COMMENTS

In this study, the authors show that subarachnoid hemorrhage (SAH) increases hypoxia inducible factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF) protein in the brainstem of rats. If these are further increased by deferoxamine (DFO), vasospasm can be attenuated. The authors correctly note that several possible mechanisms could be involved, such as increased HIF-1α protein, stabilization of HIF-1α in various protein complexes, and direct beneficial effects of DFO independent of HIF-1α and VEGF. Approximately 10 years ago, there was interest in iron chelators as a treatment for vasospasm, but this was never pursued clinically due to conflicting preclinical data, possible toxic effects, and, I suppose, the lack of a clinician to drive the study.

There continue to be some methodological flaws in vasospasm studies, including this one, that need to be known to researchers in the field. These include lack of blinding and randomization, the use of β-actin as an internal standard for molecular analysis, and the use of laser Doppler to compare blood flow between groups and/or over time (1). I have done them all wrong myself, but it would be helpful to not repeat them. Also, there seems to be a trend toward showing a smaller and smaller portion of the gel in molecular studies. It is more comforting, at least for the reviewer, to see the whole gel.

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Toronto, Canada

1. Ohkuma H, Tsurutani H, Suzuki S: Changes of beta-actin mRNA expression in canine vasospastic basilar artery after experimental subarachnoid hemorrhage. **Neurosci Lett** 311:9–12, 2001.

The authors reported that HIF-1α protein expression increased in the brainstem after SAH and that the administration of DFO further increased the protein in the brainstem and significantly attenuated basilar artery vasospasm and reduction of brainstem blood flow in rat SAH models. The effects of DFO in a double hemorrhage model seem interesting, and further study should be performed. Although the authors examined the expression of HIF-1α and speculated the mechanisms of antivasospastic effect and blood flow recovery by DFO, their conclusion that DFO-induced increase in HIF-1α protein levels and activity exerts significant attenuation of basilar artery vasospasm and of reduction in brainstem blood flow is too strong because they did not prove any direct action of HIF-1α on vasospasm and blood flow in their experiments. Although they showed that DFO increased HIF-1α protein in the brainstem, they did not show the increase of HIF-1α in vessel walls or the direct relationship between HIF-1α and the attenuation of vasospasm and reduction in brainstem blood flow. It seems that DFO attenuated the degree of vasospasm and increased brainstem blood flow by unknown mechanisms.

Kazuhiko Nozaki
Kyoto, Japan

In this study, the authors use a rodent SAH model using a single and double injection method to look at early (10 min) and late (up to 7 d) vasospasm in rats. They studied transcription factors that may be active in the regulation of these processes. Specifically, they studied HIF-1α, which is a transcription factor with the key ability to regulate a number of other factors, many of which are neuroprotective. These are involved in oxygen hemostasis, development of ischemia, and angiogenesis, and seem to regulate many genes, including erythrocyte and VEGF, as well as glucose transporter 1. The authors show that this factor HIF-1α protein is significantly increased at 10 minutes in the single injection model and 7 days in the double injection model. Importantly, at no time is there an upregulation of messenger ribonucleic acid transcription of HIF-1α during these studies. The authors note that times at which the protein has increased are times when there is increased spasm. Their hypothesis, however, is not that HIF-1α increases spasm, but rather that it is present in reaction to the spasm that is present. They then take the experiment further by using an iron chelator DFO mesylate, which decreases the metabolism HIF-1α and requires oxygen and tissue plasminogen activators to break it down. In these circumstances, the authors note an improvement in vasospasm. They also note that the increased HIF-1α protein does correlate as well with an increase in VEGF.

The authors studied these time points based primarily on the time of clinical presence of spasm in these models, but several large periods of time are not covered. The difference between messenger ribonucleic acid and protein expression for HIF-1α is clearly an intriguing part of this article and is not fully understandable. As HIF-1α could affect so many activities after ischemia, it is not clear whether they are seeing cause or effect in the increase in both VEGF and in spasm. Finally, the discussion should give the reader some idea of where this research will be going beyond observation and how they would enhance their studies to develop a causal relationship which may show treatment benefit.

Robert J. Dempsey
Madison, Wisconsin

Hishikawa et al. have investigated the relationship between HIF-1α expression and rodent SAH-associated vasospasm. Using both single and double hemorrhage models of SAH, HIF-1α protein levels and basilar artery diameter were assessed at 10 minutes, 6 hours, and on Days 1 and 2. The effects of intraperitoneal DFO were also evaluated. Results showed HIF-1α protein levels to be significantly elevated after SAH and correlated with the degree of cerebral vasospasm. DFO administration caused increased HIF-1α protein expression and attenuated cerebral vasospasm. The authors concluded that DFO may be a promising agent in clinical SAH by ameliorating SAH-associated cerebral vasospasm via induction of HIF-1α expression.

HIF-1α regulates the expression of numerous genes, including nitric oxide (NO) synthase, during hypoxia. In addition, the amount of intracellular oxygen available for other reactions, including further NO synthesis, increases as mitochondrial oxygen consumption is blunted (3). As NO inhibits mitochondrial activity, superoxide is produced to activate HIF-1α (2). Furthermore, high-output NO synthase can stabilize HIF-1α (1). Considering these interactions, the development of HIF-1α related clinical agents may be limited by this target's nonenzymatic function and multipotent regulators.

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New York, New York

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A CACNB4 mutation shows that altered $Ca_v2.1$ function may be a genetic modifier of severe myoclonic epilepsy in infancy

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ABSTRACT

Mutations of *SCN1A*, encoding the voltage-gated sodium channel α1 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 *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 Ba^{2+} current density compared with the wild-type CACNB4. The greater $Ca_v2.1$ 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.

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* (MIM# 182389), encoding the voltage-gated sodium channel α1 subunit ($Na_v1.1$), represent the most frequent genetic cause of SMEI (Claes et al., 2001; Sugawara et al., 2002; Ohmori et al., 2002). Although the percentage of family history of convulsive disorders ranges from 25% to 71% in several studies (Dravet et al., 2005), approximately 90% of *SCN1A* mutations in SMEI probands arise *de novo* (Harkin et al., 2007). Epilepsy in relatives of probands with SMEI had the characteristics of febrile seizures or idiopathic generalized epilepsy (Benlounis et al., 2001; Singh et al., 2001). SMEI probands could therefore possibly have a genetic predisposition to convulsive disorders in addition to SCN1A mutations.

We hypothesized that modifier genes or seizure susceptibility genes that are carried from parents may contribute to the malignant phenotype of SMEI. We decided to screen the genes related to idiopathic epilepsy or febrile seizures plus for candidates of genetic modifiers. We screened the *SCN1B*, *GABRG2*, and *CACNB4* genes in 38 *SCN1A* mutation-positive probands. We identified an SMEI proband who had a *de novo* R568X mutation of the *SCN1A* gene and another R468Q mutation of the *CACNB4* gene that was inherited from his father who had a history of febrile seizures.

Materials and methods

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

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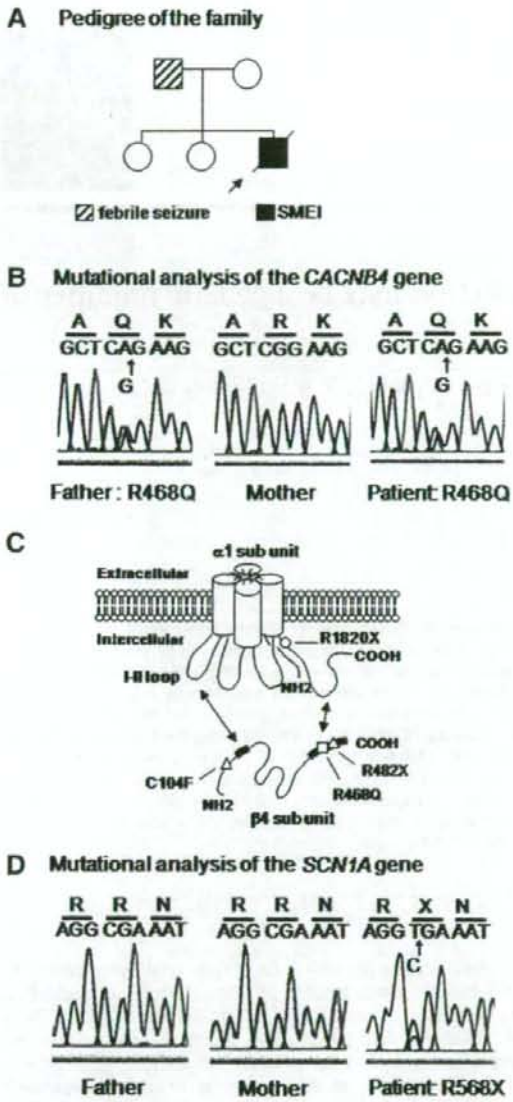

Fig. 1. Pedigree, sequencing results of the *SCN1A* gene and the *CACNB4* gene and schematic representation of the α1 and β4 subunits of the voltage-dependent calcium channel. (A) The pedigree of the proband's family. The proband with SMEI is indicated by an arrow. His father had one febrile seizure at the age of 7 years. (B) The same type of mutation of the *CACNB4* gene was found in both the proband and his father. A substitution of G with A at nucleotide position 1403 leads to amino acid change from arginine to glutamine at codon 468 of the *CACNB4* gene (GenBank accession no. NM000726). (C) Two binding regions of the β4 subunit for interaction with the α1 subunit are indicated by bold lines. Square; missense mutation in SMEI and febrile seizure; triangle; missense or nonsense mutations reported in idiopathic generalized epilepsy reported by Escayg et al. (2000); circle; nonsense mutation reported by Jouvenceau et al. (2001). (D) The proband has a *de novo* mutation of the *SCN1A* gene. A substitution of C with T at nucleotide position 1702 results in a change from arginine to stop codon at codon 568 of the *SCN1A* gene (GenBank accession no. AB093548).

were frequently provoked by 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-IRES-human 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 TGT A-3′) and SCN1A-R568X-AS1 (5′-TTG CGG CCG CTC ACC TTG GTG AAA ATA GGG A-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 pIRES-EYFP vector (BD Biosciences Clontech, Palo Alto, CA) digested with NotI.

Rat CACNB4 cDNA of pCIneo-CACNB4 (WT) was amplified by PCR with CACNB4-R468Q-S4 primer (5′-AGG GCC CAG AAG AGT AGG AAC CG-3′) and AS1 (5′-TCC CCC TGA 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 pKF18K2 digested with HincII. The cDNA region was confirmed by DNA sequencing and digested with ApaI and NotI. pCIneo-CACNB4 (R468Q) was constructed by replacement of the ApaI–NotI fragment of pCIneo-CACNB4(WT) by the missense 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 $CaCl_2$, 1 $MgCl_2$, 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 pCIneo-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 patchclamp amplifier (HEKA Electronik, 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, 4 $MgCl_2$, 5 EGTA, 2 Na_2ATP, 8 creatine-phosphate and 5 HEPES; adjusted to pH 7.3 with CsOH. The bath solution contained (in mM): 3 $BaCl_2$, 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 Ca_v β subunit with the carboxyl-terminal of Ca_v α1 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 convulsion 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 Ba^{2+} currents evoked by 30-ms depolarizing pulses from −40 to 50 mV for $Ca_v2.1$ 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 Ba^{2+} 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).

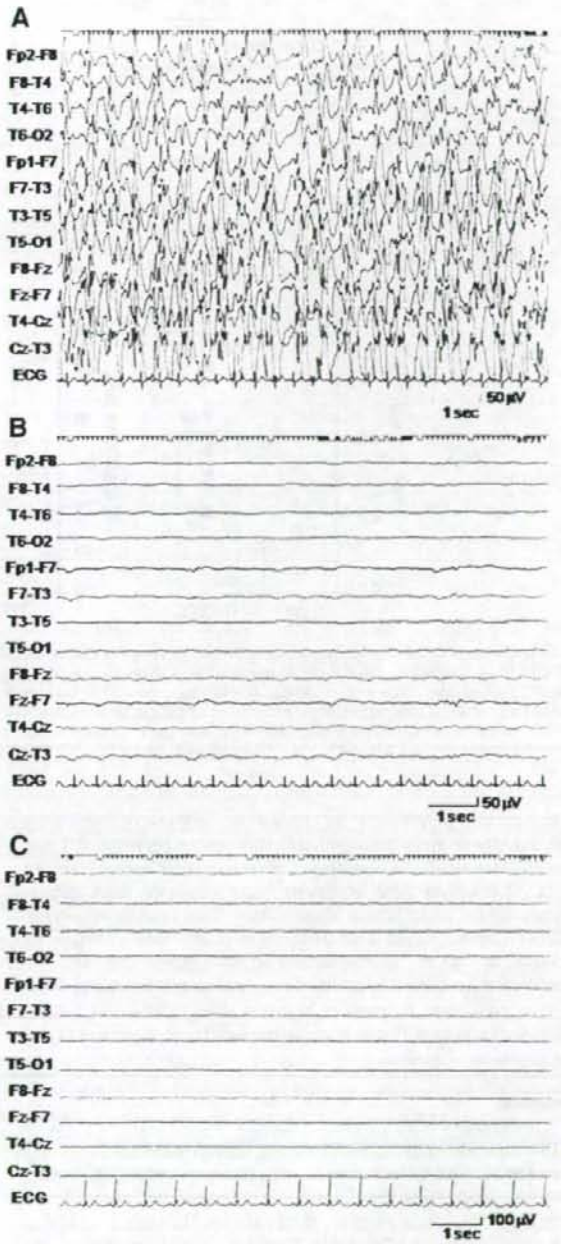

Fig. 2. The change in the EEGs after a prolonged seizure which lasted for 4 h. (A) The EEG was recorded 13 h after the convulsion began. Proband A still had intermittent seizures. Thereafter, the convulsions were finally controlled by anesthesia. (B)Although anesthesia was stopped after controlling the status epilepticus, his EEGs showed a diffuse low voltage pattern after 62 h. (C) His EEG showed no physiologic brain activity on the seventh day.

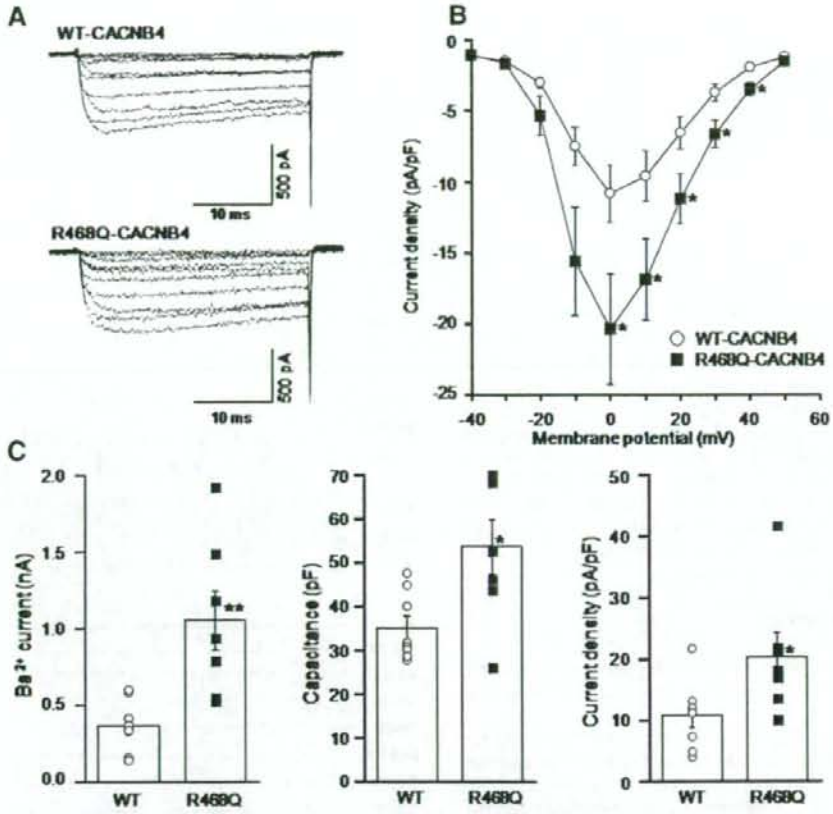

Fig. 3. Comparison of $Ca_v2.1$ currents recorded in BHK cells expressing WT-CACNB4 and R468Q-CACNB4. (A) Representative whole-cell Ba^{2+} currents. Families of Ba^{2+} currents were evoked by 30-ms depolarizing pulses from −40 to +50 mV with 10-mV increments from a holding potential of −100 mV. (B) The current–voltage relationships of $Ca_v2.1$ expressing either WT-CACNB4 (open circle, $n=8$) or R468Q-CACNB4 (filled square, $n=7$). The current density of $Ca_v2.1$ expressing R468Q-CACNB4 was significantly larger than WT between 0 and +40 mV ($P<0.05$). (C) Distribution of the peak current amplitude (left), cell capacitance (middle), and current density (right) at 0 mV. Individual values of $Ca_v2.1$ currents in the BHK cells expressing WT-CACNB4 or R468Q-CACNB4 and their means±S.E. are shown. 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. *$P<0.05$ and **$P<0.01$ versus WT-CACNB4.

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 (τ_{fast}) and slow (τ_{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 mutational screening of the *SCN1B, GABRG2*, and *CACNB4* genes in 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 (Catterall, 2000; Kandel & Siegelbaum, 2000). VDCC are composed of the pore-forming α1 subunit and the accessory subunits β, α2δ and γ (Arikkath & Campbell, 2003). Calcium channel β ($Ca_v\beta$) subunits promote functional expression of the α1 ($Ca_v\alpha_1$) subunits and increase localization of the channels at the plasma membrane. Ca_v β4 is the predominant subunit associated with $Ca_v2.1$ (P/Q type calcium channel) (Dolphin, 2003). Mutations of the calcium channel *CACNB4* and *CACNA1A* genes encoding Ca_v β4 subunit and $Ca_v2.1$, respectively, have been reported in probands with generalized epilepsy and ataxia. Some of these mutations were located in the carboxyl-terminal regions of the $Ca_v2.1$ (Jouvenceau et al., 2001) or β4 subunit (Escayg et al., 2000) (Fig. 1C). Some studies have shown that the α1 subunit interacts with the β4 subunit on the cytoplasmic loop between transmembrane domains I–II and on the carboxyl-terminal cytoplasmic region (Pragnell et al., 1994; Walker et al., 1998). These findings suggest that each carboxyl-terminal region of the α1 and β4 subunits plays a critical role in the subunit-interaction. The R468Q-CACNB4 mutation detected in our patient was also located in the carboxyl-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 neutrally 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

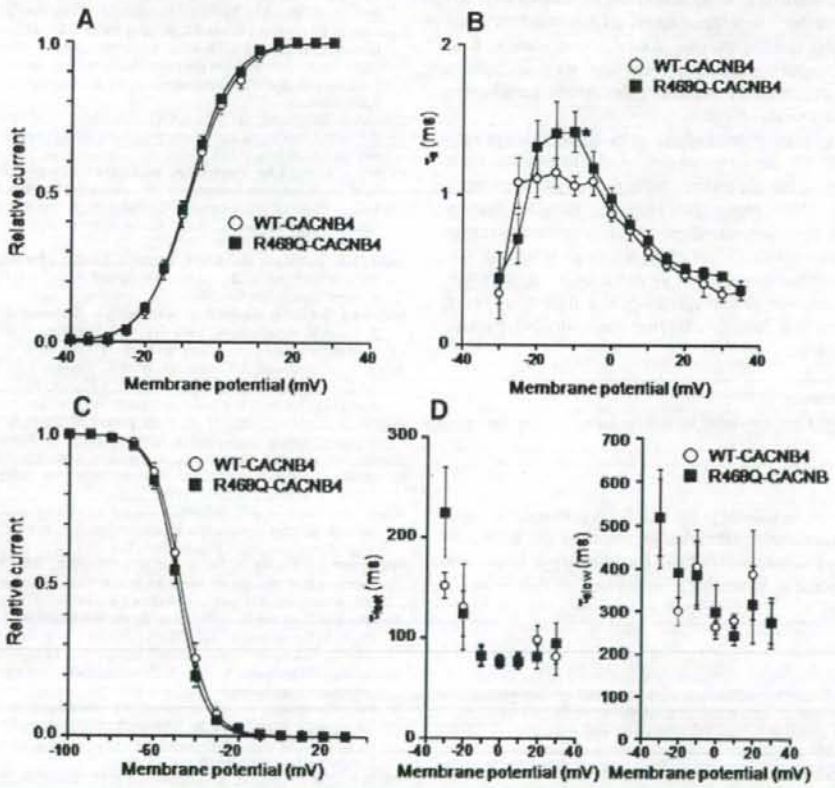

Fig. 4. (A) Voltage dependence of activation measured during voltage steps to between −40 and +30 mV from a holding potential of −100 mV. The amplitudes of the tail currents were normalized to the maximal tail current amplitude. The mean values were plotted against test pulse potentials and fitted to 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. Ba^{2+} 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) Ba^{2+} currents were evoked by 2-s test pulse. Current decay was fitted by a sum of two exponential functions. The mean inactivation time constants, τ_{fast} (left) and τ_{slow} (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.

in one mutation (Escayg 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 $Na_v1.1$ channels in inhibitory neurons is the cause of seizures in a model mouse harboring a nonfunctional SCN1A mutation (Yu et al., 2006). Our electrophysiological analysis of R568X-SCN1A demonstrated no greater current than background activity. The proband likely had the same molecular mechanism, namely reduced GABAergic inhibition.

How does $Ca_v2.1$ channel dysfunction affect the severity of epilepsy in the SMEI proband harboring the nonfunctional $Na_v1.1$? $Ca_v2.1$ channels are localized in high density in the presynaptic active zone (Westenbroek et al., 1995; Wu et al., 1999). The depolarization of the terminal membrane causes $Ca_v2.1$ 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 $Ca_v2.1$ 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 $Na_v1.1$. 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 $Ca_v2.1$ 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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