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CACNA1A variants contribute to severity of seizures in Dravet syndrome

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Abstract

Dravet syndrome is an intractable epileptic syndrome beginning in the first year of life. *De novo* mutations of *SCN1A*, which encode the $Na_v1.1$ neuronal voltage-gated sodium channel, are considered a major cause of Dravet syndrome. We investigated genetic modifiers of this syndrome.

We performed a mutational analysis of all coding exons of *CACNA1A* in 48 patients with Dravet syndrome. To assess the effects of *CACNA1A* variants on *SCN1A* mutations, we compared clinical features in two genotype groups; patients harboring *SCN1A* mutations but no *CACNA1A* variants (n = 20), and patients with *SCN1A* mutations plus *CACNA1A* variants (n = 20). *CACNA1A* variants which were detected in the patients were studied using heterologous expression of recombinant human $Ca_v2.1$ in HEK 293 cells and whole-cell patch-clamp recording.

Nine variants including six novel variants were detected in 21 (43.8%) of 48 patients. One double heterozygous variant, R1126H + R2201Q, was significantly more frequent in patients with Dravet syndrome than healthy individuals. The patients harboring *SCN1*A mutations and *CACNA1A* variants had an earlier onset of seizures and more frequent prolonged-seizures before 1-year-of-age than the patients with only *SCN1*A mutations. The electrophysiological properties of four of the five novel $Ca_v2.1$ channel

variants exhibited biophysical changes consistent with gain-of-function. We conclude that $Ca_v2.1$ channel variants occur in some Dravet syndrome patients and could be potential genetic modifiers.

Introduction

Dravet syndrome (or severe myoclonic epilepsy in infancy, SMEI, MIM# 607208) is an intractable epileptic syndrome characterized by various types of seizures beginning in the first year of life with prolonged seizures which are often provoked by fever (Dravet *et al*., 2005). *De novo* mutations of *SCN1A*, which encodes the $Na_v1.1$ neuronal voltage-gated sodium channel are detected in approximately 70-80% of the patients with Dravet syndrome, therefore, *SCN1A* mutations have been considered to be a major cause of this syndrome (Claes *et al*., 2001; Ohmori *et al*., 2002; Depienne *et al*., 2009). However, several studies have suggested that environmental factors and genetic modifiers could influence the clinical phenotype of Dravet syndrome. The patients with the same mutation of *SCN1A* often show different severities of epilepsy (Depienne *et al*., 2010; Suls *et al*., 2010; Guerrini *et al*., 2010), and approximately 50% of the patients with Dravet syndrome have a family history of convulsive disorders including febrile seizures and benign epilepsy (Dravet *et al*., 2005; Hattori *et al*., 2008). Moreover, the severity of epilepsy in a SMEI mouse model harboring a truncated *SCN1A* mutation is influenced by its genetic background (Yu *et al*., 2006). The 129/SvJ mouse strain exhibited a decreased incidence of spontaneous seizures and a longer survival in

comparison to the C57BL/6J mice. Therefore, genetic background affects the *Scn1a* KO mouse phenotype.

Supporting evidence for a multifactorial etiology of Dravet syndrome has been reported (Singh *et al*., 2009). Singh *et al.* demonstrated that the patients with *SCN9A* variants develop febrile seizures, and 6 of 109 patients with Dravet syndrome had missense variants of the *SCN9A*, in addition to *de novo SCN1A* mutations. These *SCN9A* missense variants possibly contribute to Dravet syndrome in a multifactorial fashion.

A missense mutation of the *CACNB4*, which encodes the β4 subunit of the voltage-dependent calcium channel, has been detected in a patient with Dravet syndrome (Ohmori *et al*., 2008). This patient had a *de novo SCN1A* nonsense mutation and a *CACNB4* missense mutation (R468Q), which was inherited from his father with a history of a single febrile seizure. A CACNB4-R468Q electrophysiological study using a heterologous expression system revealed increased $Ca_v2.1$ (P/Q type) voltage-gated calcium channel -current density. A *CACNA1A* mutational analysis was conducted to further assess the role of Ca_v 2.1 in the patients with Dravet syndrome, because Ca_v β4 is the predominant subunit associated with Ca_v 2.1 (Dolphin, 2003).

In the present study, we detected nine variants including six novel variants of

CACNA1A in the 48 patients. This is the first report of an association between *CACNA1A* variants and Dravet syndrome.

Materials and Methods

Clinical samples

A total of 48 patients with Dravet syndrome including typical and borderline cases were analyzed for this study. 46 of which had been recruited for our previous studies (Ohmori *et al*., 2002; Hattori *et al*., 2008). All patients were screened for *SCN1A* mutations by direct sequencing of all coding exons and a multiplex ligation dependent probe amplification (MLPA) using SALSA MPLA P137 SCN1A reagent (MRC-Holland, Amsterdam, The Netherlands). Forty of the 48 patients (83.3%) had various types of mutations (**Supplementary Table 1)**. A hundred and ninety control subjects were randomly selected from healthy Japanese volunteers.

Mutational analysis of CACNA1A

Genomic DNA was extracted from peripheral blood by SDS/proteinase K treatment. All coding exons of *CACNA1A* were analyzed by direct sequencing with primers designed outside the exons. The sequences of each sample were compared with the GenBank data

base (accession number: NM_023035). A statistical analysis was performed using either the Chi-square test or Fisher's exact two-tailed test.

The study was approved by the Ethics Committee of Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences. Written informed consent was obtained from the patients' parents and all healthy participants.

Genotype and phenotype correlation

The patients were divided into four genotypic groups; a *SCN1A* mutation plus a *CACNA1A* variant (n = 20), *SCN1A* mutation but no *CACNA1A* variant (n = 20), a *CACNA1A* variant but no SCN1A mutation (n = 2), and no *SCN1A* mutation and no *CACNA1A* variant (n = 6). The first group included a previously reported patient who had SCN1A-R568X and CACNB4-R468Q, which led to increased $Ca_v2.1$ current density (Ohmori *et al*., 2008). To assess the effects of the variant $Ca_v2.1$channels on mutant $Na_v1.1$ channels, we compared clinical features between the two genotype groups, namely between the patients harboring *SCN1A* mutations but no *CACNA1A* variants (n = 20), and the patients with *SCN1A* mutations and *CACNA1A* variants (n = 20). The clinical features of the Dravet syndrome change with an increase in age, therefore, the genotype-phenotype correlation should be evaluated at the same age in all

subjects. The patients' age ranged from 4 to 43 years. Symptoms before 1 year of age, including seizure onset, total number of seizures, total number of prolonged seizures lasting for more than 10 min, and type of seizures were assessed. Clinical data were collected based on an exhaustive review of the medical records of a previous study (Hattori *et al*., 2008).

Mutagenesis and heterologous expression of human CACNA1A

Full-length human *CACNA1A* ($Ca_v2.1$) cDNA in pcDNA1.1 and rabbit α2δ subunit cDNA expression vector, pKCRα2δ, were kindly provided by Prof. T. Tanabe (Tokyo Medical and Dental University, Tokyo, Japan). G266S, R1126H, R2201Q, DQER 2202-2205 deletion, and double variant R1126H + R2201Q were introduced into *CACNA1A* cDNA in pM014X by PCR-based mutagenesis. The entire open reading frame of all cDNAs was confirmed by sequencing before use in the experiments. The *CACNA1A* cDNA contained 5' and 3' untranslated regions, thus the coding region from the first methionine to the stop codon was amplified by PCR and cloned into pMO14X mammalian expression vector. A minor-type variant of one single nucleotide polymorphism (SNP), serine (agc) at 1108 codon (rs16027), was found for sequence confirmation. Therefore, 2.75kb of *Eco*RI-*Eco*RI fragment on the cDNA vector was

substituted with same sized-DNA fragment containing the major-type glycine (ggc) at 1108 codon to produce normal CACNA1A cDNA vector.

Human *CACNB4* cDNA was amplified by PCR with a human brain cDNA library. The cDNA was confirmed by DNA sequencing, and subcloned into pIRES2-EGFP.

CACNA1A was coexpressed heterologously with accessory β4 and α2δ subunits in HEK293 cells by transient plasmid transfection using Qiagen Superfect transfection reagent (Qiagen). Approximately 3.8 μg of total DNA was transfected (plasmid mass ratio was α1: α2δ: β4= 2:1:0.8). The cells were used for the electrophysiological analysis 72 hours after transfection.

Electrophysiological study of CACNA1A

The currents from HEK293 cells were recorded with the whole-cell patch-clamp technique by using the Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA). Patch pipettes were fabricated from borosilicate glass (Warner Instrument Co., Hamden, CT, USA). Pipette resistance ranged from 2 to 3 MΩ. As a reference electrode, a 2% agar bridge with a composition similar to the bath solution was used. The series resistance was electronically compensated to >50%. All illustrated and analyzed currents were corrected for remaining capacitance and leakage currents using a –*P*/4

procedure. The pipette solution for recordings of the whole-cell currents contained 110 mM CsOH, 20 mM CsCl, 5 mM $MgCl_2$, 10 mM EGTA, 5 mM MgATP, 5 mM creatine-phosphate and 10 mM HEPES; adjusted to pH 7.35 with aspartic acid and an osmolarity of 310 mOsmol/kg. The bath solution contained 5 mM $BaCl_2$, 150 mM TEA-Cl, 10 mM glucose, and 10 mM HEPES; adjusted to pH 7.4 with TEA-OH and osmolarity of 310 mOsmol/kg. Data were sampled at 20 kHz and filtered at 5 kHz.

Voltage-dependence of activation and inactivation curves were fitted with Boltzmann functions to determine the voltages for half-maximal activation and inactivation ($V1/2$) and slope factor (k). Time constants for activation were obtained from monoexponential fits to the raw current data. Channel inactivation was evaluated by fitting the decay phase of the whole-cell current with the two-exponential function, $I/Imax = Af \times \exp(-t/\tau f) + As \times \exp(-t/\tau s) + C$, where τf and τs denote time constants (fast and slow components, respectively), A represents a fractional amplitude, and C is the level of noninactivating current.

Statistical Analysis for electrophysiological study

All electrophysiological data are presented as the mean ± SEM, and statistical comparisons were made in reference to the wild type by using unpaired Student's *t*-test.

The threshold *p* value for statistical significance was 0.05. Data analysis was performed by using the Clampfit 8.2 (Axon Instruments, Union City, CA, USA) and OriginPro 7.0 (OriginLab, Northampton, MA, USA) software packages.

Results

CACNA1A mutational analysis

Results of the *CACNA1A* genetic analysis in 48 patients with Dravet syndrome and the frequency of the variants in 190 healthy participants are summarized in **Table 1**. Nine variants were detected in 21 patients (43.8%). Three (p.E921D, p.E996V, and p.G1108S) of the 9 variants were previously reported (dbSNP: rsl6022, rsl6023, and rsl6017, respectively), whereas the remaining 6 (p.G266S, p.K472R, p.A924G, p.R1126H, p.R2201Q and p.DQER2202-2205 deletion) were novel. Electropherograms of the *CACNA1A* DNA sequence for the nine variants are shown in Fig. 1A.

Variant p.G266S was not identified in any of the 188 healthy participants (376 chromosomes). A combination of p.R1126H and p.R2201Q was found in four patients with Dravet syndrome, but this combination was not observed in any of the control individuals, suggesting that the double variant is strongly associated with Dravet

syndrome (p = 0.0015). No statistical differences were observed for the p.K472R, p.E921D, p.A924G, p.E996V, p.G1108S, and p.DQER2202-2205 deletion. The silent mutations identified in the patients are described in Supplementary Table 2.

A mutational analysis was conducted in the parents of the children with the *SCN1A* and *CACNA1A* mutations (Table 2). As far as we examined, *SCN1A* mutations were *de novo* in each case, whereas the *CACNA1A* variants were inherited from the parents. The p.R1126H + p.R2201Q and p.E921D + p.E996V combinations were detected in one parent, so both the double variants probably exist on one allele. Parents with *CACNA1A* variants suffered no neurological symptoms and had no history of neurological diseases, except the mother of ID #02-20 who had a history of several febrile seizures.

Localization of the CACNA1A variants

Variant p.G266S altered a residue within the S5-S6 pore loop of domain 1, whereas all other variants were located in an intracellular loop. Notably, most of the variants were located in the domain 2-3 loop (Fig 1B). A comparison of the amino acid sequences of the variants among various mammals is shown in Supplementary Fig. 1. All variants except p.E921D and p.A924G are conserved in mammals.

Genotype and Phenotype correlation

To assess the effects of the *CACNA1A* variants on *SCN1A* mutations, phenotypic differences between the two groups, absence (n = 20) or presence (n = 20) of *CACNA1A* variants in the patients with *SCN1A* mutations were compared (Table 3). No significant differences were observed for the total number of seizures, or type of seizures before one year of age. However, the patients with both *SCN1A* mutations and *CACNA1A* variants showed an earlier onset of seizures and significantly more frequent prolonged seizures lasting for more than 10 minutes than those who had only *SCN1A* mutations.

Biophysical properties of the novel CACNA1A variants

Variants of *CACNA1A* channels were studied under identical conditions using heterologous expression of recombinant human $Ca_v2.1$ with the β4 and α2δ accessory subunits in HEK 293 cells and whole-cell patch-clamp recording. We chose the novel variants at the conserved amino acid positions (p.G266S, p.R1126H, p.R2201Q, p.DQER2202-2205 deletion, and p.R1126H + p.R2201Q) for the electrophysiological study.

Fig. 2A illustrates representative whole-cell currents evoked by a series of

depolarizing test potentials in cells expressing either WT- $Ca_v2.1$ or each of the five variant channels. The current-voltage relationships (Fig 2B) indicated that two of the five mutant channels, DQER2202-2205 deletion and the double variant R1126H + R2201Q had a significantly greater current density at voltages from -10 and to +10mV and from -10 to +60 mV than that of WT- $Ca_v2.1$. The peak current amplitudes and peak current densities exhibited by cells expressing DQER2202-2205 deletion or the R1126H + R2201Q double variant were significantly greater than those of WT- $Ca_v2.1$ (Fig 2C).

The voltage dependence of activation and inactivation were also examined (Fig. 3). Activation was significantly shifted toward more hyperpolarizing potentials in cells expressing the G266S, R1126H, and DQER2202-2205 deletion in comparison to WT-$Ca_v2.1$, whereas the other two variants activated with the same voltage dependence as the WT channels **(**Fig 3A and Table 4). Both R1126H and DQER2202-2205 deletion had statistically increased voltage sensitivity, as suggested by a comparison of their slope factors (*k*) with those of WT (Table 4). These findings indicate that the G266S, R1126H and DQER2202-2205 deletion channels will require a lower degree of membrane depolarization to activate, and this may contribute to neuronal hyperexcitability. Furthermore, activation time constants were obtained from

single-exponential fits of the activation phase between 0 mV and +60mV (Fig 3B). G266S showed a significant decrease in the time constant for activation at 20 mV in comparison to the WT (Fig 3C), thus suggesting that this variant may conduct a greater inward current during brief membrane depolarization.

The voltage dependence of inactivation was explored by measuring the channel availability at +20 mV after a depolarizing 2-s pre-pulse to various test potentials. Fig 3D and 3E illustrate the voltage dependence of inactivation and the time constants for inactivation at 0 mV by fitting with a two-exponential function, respectively. No statistically significant differences were observed between WT and the variants. Predicted influence of biophysical properties of these variants and novel variants on $Ca_v2.1$ channel activity is summarized in Table 5.

Discussion

$Ca_v2.1$ (P/Q-type) calcium channels play a role in controlling synaptic transmission at presynaptic nerve terminals in the mammalian central nervous system. We conducted a mutational analysis of all coding exons of *CACNA1A* in 48 patients with Dravet syndrome. Nine variants, including six novel variants causing amino acid changes were identified in 21 patients with Dravet syndrome (43.8%). Based on the incidence of

variants in healthy controls, the incidence of double variant p.E921D+p.E996V, p.A924G, and p.G1108S seemed to be common polymorphisms, whereas the p.K472R, p.R2201Q, and p.DQER2202-2205 deletion seemed to be relatively rare polymorphisms. It was noteworthy that all patients with p.R1126H also had p.R2201Q, whereas none of the controls had double variants (p = 0.0015). None of the parents of the patients with Dravet syndrome had a history of neurological disorders, except one mother with febrile seizures. The effect of a single *CACNA1A* variant alone seems to be insufficient to account for the neurological symptoms.

To assess the effects of the *CACNA1A* variants on *SCN1A* mutations, we compared the clinical features of the patients harboring *SCN1A* mutations but no *CACNA1A* variants with the patients harboring *SCN1A* mutations and *CACNA1A* variants. The patients with *SCN1A* mutations and *CACNA1A* variants had a significantly earlier onset of seizures and more frequent prolonged-seizures before one year of age. This is the first study reporting evidence that *CACNA1A* variants aggravate epileptic seizures in Dravet syndrome patients with *SCN1A* mutations.

CACNA1A mutations have been linked to familial hemiplegic migraine type 1 (FHM1) (Ophoff *et al*., 1996; Ducros *et al*., 2001; Pietrobon, 2010), episodic ataxia type 2 (EA2) (Ophoff *et al*., 1996; Guida *et al*., 2001), spinocerebellar ataxia type 6

(Zhuchenko *et al*., 1997; Pulst *et al*., 2005), and a combination of epilepsy and ataxia (Jouvenceau *et al*., 2001; Rajakulendran *et al*., 2010). The majority of EA2-related *CACNA1A* mutations exhibit either a complete or almost complete loss-of-function (Pietrobon, 2010), whereas the FHM1-related *Cacna1a* mutation shows a gain-of-function effect in synaptic transmission (van den Maagdenberg *et al*., 2004; Tottene *et al*., 2009; van den Maagdenberg *et al*., 2010). In the present study, the electrophysiological properties of five novel variants were determined. Four of them exhibited a predicted gain-of-function, whereas R2201Q did not exhibit any significant difference in the parameters when compared to the WT. Interestingly, the double variant p.R1126H + p.R2201Q revealed a marked increase in current density, whereas that of the other variants were not different from the WT.

The majority of the variants found in the patients with Dravet syndrome were localized in the intracellular loop of the α1 subunit. The voltage-dependent Ca^{2+} channel function can be modified by interacting with the appropriate neuronal protein, such as the β subunit (Pragnell *et al*., 1994; Walker *et al*., 1998), synaptic protein (Rettig et al., 1996; Kim and Catterall, 1997), calmodulin (Lee et al., 1999; DeMaria et al., 2001), and G proteins (Herlitze et al., 1996) on the intracellular loop of the α1 subunit. These proteins regulate the biophysical properties of the Ca^{2+} channel and

neurotransmitter release (Catterall and Few, 2008). The double variant p.E921D + p.E996V was located at the synprint site, where is an interaction site between presynaptic proteins, including soluble *N*-ethylmaleimide sensitive actor attachment receptor (SNARE) proteins (syntaxin 1A, and synaptosome-associated protein of 25 kD (SNAP-25)). Syntaxin 1A and SNAP25 regulate $Ca_v2.1$ channels (Catterall and Few, 2008), so the double variant p.E921D + p.E996V may consequently alter the interaction with the SNARE complex and synaptic transmission. The electrophysiological properties that were demonstrated in non-neuronal cells may not be equal to those in neuronal cells. We could not determine the precise functional interaction between $Ca_v2.1$ channel and $Na_v1.1$ channel in this study. Animal models harboring double mutant channels will be helpful to elucidate the effects of $Ca_v2.1$ channel dysfunction on loss-of-function $Na_v1.1$ channels. The genetic interactions of two different mutant channels, which are linked to epilepsy and neurological disease, have been investigated by mating mutant mice (Kearney *et al*., 2006; Martin *et al*., 2007; Glasscock *et al*., 2007). Interestingly, the phenotype caused by the mutation of one channel could be altered by a second mutant channel, and is probably more complicated.

In conclusion, nine variants including six novel *CACNA1A* variants were detected in 21 of 48 (43.8%) patients with Dravet syndrome. As far as we examined, the variants

were inherited from one parent. Most of the *CACNA1A* variants were located in $Ca_v2.1$ channel intracellular loops. The majority of parents with the same variant $Ca_v2.1$ channel were asymptomatic, therefore, the effect of each variant $Ca_v2.1$ channel alone seem insufficient to account for the seizure phenotypes. However, the patients with combinations of *CACNA1A* variants and *SCN1A* mutations showed aggravated seizure phenotypes compared to the patients with only *SCN1A* mutations. The electrophysiological properties of novel variants of $Ca_v2.1$ channels exhibited predicted gain-of-function. Variants of $Ca_v2.1$ channels are potential genetic modifiers in the patients with Dravet syndrome.

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References

Catterall WA and Few AP, 2008. Calcium channel regulation and presynaptic plasticity. Neuron 59: 882-901.

Claes L, et al., 2001. De novo mutations in the sodium-channel gene SCN1A cause severe myoclonic epilepsy of infancy. Am J Hum Genet. 68: 1327-1332.

DeMaria CD, et al., 2001 Calmodulin bifurcates the local Ca2+ signal that modulates P/Q-type Ca2+ channels. Nature. 411: 484-489.

Depienne C, et al., 2009. Spectrum of SCN1A gene mutations associated with Dravet syndrome: analysis of 333 patients. J Med Genet. 46: 183-191.

Depienne C, et al., 2010. Mechanisms for variable expressivity of inherited SCN1A mutations causing Dravet syndrome. J Med Genet. 47: 404-410.

Dolphin AC, 2003. Beta subunits of voltage-gated calcium channels. J Bioenerg Biomembr 35: 599-620.

Dravet C,et al., 2005. Severe Myoclonic Epilepsy in Infants. Epileptic Syndromes in Infancy, Childhood and Adolescence, 4th ed. John Libbey, London. pp. P89–P113.

Ducros A, et al., 2001. The clinical spectrum of familial hemiplegic migraine associated with mutations in a neuronal calcium channel. N Engl J Med 345: 17-24.

Glasscock E, et al., 2007. Masking epilepsy by combining two epilepsy genes. Nat Neurosci 10:1554-1558.

Guerrini R, et al., 2010. Variable epilepsy phenotypes associated with a familial intragenic deletion of the SCN1A gene. Epilepsia 51: 2474-2477.

Guida S, et al. 2001. Complete loss of P/Q calcium channel activity caused by a CACNA1A missense mutation carried by patients with episodic ataxia type 2. Am J Hum Genet 68: 759-764.

Hattori J, et al., 2008. A screening test for the prediction of Dravet syndrome before one year of age. Epilepsia 49:626-633.

Herlitze S, et al., 1996. Modulation of Ca2+ channels by G-protein beta gamma subunits. Nature. 380: 258-262.

Jouvenceau A, et al., 2001. Human epilepsy associated with dysfunction of the brain P/Q-type calcium channel. Lancet 358:801-807.

Kearney JA, et al., 2006. Severe epilepsy resulting from genetic interaction between Scn2a and Kcnq2. Hum Mol Genet 15: 1043-1048.

Kim DK and Catterall WA. 1997. Ca2+-dependent and -independent interactions of the isoforms of the alpha1A subunit of brain Ca2+ channels with presynaptic SNARE proteins. Proc Natl Acad Sci U S A. 94:14782-14786.

Lee A, et al., 1999. Ca2+/calmodulin binds to and modulates P/Q-type calcium channels. Nature. 399: 155-159.

Martin MS, et al., 2007 . The voltage-gated sodium channel Scn8a is a genetic modifier of severe myoclonic epilepsy of infancy. Hum Mol Genet 16: 2892-2899.

Ohmori I, et al., 2008. A CACNB4 mutation shows that altered Ca(v)2.1 function may be a genetic modifier of severe myoclonic epilepsy in infancy. Neurobiol Dis 32: 349-354.

Ophoff RA, et al., 1996. Familial hemiplegic migraine and episodic ataxia type-2 are caused by mutations in the Ca^{2+} channel gene CACNL1A4. Cell 87: 543-552.

Pietrobon D. 2010. CaV2.1 channelopathies. Pflugers Arch 460: 375-393.

Pragnell M, et al., 1994. Calcium channel beta-subunit binds to a conserved motif in the I-II cytoplasmic linker of the alpha 1-subunit. Nature 368: 67-70.

Pulst SM, et al., 2005. Spinocerebellar ataxia type 2: polyQ repeat variation in the CACNA1A calcium channel modifies age of onset. Brain 128: 2297-2303.

Rajakulendran S, et al., 2010. Genetic and functional characterisation of the P/Q calcium channel in episodic ataxia with epilepsy. J Physiol 588:1905-1913.

Rettig J, et al., 1996. Isoform-specific interaction of the alpha1A subunits of brain Ca2+ channels with the presynaptic proteins syntaxin and SNAP-25. Proc Natl Acad Sci U S A. 1996; 93: 7363-7368

Singh NA, et al., 2009. A role of SCN9A in human epilepsies, as a cause of febrile seizures and as a potential modifier of Dravet syndrome. PLoS Genet 5:e1000649.

Suls A, et al., 2010. Four generations of epilepsy caused by an inherited microdeletion of the SCN1A gene. Neurology 75: 72-76.

Tottene A, et al., 2009. Enhanced excitatory transmission at cortical synapses as the basis for facilitated spreading depression in Ca(v)2.1 knockin migraine mice. Neuron 61: 762-773.

Yu FH, et al., 2006. Reduced sodium current in GABAergic interneurons in a mouse model of severe myoclonic epilepsy in infancy. Nat Neurosci 9: 1142-1149.

van den Maagdenberg et al., 2004. A Cacna1a knockin migraine mouse model with increased susceptibility to cortical spreading depression. Neuron 41: 701-710.

van den Maagdenberg AM, et al., 2010. High cortical spreading depression susceptibility and migraine-associated symptoms in Ca(v)2.1 S218L mice. Ann Neurol 67: 85-98.

Walker D, et al., 1998. A beta 4 isoform-specific interaction site in the carboxyl-terminal region of the voltage-dependent Ca2+ channel alpha 1A subunit. J Biol Chem. 273: 2361-2367.

Zhuchenko O, et al., 1997. Autosomal dominant cerebellar ataxia (SCA6) associated with small polyglutamine expansions in the alpha 1A-voltage-dependent calcium channel. Nat Genet 15:62-69.

Figure legends

Figure 1: Mutations of the *CACNA1A* gene in patients with SMEI. (A) Electropherogram of *CACNA1A* gene DNA sequence from the patients. (B) A schematic diagram illustrating the transmembrane topology of the voltage-gated calcium channel and location of variants identified in this study.

Figure 2: Comparison of Cav2.1 currents recorded in HEK293 cells expressing WT-CACNA1A and mutant channels. (A) Representative WT and mutant whole-cell Ba^{2+} currents. Whole-cell currents recorded from HEK293 cells transiently expressing the indicated alleles during voltage steps to various potentials between −40 to +60 mV with 10-mV increments from a holding potential of −100 mV. Vertical and horizontal scale bars represent 0.4 nA and 10 ms, respectively. (B) The current–voltage relationships of whole-cell Ba^{2+} currents from transiently transfected HEK293 cells. The currents were elicited by test pulses to various potentials (B, inset) and normalized to cell capacitance (WT-CACNA1A, *n*=16; G266S, *n*=11; R1126H, *n*=10, R2201Q, *n*=8; Deletion2202-2205, *n*=8; R1126H+R2201Q, *n*=10). The current density of deletion2202-2205 and double mutation R1126H + R2201Q are significantly larger than WT between -10 and +10mV and -20 and +60 mV, respectively (*p<0.05). (C)

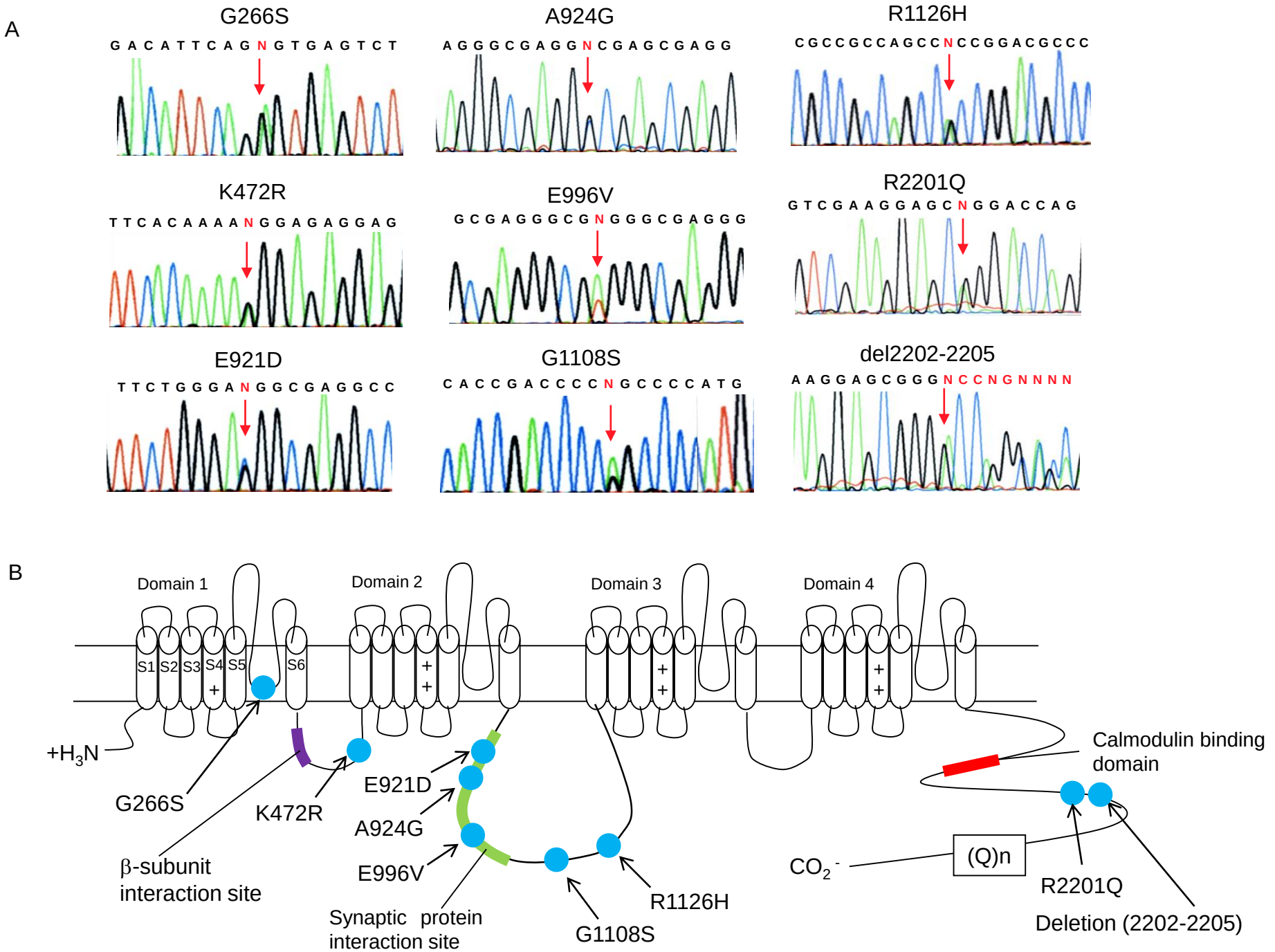
Distribution of the peak current amplitude (left), cell capacitance (middle), and current density (right) at 10 mV. *p<0.05 and **p<0.01 versus WT-CACNA1A.

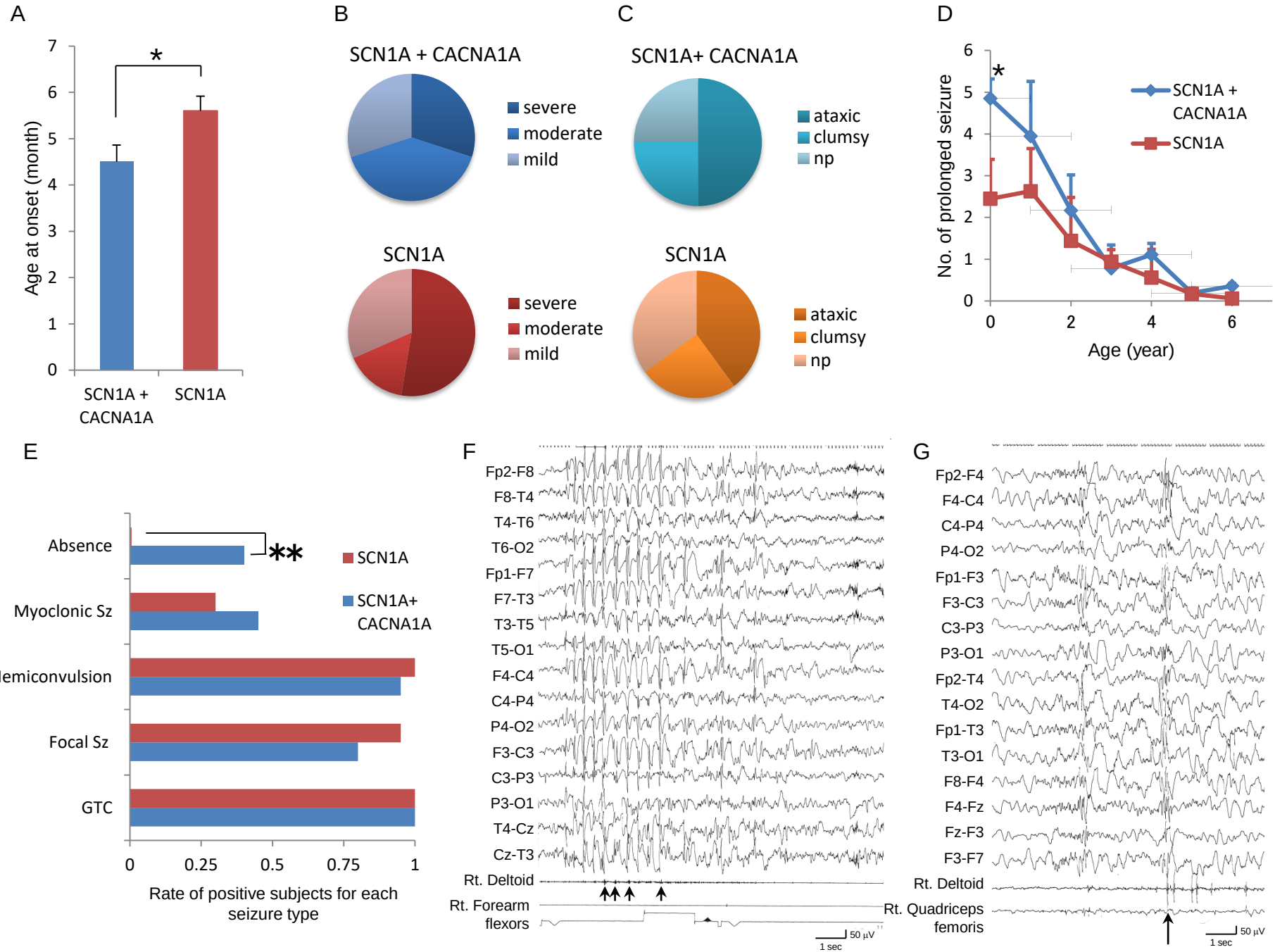
Figure 3: (A) Voltage dependence of activation. The voltage dependence of channel activation was estimated by measuring peak Ba^{2+} current during a variable test potential from holding potential of -100 mV. The current of each membrane potential was normalized to the maximum Ba^{2+} conductance. (B) Voltage dependence of activation time constants for WT-CACNA1A and mutants. The activation time constants were obtained from single-exponential fits to raw current traces at test potentials from 0 mV to 60 mV. (C) The activation time constants at +20 mV for WT and mutants. G266S showed a statistically significant decrease in activation time constant. (D) Voltage dependence of inactivation. The two-pulse protocol illustrated by the inset was used to examine the channel availability after conditioning at various potentials. The currents were normalized to the peak current amplitude. (WT-CACNA1A, n=10; G266S, n=10; R1126H, n=8, R2201Q, n=10; Deletion2202-2205, n=9; R1126H+R2201Q, n=10). (E) Ba^{2+} currents were evoked by 2-s test pulse. The current decay was fitted by two exponential functions. The mean inactivation time constants, τ_{fast} (left) and τ_{slow} (right), were plotted as a function of test potential at 0 mV. The data are expressed as the mean

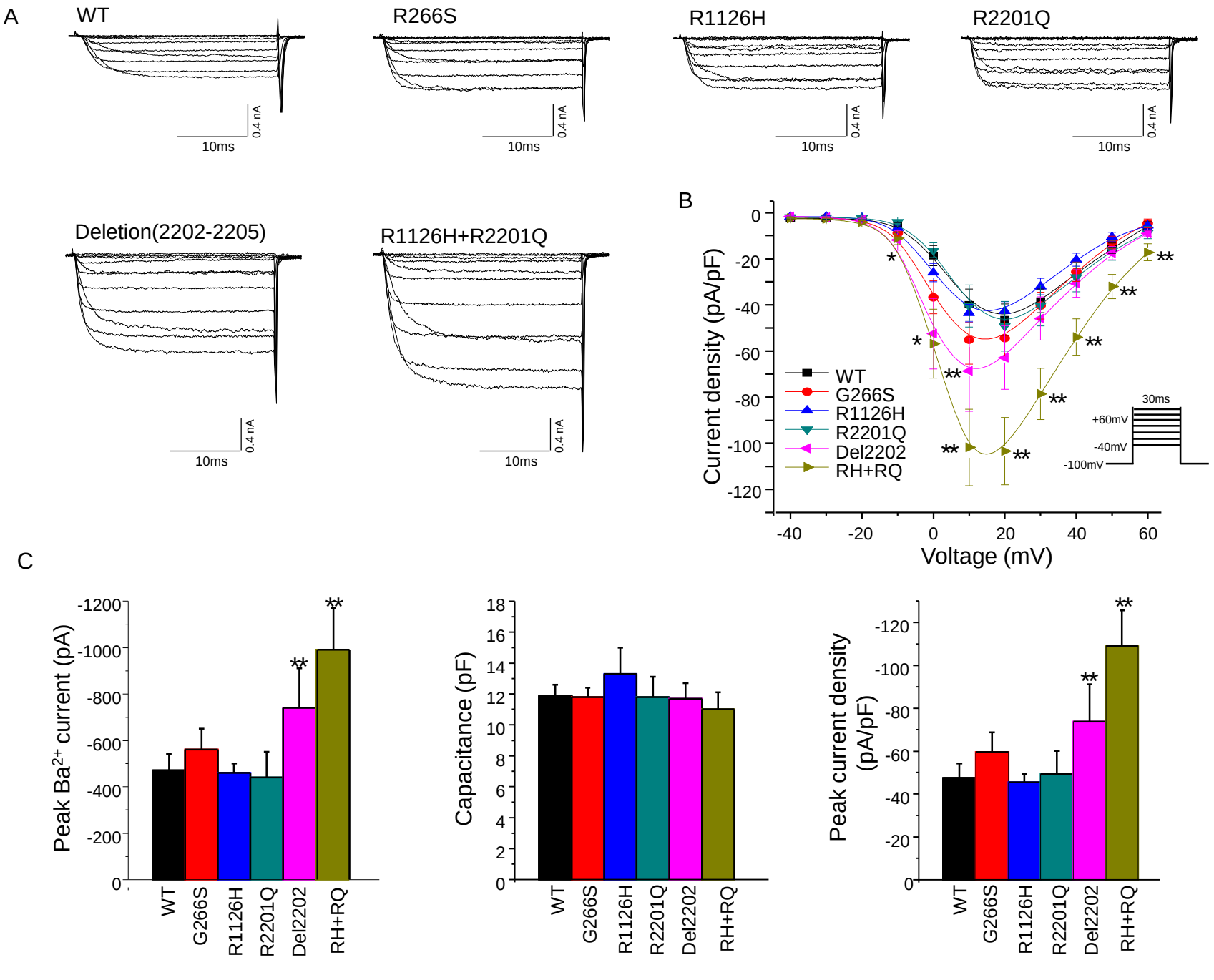
±SEM. *p<0.05 versus WT-CACNA1A.

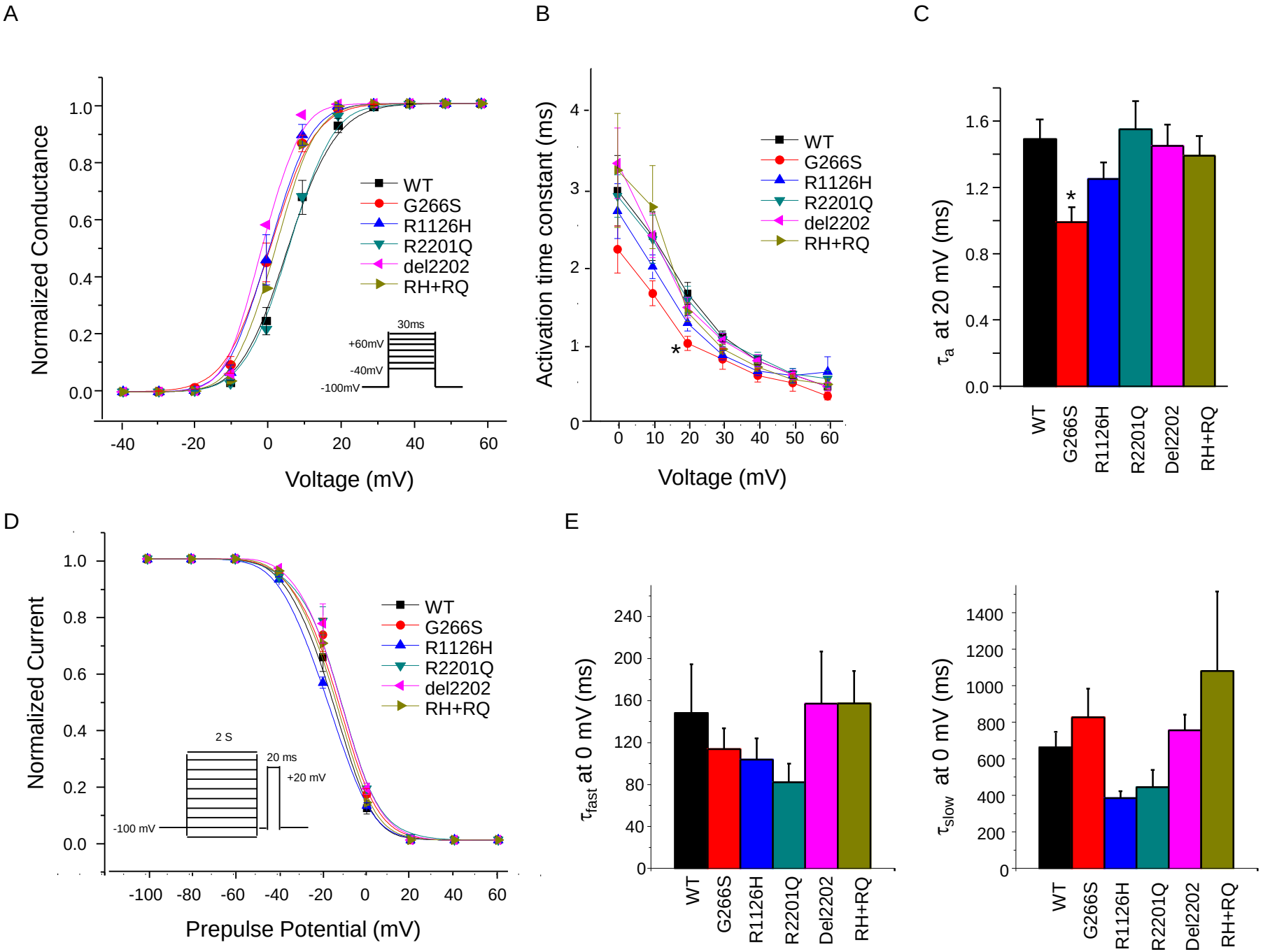
Suppl. Fig. 1 legends

Alignment of amino acid sequences in Cav2.1 calcium channel gene. The arrows indicate heterozygous variant sites. GenBank Accession Nos., from top to bottom, are NM_023035, NP_001068597, NM_001101693, NP_037050, and NP_031604.


A
G266S
G A C A T T C A G N G T G A G T C T
A924G
A G G G C G A G G N C G A G C G A G G
R1126H
C G C C G C C A G C C N C C G G A C G C C C
K472R
T T C A C A A A A N G G A G A G G A G
E996V
G C G A G G G C G N G G G C G A G G G
R2201Q
G T C G A A G G A G C N G G A C C A G
E921D
T T C T G G G A N G G C G A G G C C
G1108S
C A C C G A C C C C N G C C C C A T G
del2202-2205
A A G G A G C G G G N C C N G N N N N
B
Domain 1
Domain 2
Domain 3
Domain 4
S1
S2
S3
S4
S5
S6
+H3N
G266S
K472R
β-subunit
interaction site
E921D
A924G
E996V
Synaptic protein
interaction site
G1108S
R1126H
Calmodulin binding
domain
CO2 -
(Q)n
R2201Q
Deletion (2202-2205)


A
Age at onset (month)
SCN1A + CACNA1A
SCN1A
B
SCN1A + CACNA1A
severe
moderate
mild
SCN1A
severe
moderate
mild
C
SCN1A+ CACNA1A
ataxic
clumsy
np
SCN1A
ataxic
clumsy
np
D
No. of prolonged seizure
Age (year)
SCN1A + CACNA1A
SCN1A
E
Absence
Myoclonic Sz
Hemiconvulsion
Focal Sz
GTC
SCN1A
SCN1A+ CACNA1A
Rate of positive subjects for each seizure type
F
Fp2-F8
F8-T4
T4-T6
T6-O2
Fp1-F7
F7-T3
T3-T5
T5-O1
F4-C4
C4-P4
P4-O2
F3-C3
C3-P3
P3-O1
T4-Cz
Cz-T3
Rt. Deltoid
Rt. Forearm flexors
50 μV
1 sec
G
Fp2-F4
F4-C4
C4-P4
P4-O2
Fp1-F3
F3-C3
C3-P3
P3-O1
Fp2-T4
T4-O2
Fp1-T3
T3-O1
F8-F4
F4-Fz
Fz-F3
F3-F7
Rt. Deltoid
Rt. Quadriceps femoris
50 μV
1 sec


A
WT
R266S
R1126H
R2201Q
Deletion(2202-2205)
R1126H+R2201Q
0.4 nA
10ms
B
Current density (pA/pF)
Voltage (mV)
WT
G266S
R1126H
R2201Q
Del2202
RH+RQ
30ms
+60mV
-40mV
-100mV
C
Peak Ba2+ current (pA)
Capacitance (pF)
Peak current density (pA/pF)


A
Normalized Conductance
Voltage (mV)
WT
G266S
R1126H
R2201Q
del2202
RH+RQ
30ms
+60mV
-40mV
-100mV
B
Activation time constant (ms)
Voltage (mV)
C
τa at 20 mV (ms)
WT
G266S
R1126H
R2201Q
Del2202
RH+RQ
D
Normalized Current
Prepulse Potential (mV)
2 S
20 ms
+20 mV
-100 mV
E
τfast at 0 mV (ms)
τslow at 0 mV (ms)

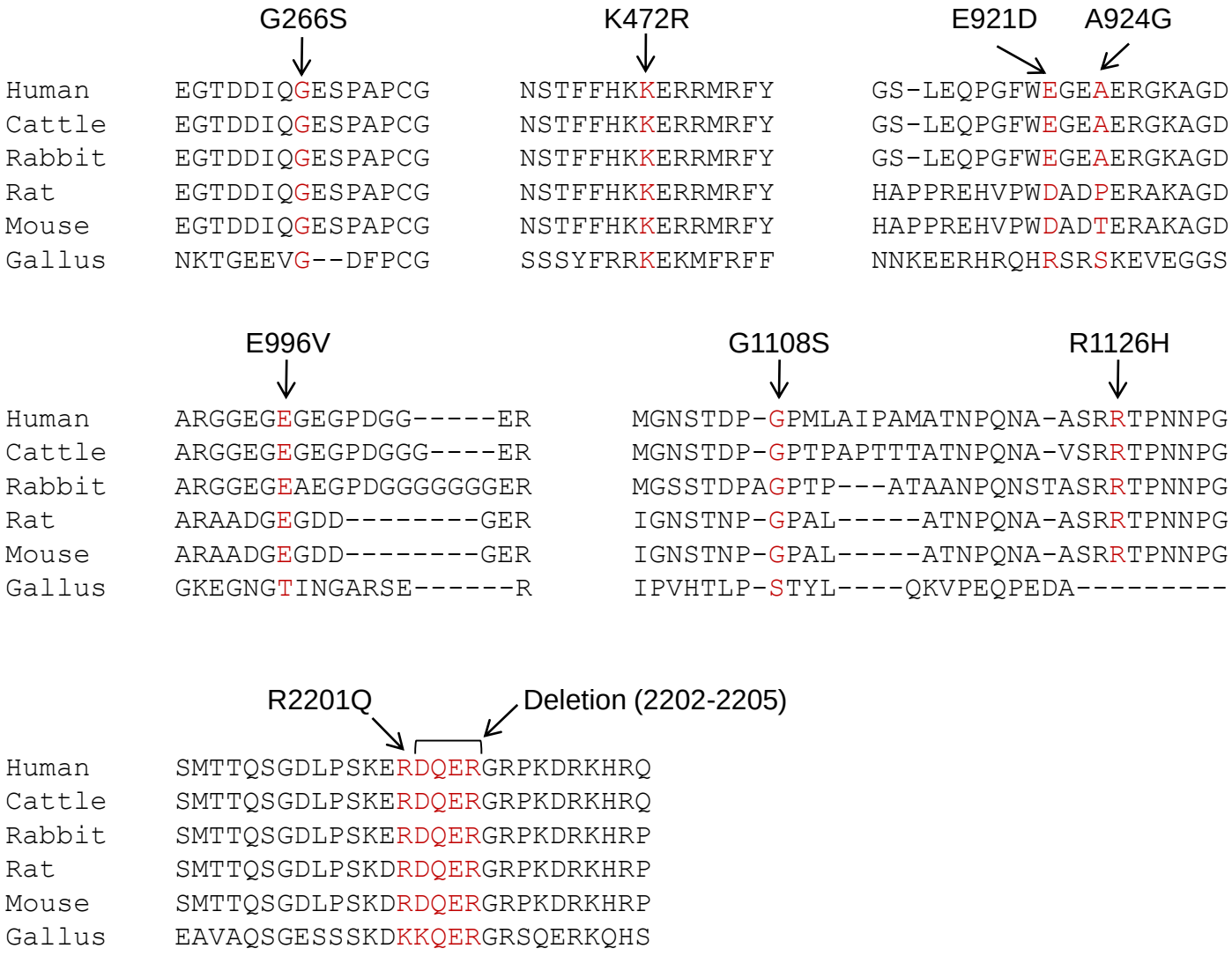

G266S
K472R
E921D
A924G
Human EGTDDIQGESPAPCG NSTFFHKKERRMRFY GS-LEQPGFWEGEAERGKAGD
Cattle EGTDDIQGESPAPCG NSTFFHKKERRMRFY GS-LEQPGFWEGEAERGKAGD
Rabbit EGTDDIQGESPAPCG NSTFFHKKERRMRFY GS-LEQPGFWEGEAERGKAGD
Rat EGTDDIQGESPAPCG NSTFFHKKERRMRFY HAPPREHVPWDADPERAKAGD
Mouse EGTDDIQGESPAPCG NSTFFHKKERRMRFY HAPPREHVPWDADTERAKAGD
Gallus NKTGEEVG--DFPCG SSSYFRRKEKMFRFF NNKEERHRQHRSRSKEVEGGS
E996V
G1108S
R1126H
Human ARGGEGEGEGPDGG-----ER MGNSTDP-GPMLAIPAMATNPQNA-ASRRTPNNPG
Cattle ARGGEGEGEGPDGGG----ER MGNSTDP-GPTPAPTTTATNPQNA-VSRRTPNNPG
Rabbit ARGGEGEAEGPDGGGGGGGER MGSSTDPAGPTP---ATAANPQNSTASRRTPNNPG
Rat ARAADGEGDD--------GER IGNSTNP-GPAL-----ATNPQNA-ASRRTPNNPG
Mouse ARAADGEGDD--------GER IGNSTNP-GPAL-----ATNPQNA-ASRRTPNNPG
Gallus GKEGNGTINGARSE------R IPVHTLP-STYL----QKVPEQPEDA---------
R2201Q
Deletion (2202-2205)
Human SMTTQSGDLPSKERDQERGRPKDRKHRQ
Cattle SMTTQSGDLPSKERDQERGRPKDRKHRQ
Rabbit SMTTQSGDLPSKERDQERGRPKDRKHRP
Rat SMTTQSGDLPSKDRDQERGRPKDRKHRP
Mouse SMTTQSGDLPSKDRDQERGRPKDRKHRP
Gallus EAVAQSGESSSKDKKQERGRSQERKQHS

Table 1

Table 1 Mutational analysis of CACNA1A gene

Exon	Nucleotide Substitution	Amino Acid Substitution	Dravet (n=48)		Control (n=188-190)			Comments and references
			Frequency		Frequency		*p* value	
6	c.876A>G	p.G266S	1/48	(2.1%)	0/188	(0%)	0.203	Novel variant
11	c.1415A>G	p.K472R	1/48	(2.1%)	1/188	(0.5%)	0.366	Novel variant
19	c.2762A>C	p.E921D	11/48	(22.9%)	49/188	(26.1)%	0.655	Four of the 49 control individuals had homozygous variant. Previously reported polymorphism (dbSNP: rs16022).
19	c.2771C>G	p.A924G	1/48	(2.1%)	7/190	(3.7%)	1.0	Novel variant. Three of the seven control individuals had homozygous variant.
19	c.2987A>T	p.E996V	11/48	(22.9%)	49/188	(26.1%)	0.655	Four of the 49 control individuals had homozygous variant. Previously reported polymorphism (dbSNP: rs16023).
20	c.3322G>A	p.G1108S	3/48	(6.3%)	16/189	(8.5%)	0.772	One of the 16 control individual had homozygous variant. Previously reported polymorphism (dbSNP: rs16027). A small increment in current density (Rajakulendran et al., 2010)
20	c.3377G>A	p.R1126H	4/48	(8.3%)	1/188	(0.5%)	0.0066	Novel variant. All individual with minor variant were heterozygous.
47	c.6602G>A	p.R2201Q	4/48	(8.3%)	4/189	(2.1%)	0.055	Novel variant. All individual with minor variant were heterozygous.
47	c.6605-6616del	p.DQER2202-2205del	1/48	(2.1%)	3/190	(1.6%)	1.0	Novel variant
Frequency of combined variants			Dravet		Control			Comments and references
			Frequency		Frequency		*p* value	
19	c.2762A>C +c.2987A>T	E921D+E996V	11/48	(22.9%)	49/188	(26.1%)	0.2	Previously reported polymorphism. A reduction of current density and depolarizing shift in activation (Rajakulendran et al., 2010)
20+47	c.3377G>A +c.6602G>A	R1126H +R2201Q	4/48	(8.3%)	0/188	(0%)	0.0015	Both variants were likely to be located on the same allele in all of the four patients. All individual with minor variant were heterozygous.

Table 2

Table 2: Inheritance of *CACNA1A* variants and *SCN1A* mutations

Patients	*CACNA1A* gene						*SCN1A* gene	
	Variants				Inheritance		Mutations	Inheritance
					Father	Mother		*De novo?*
03-8	p.G266S				p.G266S	-	p.G177R ¶	Yes
05-46	p.K472R				NA	NA	p.W738fsX746 ¶	NA
01-55	p.A924G				-	p.A924G	p.V1390M *	Yes
04-12	p.E921D	p.E996V			NA	NA	p.V212A ¶	NA
02-23	p.E921D	p.E996V			p.E921D + p.E996V	-	p.R377L ¶	Yes
01-28	p.E921D	p.E996V			NA	NA	Deletion of exon 10 ¶	NA
01-19	p.E921D	p.E996V			-	p.E921D + p.E996V	p.P707fsX714 *	Yes
01-29	p.E921D	p.E996V			p.E921D + p.E996V	-	p.R865X *	Yes
01-49	p.E921D	p.E996V			NA	NA	p.F902C *	NA
01-7	p.E921D	p.E996V			p.E921D + p.E996V	p.E921D + p.E996V	p.T1082fsX1086 *	Yes
02-27	p.E921D	p.E996V			NA	NA	p.Q1277X ¶	NA
01-4	p.E921D	p.E996V			p.E921D + p.E996V	-	p.Q1450R *	Yes
02-20	p.E921D	p.E996V			NA	p.E921D + p.E996V	p.A1685D ¶	NA
02-2	p.E921D	p.E996V	p.R1126H	p.R2201Q	NA	p.R1126H + p.R2201Q	p.T1909I *	NA
06-12	p.R1126H	p.R2201Q			-	p.R1126H + p.R2201Q	p.G163E ¶	Yes
01-16	p.R1126H	p.R2201Q			p.R1126H + p.R2201Q	-	p.R501fsX543 *	Yes
02-24	p.R1126H	p.R2201Q			-	p.R1126H + p.R2201Q	p.S1574X *	Yes
05-06	p.DQER2202-2205del				NA	-	negative	NA
01-22	p.G1108S				-	p.G1108S	p.R712X *	Yes
01-35	p.G1108S				NA	p.G1108S	p.R1648C *	NA
21	p.G1108S				NA	NA	negative	NA

NA; Agreement of the mutational analysis was not available, -; the same mutations were not detected. Sequences of each sample were compared with the GenBank data base (accession numbers: NM_023035 and AB093548). *, ¶These mutations were previously reported in our paper, BBRC (2002)* and Epilpesia (2008)¶

Table 3

Table 3: Genotype-phenotype correlation before one year of age

Genotype	N	*SCN1A* mutations		Seizure onset mean±SEM (months)	Total no. of seizures mean±SEM	Total no. of prolonged (>10min) seizures mean±SEM	Type of Seizures			
		missense N (%)	Truncation, deletion N (%)				GTC (%)	CPS (%)	Hemi-convulsion (%)	Myoclonic seizure (%)
SCN1A mutations + No *CACNA1A* variants	20	9 (45%)	11 (55%)	5.6±0.3	10.2±1.2	2.5±0.4	95	45	50	15
SCN1A mutations + *CACNA1A* variants	20	10 (50%)	10 (50%)	4.6±0.4*	11.3±1.4	4.7±0.7*	95	30	80	10

GTC: generalized tonic-clonc seizure, CPS: complex partial seizure *$p<0.05$ versus the patients with *SCN1A* mutations + no *CACNA1A* variants

Table 4

Table 4. Biophysical parameters for activation and inactivation

	Activation			Inactivation		
	$V_{1/2}$ (mV)	*k* (mV)	n	$V_{1/2}$ (mV)	*k* (mV)	n
WT-CACNA1A	6.3±1.3	4.3±0.2	16	-16.9±1.5	-4.5±0.6	10
G266S	1.0±1.2**	4.3±0.4	11	-13.8±1.6	-5.5±0.3	10
R1126H	0.4±1.6**	3.3±0.3*	10	-18.9±0.6	-6.1±0.7	8
R2201Q	6.4±1.5	4.1±0.2	8	-13.4±1.7	-5.7±0.4	10
Deletion2202-2205	1.3±1.4*	3.4±0.2*	8	-13.3±1.2	-4.7±0.6	9
R1126H+R2201Q	2.6±1.1	3.5±0.2	10	-15.2±0.9	-5.4±0.1	10

$V_{1/2}$, half-maximal voltage activation and inactivation; *k*, slope factor. Statistical coparison between WT-CACNA1A and mutant channels was performed by Student's *t* test (*P<0.05 and **P<0.01 versus WT-CACNA1A).

Table 5

Table 5. Predicted influence of biophysical properties on $Ca_v2.1$ channels activity

	CACNA1A				
Biophysical property	G266S	R1126H	R2201Q	Del 2202-2205	R1126H+ R2201Q
Peak current density	-	-	-	↑	↑
Activation $V_{1/2}$	↑	↑	-	↑	-
Activation slop factor	-	↑	-	↑	-
Inactivation $V_{1/2}$	-	-	-	-	-
Inactivation slope factor	-	-	-	-	-

↑, predicted gain of channel activity, ↓, predicted loss of channel activity, -, no predicted change in channel activity.

Suppl. Table 1

Supplementary table 1: SCN1A mutations in patients with Dravet syndrome

	SCN1A mutations			Family history
	Nucleotide Substitution	Amino Acid Substitution		
06-12	c.488G>A	p.G163E	¶	Maternal aunt:FS
03-8	c.529G>A	p.G177R	¶	negative
04-12	c.635T>C	p.V212A	¶	negative
02-23	c.1130G>T	p.R377L	¶	negative
01-40	c.1502deletionG	p.R501fsX543	*	Paternal aunt:Ep
01-16	c.1641insA	p.K547fsX570	*	negative
05-18	c.1702C>T	p.R568X	*	negative
01-56	c.1820deletionC	p.S607fsX622	*	negative
01-19	c.2120deletionC	p.P707fsX714	*	negative
01-22	c.2134C>T	p.R712X	*	Father:FS
05-46	c.2213deletionG	p.W738fsX746	¶	negative
03-22	c.2362G>A	p.E788K	¶	negative
01-29	c.2593C>T	p.R865X	*	Brother:FS
01-49	c.2705T>G	p.F902C	*	Brothers:FS, Maternal grandfather:convulsion
02-3	c.2791C>T	p.R931C	*	negative
01-37	c.2791C>T	p.R931C	*	negative
05-52	c.2970G>T	p.L990F	¶	Father&paternal uncle: FS, Maternal uncle:FS
01-46	c.3006deletionC	p.A1002fsX1009	*	Paternal uncle:FS
01-1	c.3079A>T	p.K1027X	*	Paternal aunt:FS
22	c.3170-31833del (AGAAAGACAGTTGT) ins (TCATTCTGTATG)	p.K1057fsX1073	Novel	negative
01-7	c.3245deletionC	p.T1082fsX1086	*	Maternal cousin:convulsion
01-25	c.3794T>C	p.L1265P	*	negative
02-9	c.3812G>A	p.W1271X	*	Maternal cousin:FS
02-27	c.3829C>T	p.Q1277X	¶	Sister:convulsion
02-6	c.3867-3869deletionCTT	p.1289delF	*	Father, sister:FS
01-55	c.4168G>A	p.V1390M	*	negative
03-66	IVS21-2A>G	Intron 21 splicing error	¶	Maternal cousin:FS, Paternal cousin:FS
02-10	c.4286-4290del(CCACA) ins(ATGTCC)	p.A1429fsX1443	*	Maternal uncle:Ep
01-60	c.4300T>C	p.W1434R	*	negative
01-4	c.4349A>G	p.Q1450R	*	negative
05-11	c.4615A>C	p.T1539P	¶	negative
04-09	c.4721C>G	p.S1574X	¶	Mother's cousin: Ep
02-24	c.4721C>G	p.S1574X	*	negative
01-35	c.4942C>T	p.R1648C	*	negative

01-52	c.5020G>C	p.G1674R	*	negative
06-14	c.4985C>T	p.A1662V	Novel	negative
02-20	c.5054C>A	p.A1685D	¶	Mother:FS
02-14	c.5640-5645 del(AGAGAT) ins(CTAGAGTA)	p.G1880fsX1881	*	negative
02-2	c.5726C>T	p.T1909I	*	negative
01-28		Deletion of exon 10	¶	Father, Paternal grandfather:FS
02-16		negative		negative
01-32		negative		Brother:FS
01-43		negative		Mother:Ep
02-15		negative		Brother:SMEI, Paternal grandfather:Ep
03-46		negative		Maternal grandfather:Ep
05-07		negative		negative
05-06		negative		Mother, sister, maternal uncle: FS
21		negative		negative

FS; Febrile seizure, Ep; Epilepsy, NE; not examined

Sequences of each sample were compared with the GenBank data base (accession number: AB093548)

, ¶These mutations were previously reported in our paper, BBRC (2002) and Epilpesia (2008)¶

Suppl. Table 2

Supplementary table 2: Silent mutations in coding regions of the CACNA1A gene

Exon	amino acid	Allele Frequency			SNP reference
6	p.292E	GAA/GAA 40	GAA/GAG 7	GAG/GAG 1	rs16006
8	p.394E	GAA/GAA 22	GAA/GAG 21	GAG/GAG 5	rs2248069
12	p.524I	ATT/ATT 47	ATT/ATA 1	 0	rs16010
16	p.697T	ACG/ACG 21	ACG/ACA 25	ACA/ACA 2	rs16016
19	p.1019R	AGG/AGG 37	AGG/AGA 11	AGA/AGA 0	rs16025
23	p.1287F	TTT/TTT 40	TTT/TTC 8	TTC/TTC 0	rs16030
27	p.1454T	ACC/ACC 47	ACC/ACT 1	ACT/ACT 0	-
28	p.1468S	TCG/TCG 47	TCG/TCA 1	TCA/TCA 0	-
39	p.1884V	GTC/GTC 46	GTC/GTT 2	GTT/GTT 0	rs17846921
47	p.2219H	CAT/CAT 2	CAT/CAC 17	CAC/CAC 29	rs16051