than causal actions to epileptic seizures, and these changes in the hippocampus themselves also support the occurrence of epileptic causal changes in these mice.

In summary, our results of mutation analyses of *CUX* family genes in patients with epilepsies including TLE and the functional and mouse model analyses suggest that *CUX* family gene deficiency is one of the bases for TLE and that increase of cell number in the entorhinal cortex projection neurons and resultant increase of glutamatergic synaptic transmission to hippocampus is a possible pathological mechanism for TLE. Further investigations using mouse models with heterozygous missense variants, which have been identified in TLE patients are required to clarify whether the variants are true loss-of-function mutations and contribute to the TLE pathology.

Data availability

All data generated or analyzed during this study are included in this published article and its Supplementary Information File.

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Author contributions

T.S. and K.Y. designed the experiments; T.S., T.T., G.S., C.D., Y.J.P. and M.R. performed statistical analyses; T.S., I.O., S.H., K.H., M.U., Y.T., M.M., S.F., H.Os., H.Og., M.O., A.I., S.H., S.K., Y.I. and K.Y. managed DNA samples; T.S., I.O., S.H., K.H., M.U., Y.T., M.M., S.F., H.Os., H.Og., M.O., A.I., S.H., S.K., Y.I. and K.Y. recruited case and control samples; T.S. and K.Y. performed targeted sequencing analyses; T.S., C.D., Y.J.P., A.W.M., and K.Y. performed functional analyses; T.S., G.S., T.T., H.M., A.S., and K.Y. performed mouse analyses; and T.S., R.M. and K.Y. wrote the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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