Neurobiology of Disease 50 (2013) 209–217

Contents lists available at SciVerse ScienceDirect

Neurobiology of Disease

journal homepage: www.elsevier.com/locate/ynbdi

CACNA1A variants may modify the epileptic phenotype of Dravet syndrome

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ARTICLE INFO

Article history:
Received 27 April 2012
Revised 4 October 2012
Accepted 19 October 2012
Available online 25 October 2012

Keywords:
CACNA1A
Epilepsy
SCN1A
Electrophysiology
Molecular genetics

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 the major cause of Dravet syndrome. In this study, we investigated genetic modifiers of this syndrome.

We performed a mutational analysis of all coding exons of *CACNA1A* in 48 subjects with Dravet syndrome. To assess the effects of *CACNA1A* variants on the epileptic phenotypes of Dravet syndrome, we compared clinical features in two genotype groups: 1) subjects harboring *SCN1A* mutations but no *CACNA1A* variants (n = 20) and 2) subjects with *SCN1A* mutations plus *CACNA1A* variants (n = 20). *CACNA1A* variants detected in patients were studied using heterologous expression of recombinant human $Ca_v2.1$ in HEK 293 cells and whole-cell patch-clamp recording.

Nine *CACNA1A* variants, including six novel ones, were detected in 21 of the 48 subjects (43.8%). Based on the incidence of variants in healthy controls, most of the variants seemed to be common polymorphisms. However, the subjects harboring *SCN1A* mutations and *CACNA1A* variants had absence seizures more frequently than the patients with only *SCN1A* mutations (8/20 *vs.* 0/20, $p = 0.002$). Moreover, the former group of subjects exhibited earlier onset of seizures and more frequent prolonged seizures before one year of age, compared to the latter group of subjects. The electrophysiological properties of four of the five novel $Ca_v2.1$ variants exhibited biophysical changes consistent with gain-of-function. We conclude that *CACNA1A* variants in some persons with Dravet syndrome may modify the epileptic phenotypes.

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 that 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 to 80% of subjects with Dravet syndrome. *SCN1A* mutations have therefore been considered to be the major cause of this syndrome (Claes et al., 2001; Depienne et al., 2009; Ohmori et al., 2002). However, several studies have suggested that environmental factors and genetic modifiers may influence the clinical phenotype of Dravet syndrome. Subjects with the same *SCN1A* mutation often show different severities of epilepsy (Guerrini et al., 2010; Suls et al., 2010), and approximately 50% of subjects 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, genetic background influences the severity of epilepsy in a SMEI mouse model harboring a truncated *SCN1A* mutation (Yu et al., 2006). The 129/SvJ mouse strain exhibited a decreased incidence of spontaneous seizures and survived longer compared to C57BL/6J mice, indicating that genetic background affects the *Scn1a* knockout (KO) mouse phenotype.

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

A missense mutation of *CACNB4*, which encodes the β4 subunit of the voltage-dependent calcium channel, was detected in one person out of the 38 subjects with Dravet syndrome (Ohmori et al., 2008). This person had a *de novo SCN1A* nonsense mutation and a *CACNB4* missense mutation (R468Q), which was inherited from his father who had a history of a single febrile seizure. A previous electrophysiological study of heterologously expressed CACNB4-R468Q revealed increased $Ca_v2.1$ (P/Q type) voltage-gated calcium channel current density. However, the detection rate for *CACNB4* variants and mutations was low, so we decided to perform mutational analysis of other genes.

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Available online on ScienceDirect (www.sciencedirect.com).
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http://dx.doi.org/10.1016/j.nbd.2012.10.016

Ca_v β4 is the predominant subunit associated with Ca_v 2.1 (Dolphin, 2003), we screened *CACNA1A* genetic variants to further assess the role of Ca_v 2.1 as a potential genetic modifier of Dravet syndrome.

We detected nine *CACNA1A* variants, including six that were novel, in the 48 subjects. Some variants exhibited properties consistent with gain-of-function. This association between *CACNA1A* variants and clinical phenotypes of Dravet syndrome is consistent with the effect of a genetic modifier.

Materials and methods

Clinical samples

A total of 48 subjects with Dravet syndrome, including typical and borderline cases, were analyzed for this study. Of this 48-subject cohort, 46 had been recruited for our previous studies (Hattori et al., 2008; Ohmori et al., 2002). One 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 subjects 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 subjects (83.3%) had various types of mutations (Supplementary Table 1). Coding exons of *CACNA1A* were analyzed by direct sequencing with primers designed outside the exons. The sequences of each sample were compared with those in the GenBank database (accession number: NM_023035). The frequencies of the *CACNA1A* variants identified in the subjects with Dravet syndrome were studied in the 190 healthy controls. 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 clinical features of Dravet syndrome dramatically change as age increases, so genotype–phenotype correlations should be evaluated in subjects of the same age. We assessed age at seizure onset, cognitive development at 6 years of age, cerebellar symptoms at 6 years of age, total number of prolonged seizures that were longer than 10 min, and types of seizure evident by 6 years of age. Clinical data were collected based on an exhaustive review of the medical records and interviews. Intellectual levels at 6 years of age were classified into three groups: mild cognitive impairment, moderate cognitive impairment, and severe cognitive impairment. The severe cognitive impairment group included subjects with no language ability, those who could speak several words, and those who could hold basic conversations. We carried out WISC-R (n = 2), WISC-III (n = 3), the Tanaka-Binet test (n = 1), or the Kaufman Assessment Battery for Children (n = 2) whenever possible. Subjects whose IQ was in the range of 35 to 50 were placed in the moderate cognitive impairment group, and those with an IQ of 50 to 70 were placed in the mild cognitive impairment group. Cerebellar symptoms at 6 years of age were assessed by child neurologists. We intensively examined EEGs whenever absence seizures and myoclonic seizures were suspected. All subjects with absence seizures and myoclonic seizures were confirmed by ictal simultaneous video-EEG-electromyography (EMG) recordings. The average number of EEG recordings prior to 6 years of age was 33.6 ± 5.0/subject. Correlation of genotype (*CACNA1A* variants and *SCN1A* mutations) and clinical features were also investigated.

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 Professor 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.

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, Venlo, The Netherlands). 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 h after transfection.

Electrophysiological study of CACNA1A

The currents from HEK293 cells were recorded with the whole-cell patch-clamp technique using an 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 that of 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 used when recording 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. The solution was 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, and was adjusted to pH 7.4 with TEA-OH and an 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 using Boltzmann functions to determine the voltages for half-maximal activation and inactivation ($V1/2$) and slope factor (k). Time constants for the 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 following 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 non-inactivating current.

Statistical analysis for electrophysiological study

All electrophysiological data are presented as the mean ± standard error of the mean (SEM), and statistical comparisons were made in reference to the wild type (WT) by using unpaired Student's *t*-test. The threshold *p* value for statistical significance was 0.05. Data analysis was performed using Clampfit 8.2 (Axon Instruments, Union City, CA, USA) and OriginPro 7.0 (OriginLab, Northampton, MA, USA) software packages.

Results

CACNA1A mutational analysis

The results of the *CACNA1A* genetic analysis in the 48 subjects with Dravet syndrome are summarized in Table 1. The frequencies of *CACNA1A* variants identified in the subjects with Dravet syndrome were studied in the 190 healthy controls. Nine variants

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	
20	c.3377G>A	p.R1126H	–	–	1/188	(0.5%)	–	Novel variant. All individuals with minor variant were heterozygous.
47	c.6602G>A	p.R2201Q	–	–	4/189	(2.1%)	–	Novel variant. All individuals with minor variant were heterozygous.
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
47	c.6605–6616del	p.DQER2202–2205del	1/48	(2.1%)	3/190	(1.6%)	1.0	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 individuals had homozygous variant. Previously reported polymorphism (dbSNP: rs16027). A small increment in current density (Rajakulendran et al., 2010)
Frequency of combined variants			Dravet		Control			Comments and references
			Frequency		Frequency		*p* value	
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 individuals with minor variant were heterozygous.
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)

were detected in 21 subjects (43.8%), and eight variants were detected in healthy participants (39.6%). No statistical differences were observed for the p.K472R, p.E921D, p.A924G, p.E996V, p.G1108S, and p.DQER2202-2205 deletion. A combination of p.R1126H and p.R2201Q was found in four subjects with Dravet syndrome, but this combination was not observed in any of the control individuals, suggesting that the double variant was over represented in Dravet syndrome ($p = 0.0015$).

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. Synonymous (silent) variants identified in the subjects are provided in Supplementary Table 2.

A mutational analysis was conducted in the parents of the children with the *SCN1A* and *CACNA1A* mutations (Table 2). *SCN1A* mutations appeared *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 double variants probably exist on one allele. Parents with *CACNA1A* variants suffered no neurological symptoms and had no history of neurological disease, except for the mother of ID 02-20 who had a history of several febrile seizures, and the father of ID 01-12 who had a history of a single febrile seizure.

Genotype and phenotype correlation

The subjects were divided into four genotypic groups: 1) *SCN1A* mutation but no *CACNA1A* variant (n = 20); 2) *SCN1A* mutation plus a *CACNA1A* variant (n = 20); 3) *CACNA1A* variant but no *SCN1A* mutation (n = 2); and 4) no *SCN1A* mutation and no *CACNA1A* variant (n = 6). Regarding the type of *SCN1A* mutations, the first group consisted of subjects with 9 missense mutations and 11 truncation or deletion mutations, and the second group consisted of subjects with 10 missense mutations and 10 truncation mutations. The second group included a previously reported person 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 *CACNA1A* variants on the clinical severity of Dravet syndrome, phenotypic differences between the two groups, namely, absence (n = 20) or presence (n = 20) of *CACNA1A* variants in subjects with *SCN1A* mutations were analyzed (Fig. 2). The subjects with both a *SCN1A* mutation and one or more *CACNA1A* variants showed an earlier onset of seizures ($p = 0.03$) (Fig. 2A). The number of prolonged seizures during each year was counted, based on medical records, prior to 6 years of age. Frequency decreased with age in both groups (Fig. 2D). The subjects with *CACNA1A* variants had significantly more frequent prolonged seizures than those without *CACNA1A* variants before one year of age ($p = 0.03$). There were no statistical differences after two years of age. Regarding the appearance of type of seizures prior to 6 years of age, the subjects with both *SCN1A* mutations and *CACNA1A* variants had significantly more frequent absence seizures than subjects who had only *SCN1A* mutations (8/20, 40% *vs.* 0/20, 0%, $p = 0.002$) (Figs. 2E and F). Absence seizures in Dravet syndrome typically appear between the age of 1 and 4 years. However, we could not exclude the possibility that absence seizures may have appeared after 6 years of age in some subjects. No significant differences were observed for other types of seizures, including generalized tonic clonic seizures, focal seizures, hemiconvulsions, and myoclonic seizures (Fig. 2E), or concerning intellectual level and cerebellar symptoms at 6 years of age (Figs. 2B and C).

Regarding the correlation of the number of *CACNA1A* variants and phenotype, seven subjects had one variant, 13 subjects had two variants, and one subject had four variants. We did not find any statistically significant clinical differences pertaining to the number of *CACNA1A* variants. As for absence seizures, two subjects who had only *CACNA1A* variants did not have absence seizures prior to 6 years of age.

Biophysical properties of the novel CACNA1A variants

The 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.

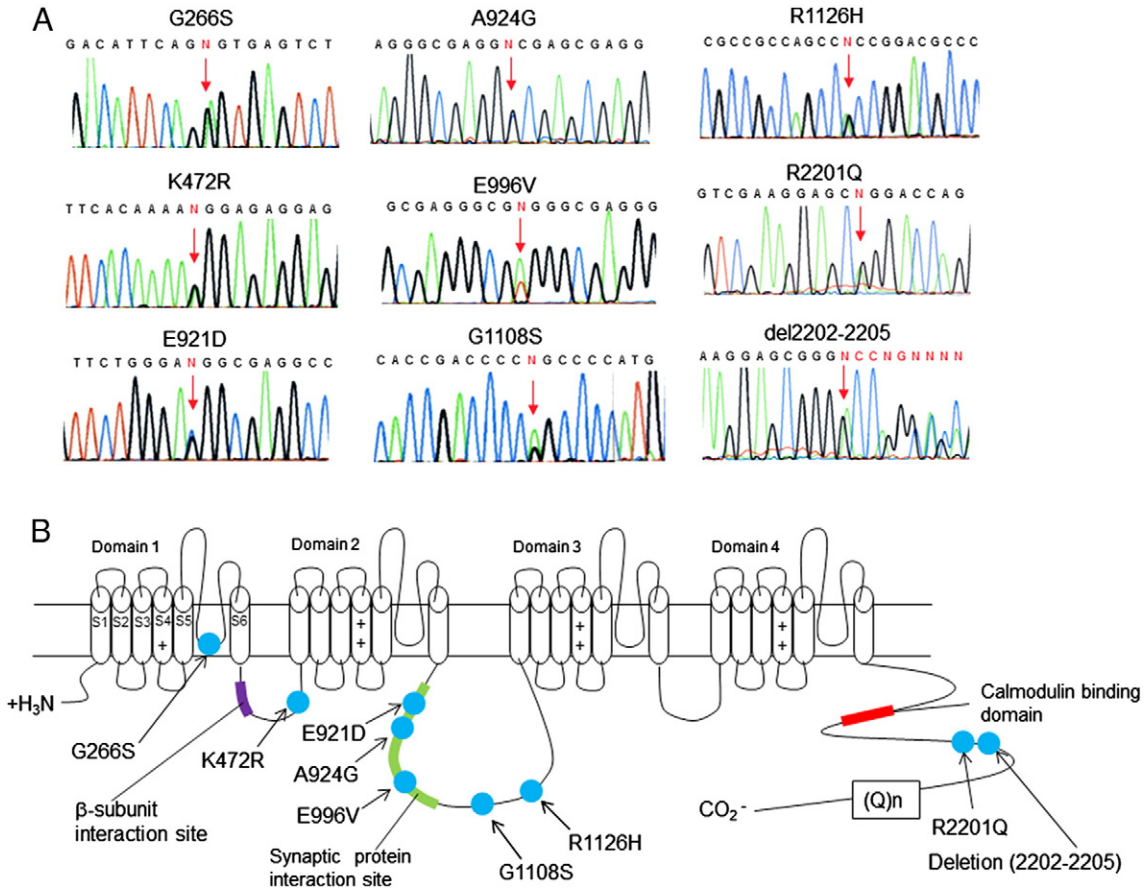

Fig. 1. Mutations of the *CACNA1A* gene in subjects with Dravet syndrome. (A) Electropherogram of *CACNA1A* gene DNA sequence from subjects. (B) Schematic diagram illustrating the transmembrane topology of the voltage-gated calcium channel and location of variants identified in this study.

Fig. 3A 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. 3B) indicate that two of the five mutant alleles, DQER2202–2205 deletion and the double variant R1126H + R2201Q, showed significantly greater current densities, at voltages from −10

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 genetic analysis was not available, -; the same variants 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, Ohmori et al. (2002)* and Hattori et al. (2008)¶.

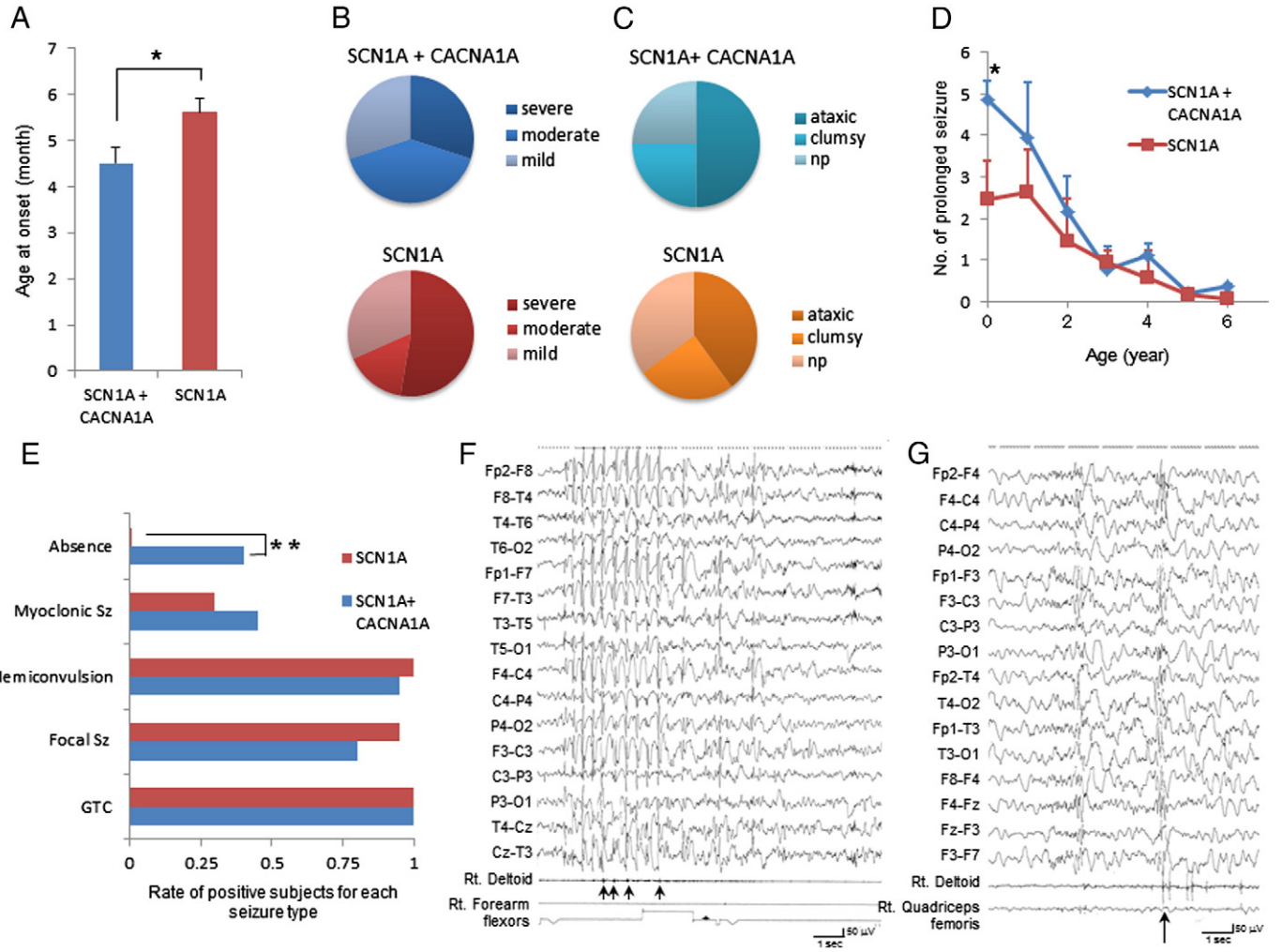

Fig. 2. Genotype–phenotype correlation. Comparison of phenotypic differences between two groups of patients with *SCN1A* mutations: 1) with *CACNA1* variants (n = 20) and 2) without *CACNA1A* variants (n = 20). (A) Age at seizure onset in subjects with *CACNA1A* variants was earlier than in those without *CACNA1A* variants (4.5 ± 0.36 months *vs.* 5.6 ± 0.32 months, $p = 0.028$). (B) There was no significant difference in subject's intellectual levels between the two groups in terms of the absence or presence of *CACNA1A* variants. (C) Ataxic gait and clumsiness were observed in approximately two-thirds of subjects in both groups. (D) Number of prolonged seizures up to 6 years of age was counted based on medical records. Subjects with *CACNA1A* variants had significantly more frequent prolonged seizures than those without *CACNA1A* variants before one year of age ($p = 0.03$). (E) Types of seizures prior to 6 years of age were assessed. Each individual had various types of seizures. GTC (generalized tonic clonic seizures), focal seizures, and hemiconvulsions were observed in more than 80% of subjects in both groups. Myoclonic seizures were observed in 9 of 20 subjects (45%) in subjects with *CACNA1A* variants, and in 6 of 20 subjects (30%) who lacked *CACNA1A* variants. Absence seizures were only observed in subjects with *CACNA1A* variants (8/20, 40% *vs.* 0/20, 0%, $p = 0.002$). (F) Ictal EEG of absence seizure in a subject with *SCN1A* mutation and *CACNA1A* variants (patient ID 02-2). Vacant staring with myoclonic movements of the eyelids and the shoulders (small arrows) was evoked by hyperventilation. The EMG on the right deltoid muscle showed myoclonic jerks of the right shoulder. The absence seizure was accompanied by diffuse 3 c/s spike-wave bursts on EEG. (G) Ictal EEG of myoclonic seizure during sleep in a patient with *SCN1A* mutation alone (patient ID 01-25). Myoclonic jerks of the upper limbs (arrow) were accompanied by polyspike-waves on EEG.

to +10 mV and from −10 to +60 mV than those of WT-$Ca_v2.1$, respectively (Fig. 3B).

The voltage dependence of activation and inactivation is also examined (Fig. 4). Activation was shifted significantly toward more hyperpolarizing potentials in cells expressing the G266S, R1126H, and DQER2202–2205 deletion, and showed significantly steeper voltage dependence, as suggested by a comparison of their slope factors (k) with those of WT (Table 3). These findings indicate that the G266S, R1126H, and DQER2202–2205 deletion channels 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 +60 mV (Fig. 4B). G266S showed a significant decrease in the time constant for activation at 20 mV compared to that of the WT (Fig. 4C), 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. Figs. 4D and E respectively illustrate the voltage dependence of inactivation and the time constants for inactivation at 0 mV, by fitting with a two-exponent function. No statistically significant differences were observed between WT and the variants. The predicted influence of variant biophysical properties on $Ca_v2.1$ channel activity is summarized in Table 4.

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 subjects with Dravet syndrome. Nine nonsynonymous variants, including six novel alleles, were identified in 21 subjects with Dravet syndrome (43.8%). Based on the

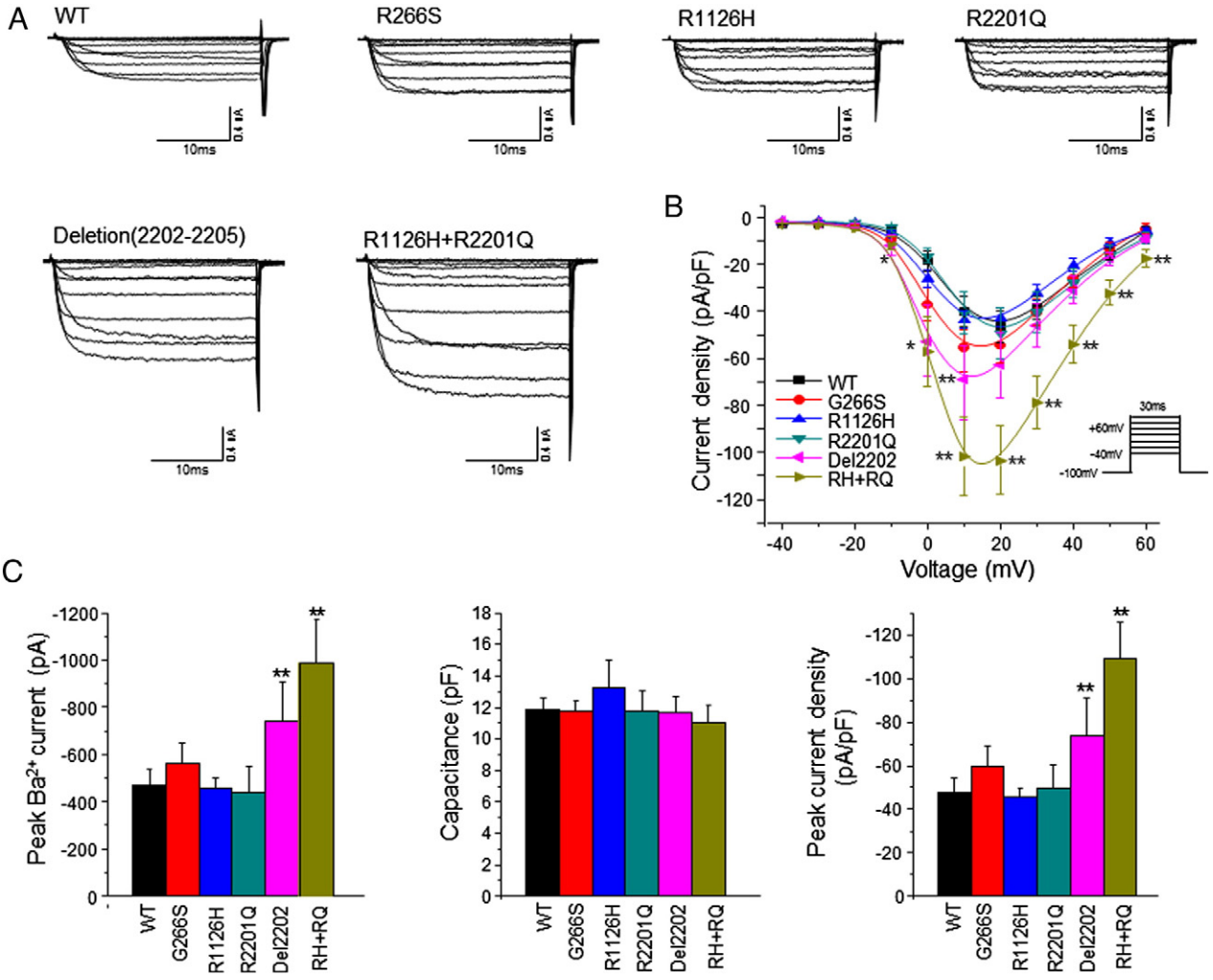

Fig. 3. Comparison of Cav2.1 currents recorded in HEK293 cells expressing WT-*CACNA1A* and mutant channels. (A) Representative WT and mutant whole-cell Ba^{2+} currents. Whole-cell currents recorded from HEK293 cells transiently expressing the indicated alleles, from a holding potential of −100 mV to voltages between −40 and +60 mV in 10-mV increments. Vertical and horizontal scale bars represent 0.4 nA and 10 ms, respectively. (B) Current–voltage relationships of whole-cell Ba^{2+} currents from transiently transfected HEK293 cells. 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; DQER2202–2205 deletion, n = 8; R1126H + R2201Q, *n* = 10). The current densities of DQER2202–2205 deletion and double mutation R1126H + R2201Q were significantly larger than WT between −10 and +10 mV, and between −20 and +60 mV, respectively (*$p<0.05$). (C) Distribution of peak current amplitude (left), cell capacitance (middle), and current density (right) at 10 mV. *$p<0.05$ and **$p<0.01$ *vs.* WT-*CACNA1A*.

incidence of variants in healthy controls, the incidence of double variant p.E921D + p.E996V, p.A924G, and p.G1108S seemed to be similar to that for common polymorphisms, whereas the p.K472R, p.R2201Q, and p.DQER2202–2205 deletion seemed to be relatively rare variants. It is noteworthy that all subjects with p.R1126H also had p.R2201Q, whereas none of the controls had double variants ($p = 0.0015$). However, it is not known whether the controls have any other variants, as we did not examine all coding exons in the controls. *CACNA1A* mutations have been linked to familial hemiplegic migraine type 1 (FHM1) (Ducros et al., 2001; Ophoff et al., 1996; Pietrobon, 2010), episodic ataxia type 2 (EA2) (Guida et al., 2001; Ophoff et al., 1996), spinocerebellar ataxia type 6 (Ducros et al., 2001; Zhuchenko et al., 1997), and a combination of epilepsy and ataxia (Jouvenceau et al., 2001; Rajakulendran et al., 2010). None of the parents of the subjects with Dravet syndrome had a significant history of neurological disorders. The effect of these *CACNA1A* variants alone seems to be insufficient to account for the neurological symptoms.

To assess the effects of the *CACNA1A* variants on the epileptic phenotypes of Dravet syndrome, we compared the clinical features of the patients with *SCN1A* mutations but no *CACNA1A* variants with those of the patients harboring *SCN1A* mutations as well as *CACNA1A* variants. It is remarkable that absence seizures were observed only in the subjects with *CACNA1A* variants in addition to *SCN1A* mutations. Absence seizures in Dravet syndrome are often accompanied by repetitive myoclonic jerks and/or eyelid myoclonias. The *Scn1a* KO mouse exhibits phenotypic features of Dravet syndrome, such as hyperthermia-induced seizures, spontaneous generalized tonic clonic seizures, myoclonic seizures, and ataxia. However, absence seizures were not reported in these mice (Ogiwara et al., 2007; Yu et al., 2006). Modifier genes may be required generate absence seizures in the *Scn1a* KO mouse. In this study, absence seizures were observed in some subjects with *SCN1A* mutations and *CACNA1A* variants (8/20, 40%) but not in any subjects with *SCN1A* mutations alone (0/20, 0%, $p = 0.002$). *CACNA1A* mutations have been linked to absence seizures in both humans and rodents (Zamponi et al., 2010). Thus, the *CACNA1A* variants may affect the appearance of absence seizures in Dravet syndrome.

In addition, the subjects with *SCN1A* mutations and *CACNA1A* variants showed significantly earlier onset of seizures and more frequent prolonged seizures before one year of age, although the differences were small. We could not find differences in the prevalence of cognitive and motor deficits between the two groups at 6 years of age.

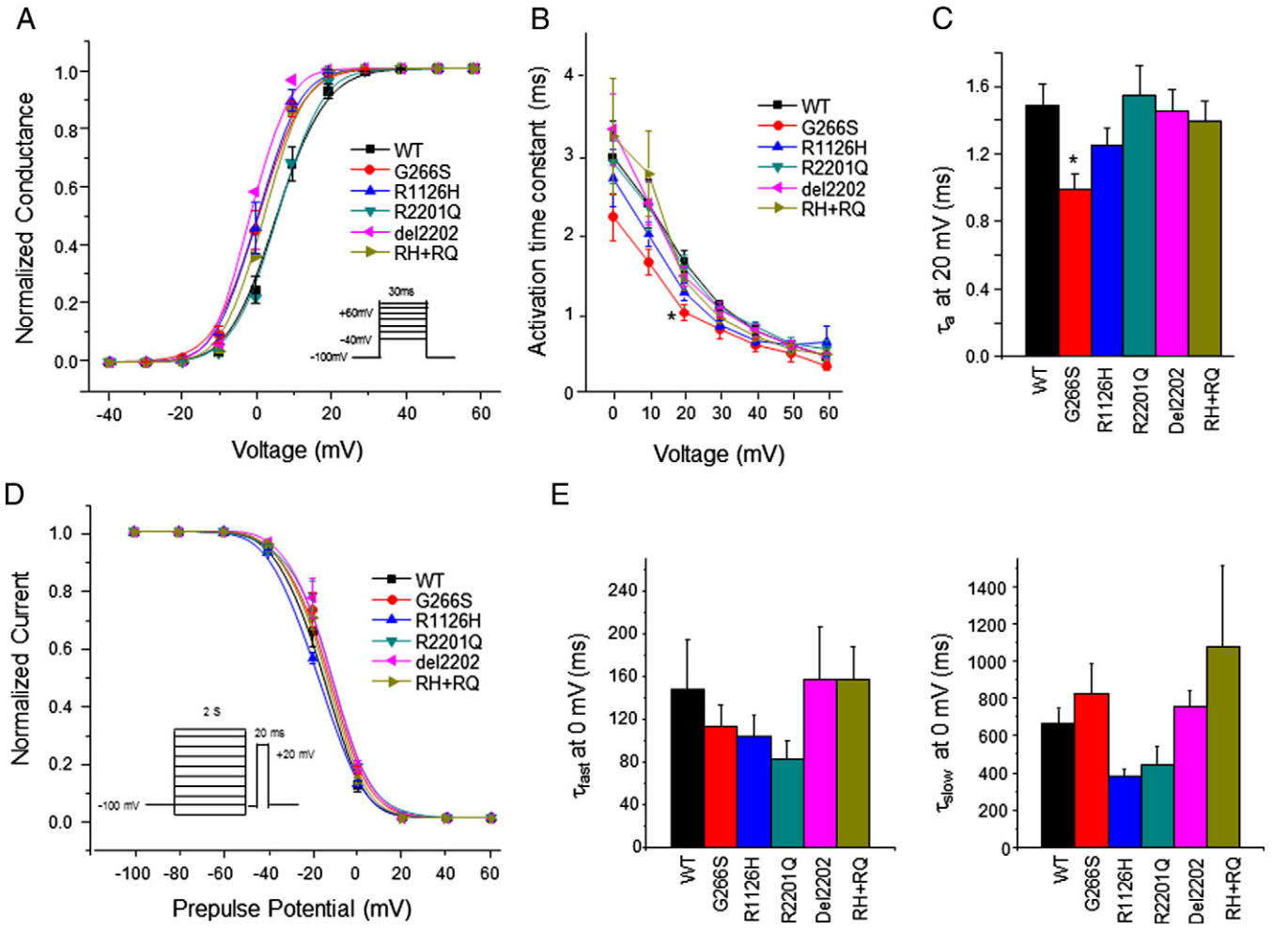

Fig. 4. (A) Voltage dependence of activation. The voltage dependence of channel activation was estimated by measuring peak Ba^{2+} current when exposed to different test potentials from a holding potential of −100 mV. The current of each membrane potential was normalized to the maximum Ba^{2+} conductance. (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 to 60 mV. (C) 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 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; DQER2202–2205 deletion, n = 9; R1126H + R2201Q, n = 10). (E) Ba^{2+} currents were evoked by 2-s test pulses. Current decay was fitted using 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. $^{*}p<0.05$ *vs.* WT-*CACNA1A*.

Because cognitive decline and ataxia are progressive until adulthood in some subjects with Dravet syndrome (Akiyama et al., 2010), our results may be subject to change with longer follow-up. However, the possibility of a sole *SCN1A*-mutation effect as a cause of phenotypic differences could not be excluded, even though the ratio of mutation types was similar for subjects in group 1 (9 missense mutations and 11 truncated/deletion mutations) and group 2 (10 missense mutations and 10 truncated mutations).

Table 3
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 comparison between WT-*CACNA1A* and variant channels was performed by Student's t test (*P<0.05 and **P<0.01 *vs.* WT-*CACNA1A*).

In the present study, we determined the electrophysiological properties of five novel variants. Four alleles exhibited gain-of-function, whereas R2201Q did not exhibit any significant difference in parameters when compared to the WT. Interestingly, the double variant p.R1126H + p.R2201Q revealed much greater current density, whereas that of the other variants was the same as the WT. Electrophysiological properties of common variants p.E921D + p.E996V and p.G1108S have been reported (Rajakulendran et al., 2010), and the former showed a decreased current density and depolarizing shift in activation, while the latter exhibited a small increment in current density. The channel function of *CACNA1A* variants in Dravet syndrome showed a range of electrophysiological properties in the recombinant assay. A similar diversity was observed in missense *SCN1A* mutations in Dravet syndrome (Rhodes et al., 2004). The inability to maintain neuronal homeostasis at appropriate levels for a variety of molecular processes is sufficient to cause common neuropsychiatric phenotypes, including mental retardation, autism, and epilepsy (Ramocki and Zoghbi, 2008). The loss or gain of protein molecular functions revealed in recombinant assays can indicate

Table 4
Predicted influence of biophysical properties on $Ca_v2.1$ channels activity.

	CACNA1A					CACNB4	*CACNA1A*	
Biophysical property	G266S	R1126H	R2201Q	Del 2202–2205	R1126H + R2201Q	R468Q	E921D + E996V	G1108S
Peak current density	-	-	-	↑	↑	↑	↓	↑
Activation $V_{1/2}$	↑	↑	-	↑	-	-	↓	-
Activation slop factor	-	↑	-	↑	-	-		
Inactivation $V_{1/2}$	-	-	-	-	-	-	-	-
Inactivation slope factor	-	-	-	-	-	-		
References	The present study					Ohmori et al. (2008)	Rajakulendran et al. (2010)	

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

conditions that are deleterious to the nervous system (Ramocki and Zoghbi, 2008).

The majority of the variants found in our subjects with Dravet syndrome were localized to the intracellular loop of the α1 subunit. The voltage-dependent Ca^{2+} channel function can be modified by interacting with other neuronal proteins such as the Ca_v β subunit (Pragnell et al., 1994; Walker et al., 1998), a synaptic protein (Kim and Catterall, 1997; Rettig et al., 1996), calmodulin (DeMaria et al., 2001; Lee et al., 1999), and G proteins (Herlitze et al., 1996) through 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 an interaction site for presynaptic proteins, including SNARE proteins (syntaxin 1A, and a synaptosome-associated protein of 25 kD, SNAP-25) occurs. 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 thereby affect synaptic transmission. Further functional studies using neuronal cells from *Cacna1a*-variant knockin mice are needed in the future. 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 (Glasscock et al., 2007; Kearney et al., 2006; Martin et al., 2007). Interestingly, the phenotype caused by the mutation of one channel could be altered by a second mutant channel, pointing to complex inter-relationships.

In conclusion, nine *CACNA1A* variants, including six novel ones, were detected in 21 of 48 subjects (43.8%) with Dravet syndrome. The variants were inherited from one parent. The majority of parents with the same variant $Ca_v2.1$ channel were asymptomatic, so the effect of each variant $Ca_v2.1$ channel alone seems insufficient to account for the seizure phenotypes. However, the subjects with combinations of *CACNA1A* variants and *SCN1A* mutations showed higher incidence of absence seizures, earlier onset, and more frequent prolonged seizures compared to subjects with only *SCN1A* mutations. The electrophysiological properties of novel variants of $Ca_v2.1$ channels exhibited gain-of-function. Variants of $Ca_v2.1$ channels are potential genetic modifiers in subjects with Dravet syndrome.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nbd.2012.10.016.

Conflict of interest statement

None declared.

Role of funding source

This work was supported by a Grant-in-Aid for Scientific Research (B) from the Japanese Ministry of Education, Culture, Sports, Science and Technology (Grant No. 21390312 to I.O.).

Acknowledgments

The authors are grateful to the patients and their families for their cooperation in this study. We also wish to thank Dr. Alfred L. George, Jr., for his critical reading of the manuscript and expert advice.

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