A CACNB4 mutation shows that altered \( \text{Ca}_\text{v}2.1 \) function may be a genetic modifier of severe myoclonic epilepsy in infancy

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Introduction

Severe myoclonic epilepsy in infancy (SMEI or Dravet syndrome; MIM# 607208) is a malignant epileptic syndrome beginning in the first year of life (Dravet et al., 2005). Prolonged, generalized, or unilateral clonic seizures are typically triggered by fever. The seizures often evolve into life-threatening status epilepticus. In the second year of life, other types of afebrile seizures appear, including myoclonic, absence, generalized tonic-clonic, and partial seizures. Development is normal in the first year of life followed by developmental slowing and regression. A family history of epilepsy or febrile convulsions is often observed (Commission on classification and terminology of the international league against epilepsy, 1989).

Mutations of SCN1A, encoding the voltage-gated sodium channel \( \alpha \text{1} \) subunit, represent the most frequent genetic cause of severe myoclonic epilepsy in infancy (SMEI). The purpose of this study was to determine if mutations in other seizure susceptibility genes are also present and could modify the disease severity. All coding exons of SCN1B, GABRG2, and CACNB4 genes were screened for mutations in 38 SCN1A-mutation-positive SMEI probands. We identified one proband who was heterozygous for a \textit{de novo} SCN1A nonsense mutation (R568X) and another missense mutation (R468Q) of the CACNB4 gene. The latter mutation was inherited from his father who had a history of febrile seizures. An electrophysiological analysis of heterologous expression system exhibited that R468Q-CACNB4 showed greater \( \text{Ba}^{2+} \) current density compared with the wild-type CACNB4. The greater \( \text{Ca}_\text{v}2.1 \) currents caused by the R468Q-CACNB4 mutation may increase the neurotransmitter release in the excitatory neurons under the condition of insufficient inhibitory neurons caused primarily by the SCN1A mutation.

Subjects

A total of 38 previously reported SMEI probands with SCN1A mutations were recruited for this study (Hattori et al., 2008). The probands with SMEI fulfilled the following criteria: normal development before seizure onset, the occurrence of either generalized, unilateral, or partial seizures during the first year of life, seizures that
were frequently provoked by the fever, the presence of myoclonic seizures or segmental myoclonus, diffuse spike-waves or focal spikes on EEG during the clinical course, intractable epilepsy, and gradual evidence of psychomotor delay after two years of age. Two hundred control subjects were randomly selected from Japanese healthy volunteers.

Mutational analysis

Genomic DNA was extracted from peripheral blood by a standard method. All coding exons of the SCN1B, GABRG2, and CACNB4 genes were analyzed by direct sequencing (primer sequences are available on request). The mutational analysis of the SCN1A gene was described in our previous paper (Ohmori et al., 2002). Briefly, PCR products were purified with exonuclease I and shrimp alkaline phosphatase (USB corporation, Cleveland, OH), reacted with a Big Dye Terminator (Applied Biosystems, Foster City, CA), and analyzed on an ABI 3100 sequencer (Applied Biosystems, Foster City, CA). 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.

Mutagenesis

The plasmids pCMV Script full-length human SCN1A and pCD8-IRESHuman SCN1B were kindly provided by Dr. Al George (Vanderbilt University, Nashville, TN). SCN1A cDNA was amplified by PCR with SCN1A-NOT-S1 primer (5'-AAG CGG CCG CAT GGA GCA AAC AGT GCT GCT TGT A-3') and SCN1A-R568X-AS1 (5'-TTG CCC CAC TTC ACC TGG GAA ATA GGG G-3') to produce the nonsense mutation R568X. The product was treated with T4 DNA polymerase and T4 poly nucleotide kinase, and ligated to pBluescript KS digested with EcoRV. The cDNA region was confirmed by DNA sequencing, digested with NotI, and subcloned into pRES-EYFP vector (BD Biosciences Clontech, Palo Alto, CA) digested with NotI.

Rat CACNB4 cDNA of pCneo-CACNB4 (WT) was amplified by PCR with CACNB4-R468Q-S4 primer (5'-AGG GCC CAG AAG AGT AGG AAC CG-3') and AS1 (5'-GCC TTC CCA ACC TGA AAC AT-3') located on the plasmid, to produce the R468Q missense mutation. The product was treated with T4 DNA polymerase and T4 poly nucleotide kinase, and ligated to pBluescript KS digested with EcoRV. The cDNA region was confirmed by DNA sequencing and digested with Apal and NotI. pCneo-CACNB4 (R468Q) was constructed by replacement of the Apal–NotI fragment of pCneo-CACNB4 (WT) by the nonsense mutation R468Q fragment.

All cDNAs were resequenced in the entire coding region or open reading frame before use in the experiments.

Electrophysiological study of SCN1A

Recombinant SCN1A was heterologously coexpressed with the human accessory β1 subunit in human embryonic kidney (HEK293) cells. Whole-cell voltage-clamp recordings were used to characterize the functional properties of the WT-SCN1A and R568X mutant sodium channel as previously described (Ohmori et al., 2008). The pipette solution consisted of (in mM): 110 CsF, 10 NaF, 20 CsCl, 2 EGTA, 10 HEPES, with a pH of 7.35 and osmolarity of 310 mOsmol/kg. The bath solution consisted of (in mM): 145 NaCl, 4 KCl, 1.8 CaCl2, 1 MgCl2, 10 HEPES, with a pH of 7.35 and osmolarity of 310 mOsmol/kg. Data analysis was performed using the Clampfit 8.2 software program (Axon Instruments, Union City, CA).

Electrophysiological study of CACNB4

Rabbit calcium channel α1A subunit (Niidome et al., 1994) and pCneo-CACNB4 (WT or R468Q) were transiently transfected to a BHK cell line having stable expression of the α2δ subunit (Wakamori et al., 1998). The currents from BHK cells were recorded at room temperature with the whole-cell patch-clamp technique by using the EPC-9 patch-clamp amplifier (HEKA Electronic, Lambrecht, Germany) as described previously (Wakamori et al., 1998). The stimulation and data acquisition were performed using the PULSE program (version 7.5, HEKA Elektronik). Patch pipettes were made from borosilicate glass capillaries (1.5 mm outer diameter; Hilgenberg, Malsfeld, Germany) using a model P-87 Flaming–Brown micropipette puller (Sutter Instrument Co., San Rafael, CA). The series resistance was electronically compensated to >50% and both the leakage and the remaining capacitance were subtracted. For recordings of the whole-cell currents, the pipette...
solution contained (in mM): 95 CsOH, 95 aspartic acid, 40 CsCl, 5 EGTA, 2 Na2ATP, 8 creatine-phosphate and 5 HEPES; adjusted to pH 7.3 with CsOH. The bath solution contained (in mM): 3 BaCl2, 148 TEA-Cl, 10 glucose, and 10 HEPES; adjusted to pH 7.4 with TEA-OH.

All data are presented as the mean ± SEM, and statistical comparisons were made in reference to the wild-type by using the unpaired Student’s t-test. The threshold P value for statistical significance was 0.05.

Results

Mutational analysis of SCN1B, GABRG2, and CACNB4 genes

There were no probands who had the SCN1B or GABRG2 mutation. We identified one CACNB4 mutation in the 38 SCN1A mutation-positive probands. This patient A had heterozygous R468Q missense mutation of the CACNB4 gene (Fig. 1B). The mutation was located in the carboxyl-terminal binding site of the Caβ subunit with the carboxyl-terminal of Caα1 subunit (Fig. 1C). The same mutation was also detected in the proband’s father. Because this mutation was not found in 200 healthy control individuals, we reasoned that it is not a polymorphism. Proband A also carried R568X-SCN1A, while this SCN1A mutation was not detected in his parents (Fig. 1D). No other family members agreed to undergo any genetic tests. The clinical characteristics of patient A were as follows.

Clinical characteristics of patient A

Patient A was a 4-year-old boy with SMEI. The pedigree of patient A is presented in Fig. 1A. His father had a febrile seizure associated with measles at the age of seven.

He was born of non-consanguineous parents without any perinatal difficulties. At three months of age, he had his first febrile seizure. An afebrile generalized tonic–clonic seizure occurred one week after the first attack. He began to have myoclonic jerks at 8 months of age. His epileptic attacks were often induced by fever and were resistant to antiepileptic drugs. A brain MRI showed no abnormalities. His total developmental quotient was 44 (Enjoji Developmental Test) at 4 years 2 months of age. An ataxic gait was observed. His clinical features fulfilled the diagnostic criteria of SMEI.

At 4 years and 6 months of age, he suffered from refractory status epilepticus associated with high fever (40 °C) caused by influenza A infection. A generalized convolution lasted for 4 h despite the administration of intravenous diazepam, phenytoin, lidocaine, and midazolam. Intermittent seizures continued over 13 h (Fig. 2A). Status epilepticus was finally controlled by thiamylal sodium anesthesia with ventilatory support. Although the thiamylal sodium was stopped after controlling the status epilepticus, his EEGs showed a progressive diffuse low voltage pattern during the following 3 days (Fig. 2B). His spontaneous respiration, response to stimuli, and response to the corneal reflex test disappeared at the fourth day. Central diabetes insipidus began to occur at the same time. His EEG showed no physiologic brain activity at the seventh day (Fig. 2C). Twenty-seven days later he died of multiple organ failure.

Biophysical properties of R568X-SCN1A mutation

Whole-cell voltage-clamp recordings were used to characterize the functional properties of WT-SCN1A and R568X-SCN1A. The R568X-SCN1A mutant exhibited no greater sodium current than background activity (data not shown).

Biophysical properties of R468Q-CACNB4 mutation

Fig. 3A illustrates representative whole-cell Ba2+ currents evoked by 30-ms depolarizing pulses from −40 to 50 mV for Caβ1.1 channels in BHK cells expressing WT-CACNB4 or R468Q-CACNB4. The current–voltage relationship (Fig. 3B) illustrates that the mutant channels had significantly greater peak Ba2+ current densities at voltages between 0 mV and 40 mV in comparison to WT-CACNB4 (P < 0.05). The peak current amplitudes (P < 0.01), cell capacitance (P < 0.05), and current densities (P < 0.05) exhibited by cells expressing R468Q-CACNB4 were significantly greater than WT-CACNB4 (Fig. 3C).
Biophysical parameters for activation and inactivation were analyzed. There were no significant differences between WT and mutant channels in the activation and inactivation curves (Figs. 4A and C). Activation time constants were obtained from single-exponential fits of activation phase during 5-ms depolarizing steps. R468Q-CACNB4 showed a small increase in the time constant for activation at $-10$ mV in comparison to WT-CACNB4 (Fig. 4B). The current decay was fitted by a sum of two exponential functions. There were no significant differences between WT-CACNB4 and R468Q-CACNB4 with regard to the inactivation fast ($\tau_{\text{fast}}$) and slow ($\tau_{\text{slow}}$) time constants (Fig. 4D).

**Discussion**

The mortality rate of SMEI during early childhood is 15–18% (Dravet et al., 2005). The causes include status epilepticus, drowning, accidents, severe infection, and sudden unexpected death. Status epilepticus is life-threatening for SMEI patients. The clinical variability such as refractoriness of epilepsy, degree of mental retardation, and ataxia is observed in SMEI. This patient, who lapsed into brain death after antiepileptic drug-resistant status epilepticus represents one of the most catastrophic outcomes observed for SMEI.

The mechanisms that produce the wide phenotypic spectrum of epilepsy associated with SCN1A mutations are not clear. Some clinical and experimental observations raise the possibility that modifier genes or seizure susceptibility genes that are inherited from parents may contribute to the malignant phenotype of SMEI. First, approximately half of the SMEI probands have a family history of convulsive disorders. Epilepsy in relatives of probands with SMEI had the characteristics of idiopathic generalized epilepsy or febrile seizures (Benlounis et al., 2001; Singh et al., 2001). Considering that 90% of SCN1A mutations in SMEI probands arise de novo (Harkin et al., 2007), SMEI probands could have genetic predisposition to convulsive disorders involving genes other than SCN1A. Second, electrophysiological studies for generalized epilepsy with febrile seizures plus (GEFS+) or SMEI-associated SCN1A mutations have demonstrated alternations of biophysical parameters that are predicted to be gain-of-function or loss-of-function (Spampanato et al., 2001; Lossin et al., 2002; Lossin et al., 2003; Rhodes et al., 2004; Rhodes et al., 2005). Nonfunctional SCN1A mutations were more frequently observed in SMEI than GEFS+ (Sugawara et al., 2003; Lossin et al., 2003; Ohmori et al., 2006), but there was no simple correlation between the severity of epilepsy and the dysfunction of mutant channels. Third, the severity of epilepsy in a mouse model of SMEI harboring a truncated SCN1A mutation was influenced by the genetic background (Yu et al., 2006; Ogiwara et al., 2007). The strain C57BL/6J mice exhibited a decreased incidence of spontaneous seizures and longer survival in comparison to 129/SvJ mice. These observations indicate that a genetic modifier
may influence the clinical severity of epilepsy associated with SCN1A mutations.

We decided to screen the genes related to idiopathic epilepsy or febrile seizures plus for candidates of genetic modifiers or for enhancing the seizure susceptibility in SMEI patients. These epilepsy-related genes include SCN1B, SCN2A, CACNB4, CACNA1A, CACNA1H, CLCN2, GABRG2, GABRA1, GABRD, GABRB3 and others (George, 2004; Ashcroft, 2006; Heron et al., 2007). When we completed the process of searching for genetic modifiers, we found one mutation in CACNB4. Because only a small proportion of patients with GEFS+ and idiopathic generalized or partial epilepsy have identifiable these genes mutations, other families with SMEI patients may have other genetic modifiers besides CACNB4.

Voltage-dependent calcium channels (VDCC) are key mediators of calcium entry into neurons in response to membrane depolarization. Calcium influx mediates a number of essential neuronal responses, such as the release of neurotransmitters from presynaptic sites, the activation of calcium dependent enzymes, and gene expression such as the release of neurotransmitters from presynaptic sites, the activation of calcium dependent enzymes, and gene expression (Catterall, 2000; Kandel & Siegelbaum, 2000). VDCC are composed of the pore-forming α1 subunit and the accessory subunits β, α2δ and γ (Arikath & Campbell, 2003). Calcium channel β (Caβ) subunits promote functional expression of the α1 (Caα) subunits and increase localization of the channels at the plasma membrane. Caα (α4 is the predominant subunit associated with Ca2,1 (P/Q type calcium channel) (Dolphin, 2003). Mutations of the calcium channel CACNB4 and CACNA1A genes encoding Caα (α4 subunit and Ca2,1, respectively, have been reported in probands with generalized epilepsy and ataxia. Some of these mutations were located in the carboxy-terminal regions of the Caα,1 (Jouvenceau et al., 2001) or α4 subunit (Escayg et al., 2000) (Fig. 1C). Some studies have shown that the α1 subunit interacts with the α4 subunit on the cytoplasmic loop between transmembrane domains I–II and on the carboxy-terminal cytoplasmic region (Pragnell et al., 1994; Walker et al., 1998). These findings suggest that each carboxy-terminal region of the α1 and α4 subunits plays a critical role in the subunit-interaction. The R468Q-CACNB4 mutation detected in our patient was also located in the carboxy-terminal binding site of the β4 subunit. This mutation has the potential to alter the interaction between the α1 and β4 subunits because it results in an amino acid change from positively charged arginine to neutral charged glutamine.

The biophysical properties of idiopathic generalized epilepsy-related CACNB4 mutations have exhibited increases in current density in two mutations and acceleration of the fast-inactivation component.

Fig. 4. (A) Voltage dependence of activation measured during voltage steps to between −40 and +30 mV from a holding potential of −100 mV. The amplitudes of the tail currents were normalized to the maximal tail current amplitude. The mean values were plotted against test pulse potentials and fitted to the Boltzmann equation. Half-maximal activation occurred at −7.90±1.12 mV with a slope factor of 5.67±0.19 (n = 7) for WT-CACNB4 and at −8.55±0.49 mV with a slope factor of 5.55±0.14 (n = 8) for R468Q-CACNB4. (B) Voltage dependence of activation time constants for WT-CACNB4 and R468Q. The activation time constants were obtained from single-exponential fits of activation phase during 5-ms depolarizing steps. R468Q-CACNB4 showed a small increase in the time constant for activation at −10 mV in comparison to WT-CACNB4. (C) Voltage dependence of the inactivation. Ba2+ currents were evoked by a 20-ms test pulse to 0 mV after 10-ms repolarization to −100 mV following 2-s holding potential displacement from −100 to +30 mV with 10-mV increments. The mean values were plotted as a function of the potentials of the 2-s holding potential displacement, and were fitted to the Boltzmann equation. The membrane potentials for half-maximal inactivation and slope factors were as follows: WT, −47.05±1.79 mV and −6.33±0.16, n = 6; R468Q, −48.80±1.29 mV and −6.23±0.18, n = 6. (D) Ba2+ currents were evoked by 2-s test pulse. Current decay was fitted by a sum of two exponential functions. The mean inactivation time constants, τinact (left) and τinact (right), were plotted as a function of test potentials from −30 to 30 mV. The data are expressed as the mean±SEM of 4 and 6 BHK cells expressing the WT-CACNB4 and the R468Q-CACNB4, respectively. *P<0.05 and **P<0.01 versus WT-CACNB4.

Table 1. Modification of the biophysical properties of WT-CACNB4 and R468Q-CACNB4 by Ba2+ extracellularly applied to BHK cells expressing the WT-CACNB4 and the R468Q-CACNB4. The mean values were plotted against test pulse potentials and fitted to the Boltzmann equation. Half-maximal activation occurred at −7.90±1.12 mV with a slope factor of 5.67±0.19 (n = 7) for WT-CACNB4 and at −8.55±0.49 mV with a slope factor of 5.55±0.14 (n = 8) for R468Q-CACNB4.
in one mutation (Escay et al., 2000). R468Q-CACNB4 in this proband also showed a greater current density in comparison to the wild-type. R468Q-CACNB4 was carried by his father with a febrile seizure. We speculate that this mutation likely promotes seizure susceptibility and worsens the SMEI phenotype caused primarily by the SCN1A mutation.

SCN1A is expressed in GABAergic interneurons, haploinsufficiency of Na\textsubscript{v}1.1 channels in inhibitory neurons is the cause of seizures in a model mouse harboring a nonfunctional Na\textsubscript{v}1.1? Ca\textsubscript{v}2.1 channels are localized in high density in the presynaptic active zone (Westerenbroek et al., 1995; Wu et al., 1999). The depolarization of the terminal membrane causes Ca\textsubscript{v}2.1 channels to open. The resulting elevation in calcium ions is the signal that causes neurotransmitter to be released from synaptic vesicles (Takahashi & Momiyama, 1993; Regehr & Mintz, 1994). Once epileptic seizure begins, the greater Ca\textsubscript{v}2.1 currents caused by the R468Q-CACNB4 mutation may increase facilitation of the neurotransmitter release in the excitatory neurons under insufficient inhibitory neurons caused by the nonfunctional Na\textsubscript{v}1.1. In a resting cell the cytoplasmic free calcium level is held extremely low. The excessive entry of calcium into neuronal cells in the condition of over-excitation of neural circuits during a prolonged seizure may lead to neuronal cell death.

This study addresses the important issue of multifactorial epilepsy in the etiology of SMEI. However, we could not elucidate how much the R468Q-CACNB4 mutation affects the severe phenotype of epilepsy with a nonfunctional SCN1A. Our results from heterologous expression systems cannot simply be generalized to native neurons because many other factors can affect the functional consequences of the mutant channels. To determine whether or not a Ca\textsubscript{v}2.1 dysfunction may exacerbate the seizure phenotype of Scn1A mutant rodents, further studies in animal models carrying such double mutant channels are thus needed.

Conflict of interest statement
We have no conflicts of interest associated with this study.

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