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Modulatory Proteins Can Rescue a Trafficking Defective Epileptogenic $Na_v1.1$ Na^+ Channel Mutant

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Familial epilepsies are often caused by mutations of voltage-gated Na^+ channels, but correlation genotype–phenotype is not yet clear. In particular, the cause of phenotypic variability observed in some epileptic families is unclear. We studied $Na_v1.1$ (SCN1A) Na^+ channel α subunit M1841T mutation, identified in a family characterized by a particularly large phenotypic spectrum. The mutant is a loss of function because when expressed alone, the current was no greater than background. Function was restored by incubation at temperature $<30°C$, showing that the mutant is trafficking defective, thus far the first case among neuronal Na^+ channels. Importantly, also molecular interactions with modulatory proteins or drugs were able to rescue the mutant. Protein–protein interactions may modulate the effect of the mutation *in vivo* and thus phenotype; variability in their strength may be one of the causes of phenotypic variability in familial epilepsy. Interacting drugs may be used to rescue the mutant *in vivo*.

Key words: **sodium; current; epilepsy; excitability; trafficking; GEFS+; SMEI; seizure; SCN1A**

Introduction

Mutations of neuronal voltage-gated Na^+ channel genes are the most common known cause of familial epilepsy. Voltage-gated Na^+ channels are complexes consisting of a principal pore-forming and voltage-sensing α subunit and accessory β subunits (see Fig. 1). Nine α subunits ($Na_v1.1$-$Na_v1.9$) and four β subunits ($\beta1$-$\beta4$ or $Na_v\beta1.1$-$Na_v\beta1.4$) have been identified thus far (Catterall et al., 2005). The primary sequence of the α subunits contains four homologous domains (DI-DIV), which each contain six predicted transmembrane segments (S1-S6), and N-terminal and C-terminal cytoplasmic domains; the β subunits contain a single transmembrane segment, an N-terminal extracellular immunoglobulin-like domain and a C-terminal short cytoplasmic domain (Catterall, 2000).

Mutations of $Na_v1.1$ (SCN1A) cause generalized epilepsy with febrile seizures plus (GEFS+) type 2, severe myoclonic epilepsy of infancy (SMEI), intractable childhood epilepsy with generalized tonic-clonic seizures (ICEGTC), and simple febrile seizures (FS); mutations of $Na_v1.2$ (SCN2A) cause benign familial neonatal-infantile seizures (BFNIS) and benign familial infantile seizures (BFIS); mutations of $\beta1$ (SCN1B) cause GEFS+ type 1 (Avanzini and Franceschetti, 2003; Scheffer and Berkovic, 2003; Noebels, 2003; George, 2005; Mantegazza et al., 2005a; Meisler and Kearney, 2005; Scalmani et al., 2006; Avanzini et al., 2007).

The genetic and functional analysis of Na^+ channel epileptogenic mutations has generated a vast amount of data, but genotype–phenotype correlation has not been clarified yet. For instance, the functional effects of some GEFS+ mutations are similar to those of SMEI mutations, a much more severe epilepsy characterized also by cognitive impairment and high mortality rate (Dravet et al., 2005). In particular, some GEFS+ missense mutations cause complete loss of function of the mutant channel, as the majority of SMEI mutations (Lossin et al., 2003). Moreover, GEFS+ families show a large phenotypic variability that ranges from mild phenotypes to very severe ones (Scheffer and Berkovic, 1997). Thus, it has been hypothesized that genetic background could modulate disease severity modifying the properties of the mutant protein (Singh et al., 2001).

We studied the M1841T GEFS+ mutation of $Na_v1.1$ α subunit ($hNa_v1.1$-M1841T), a missense mutation that replaces with a threonine the methionine at position 1841, located in a region of the C-terminal cytoplasmic domain involved in interactions with accessory subunits (Spampanato et al., 2004) (see Fig. 1). The mutation cosegregates with epileptic seizures in a GEFS+ family whose affected members show extreme phenotypes, ranging from simple febrile convulsions in the mildest case to SMEI in the most severe one (Annesi et al., 2003). Thus, the study of this mutation could shed light on the mechanism of the phenotypic variability in epilepsies caused by mutations of Na^+ channels.

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We found that $hNa_v1.1$-M1841T is a trafficking defective loss of function mutant rescued by molecular interactions with modulatory proteins and drugs. Thus, *in vivo* variability in the strength of protein–protein interactions may be one of the causes of the striking phenotypic variability observed in some epileptic families, and it might be possible to develop drugs that specifically target folding defective epileptogenic neuronal Na^+ channel mutants.

Materials and Methods

Tagging, mutagenesis, and cloning. The cDNA for human $Na_v1.1$ Na^+ channel α subunit ($hNa_v1.1$) was provided by Dr. Jeff Clare (GlaxoSmithKline, Stevenage, Herts, UK) and encodes the shorter splice variant isoform of 1998 aa (Noda et al., 1986; Schaller et al., 1992); we numbered $Na_v1.1$ protein sequence accordingly. We generated the chimeric construct $YFPhNa_v1.1$ (N-terminal tagging of $hNa_v1.1$ with yellow fluorescent protein) (Fig. 1) by a three-step strategy. In the first step, a 2100 bp 5′ fragment of $hNa_v1.1$ coding sequence was amplified by PCR using as template the plasmid pCDM8-$hNa_v1.1$, which has been described previously (Mantegazza et al., 2005a), and the following primers: 5′-ACTTAATTAATCCGGAGCCACCATGGAG (forward, which introduced the restriction site for *Bsp*EI upstream of the ATG codon) and 5′-TTGCTCGTTGCCTTTGGGAAGG (reverse). The fragment was then subcloned into pEYFP-C1 (Clontech, Palo Alto, CA) using *Bsp*EI and *Xba*I restriction sites to fuse the 5′ fragment of $hNa_v1.1$ coding sequence to YFP conserving the correct reading frame. In the second step, an AgeI restriction site was introduced in pCDM8-$hNa_v1.1$ upstream of the ATG codon by means of Quick Change XL Kit (Stratagene, La Jolla,CA),using5′-TTAATTAACCGGTCGCCACCATGG as forward primer and 5′-CATGGTGGCGACCGGTTAATTAAG as reverse primer. In the third step, the 5′ fragment of $hNa_v1.1$ fused to YFP was subcloned into the AgeI-mutated pCDM8-$hNa_v1.1$ plasmid using AgeI and *Eco*RI restriction sites. Thus, we obtained the chimera between YFP and $hNa_v1.1$ containing the whole $hNa_v1.1$ coding sequence in the plasmid pCDM8-$YFPhNa_v1.1$.

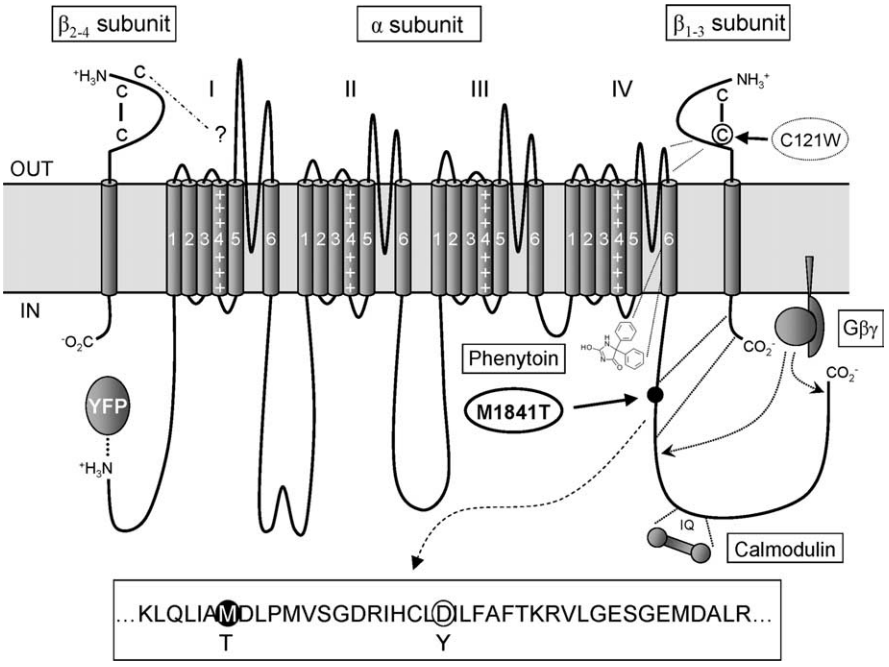

Figure 1. Schematic representation of the Na^+ channel molecular complex. The principal α subunit is shown in the center, flanked by auxiliary subunits (β2/4 on the left, β1/3 on the right); interactions with calmodulin, G-protein $\beta\gamma$ subunits, and phenytoin are also shown, as well as the N-terminal tagging of the α subunit with YFP, which we used in some of the experiments. Cylinders represent probable α-helical transmembrane segments; IN and OUT are the intracellular and extracellular sides of the membrane. The α subunit consists of four homologous domains (I–IV), each formed by six transmembrane segments (S1–S6) and N-terminal and C-terminal cytoplasmic domains; the S4 segments are the positively charged voltage sensors, the S5 and S6 segments of each homologous domain and the re-entrant loops between them form the pore (Catterall, 2000). The GEFS+ M1841T mutation of $Na_v1.1$ α subunit (Annesi et al., 2003) that we studied is depicted as a black circle. β subunits are related proteins with a large extracellular Ig-like domain, a single transmembrane segment and a short cytoplasmic C-terminal region; the Ig-like domain is stabilized by an intramolecular disulfide bridge (Catterall, 2000; Isom, 2001). Lymphocyte T-killer CD8 receptor, which we used as negative control, has a similar structure (Cole and Gao, 2004). The GEFS+ C121W mutation of β1, which we used in some of the experiments, destroys the intramolecular disulfide bridge and inhibits β1 function (Wallace et al., 1998; Meadows et al., 2002) and is depicted as a hollow circle. β2 and β4 subunits are covalently linked to the α subunit by disulfide bridges (dashed-dotted line), but the cysteine residues implicated have not been identified yet. β1 and β3 subunits have noncovalent intracellular and extracellular interactions with the α subunit (dashed lines) (McCormick et al., 1998; Qu et al., 1999; Meadows et al., 2001; Spampanato et al., 2004). M1841T lies in a 41-residue area, highlighted in the inset, comprising a cytoplasmic binding domain for β1 and, probably, β3 (Spampanato et al., 2004). The GEFS+ mutation D1855Y (Spampanato et al., 2004) is also shown in the inset; we used a different numeration than the original D1866Y, because the clone that we used codifies for a shorter $hNa_v1.1$ variant (see Results). Part of the receptor site of intracellular pore blocker drugs is located in the S6 segment of domain IV (Catterall, 2000), ~70 aa upstream of M1841T; interaction with the antiepileptic drug phenytoin is depicted. Several modulatory proteins bind Na^+ channel α subunits; the interactions that have been better characterized occur in the C-terminal cytoplasmic domain (Abriel and Kass, 2005). For instance, calmodulin binds to an IQ apo-calmodulin binding motif located 70 aa downstream of M1841T; binding site(s) of G-protein $\beta\gamma$ subunits has not been completely clarified yet, but the last 28 aa of the C-terminal domain are important and they may bind also to a QxxER domain located 36 amino acids downstream of M1841T (Mantegazza et al., 2005b).

The epileptogenic mutation M1841T was introduced both in pCDM8-$hNa_v1.1$ and in pCDM8-$YFPhNa_v1.1$ with Quick Change XL Kit (Stratagene), using the following primers: 5′-GCTCATTGCCACGGATTTGCCC (forward) and 5′-TGGGCAAATCCGTGGCAATGAGC (reverse).

$hNa_v1.1$ has high rearrangement rate when propagated in bacteria, so the plasmids containing $hNa_v1.1$ were grown at 28°C for >48 h to decrease the rearrangements, and the entire coding sequence was sequenced after each propagation.

The plasmids pSP64T-hβ1 (encoding human Na^+ channel β1 subunit) and pRc/CMV-hβ2 (encoding human Na^+ channel β2 subunit) were provided by Dr. Al George (Vanderbild University, Nashville, TN). We numbered β1 protein sequence starting with the initial methionine, thus including the leader sequence identified by Isom et al. (1992). To express with the same plasmid both the protein of interest and cyan fluorescent protein (CFP) as reporter, the cDNAs were subcloned into the bicistronic mammalian expression vector pIRES-CFP (Clontech). We subcloned hβ1 using *Not*I and *Eco*RI restriction sites, obtaining the bicistronic plasmid pIRES-CFP-β1. We excised hβ2 from pRc/CMV-hβ2 digesting with *Xba*I, blunting with T4 polymerase (NEB) and then digesting with *Not*I. The fragment was subcloned into pIRES-CFP between *Eco*RI (blunted with T4 polymerase) and *Not*I restriction sites, obtaining the plasmid pIRES-CFP-β2.

We introduced the epileptogenic mutation C121W into hβ1 (obtaining the mutant hβ1C121W) (Fig. 1 and see Fig. 5*C*) (Wallace et al., 1998) by sequential PCR with the flanking primers 5′-AGGCGTGTACGGTGGGAGGT and 5′-ACAATCTTAGCGCAGAAGTC and the mutagenic primers 5′-GAGTGGCACGTCTACCG and 5′-ACGTGCCACTCGTAGTC. The mutated PCR product was subcloned into pIRES-CFP (obtaining the plasmid pIRES-CFP-β1-C121W) and sequenced to confirm the presence of the introduced mutation and the absence of spurious mutations.

To obtain the truncated $h\beta1_{STOP}$ mutant (McCormick et al., 1998; Meadows et al., 2001) with deleted C-terminal intracellular domain (see Fig. 5*C*), we substituted with Quick Change XL Kit a stop codon (TAA)

for the isoleucine 185 codon, using pIRES-CFP-β1 as template and the mutagenic primers 5′CTACAAGAAGTAAGCTGCCGCCAC (forward) and 5′GTGGCGGCAGCTTACTTCTTGTAG (reverse), obtaining the plasmid pIRES-CFP-$\beta 1_{STOP}$.

We cloned human Na^+ channel β3 and β4 subunits from tsA-201 cells (a cell line derived from human embryonic kidney cells). Total RNA was extracted by means of RNAeasy midi kit (Qiagen, Hilden, Germany). Total RNA (1 μg), oligo(dT)12–16 (50 μM), random hexamers (30 ng/ul), and Super Script III Reverse Transcriptase (Invitrogen, San Diego, CA) were used for first strand cDNA synthesis. Reactions, including RNase H treatment to remove RNA template, were performed according to manufacture protocols. hβ3 and hβ4 cDNA was obtained by PCR amplification using the forward primers: 5′-TTGACCGAGGATATCTCTCTGTGT for hβ3 and 5′-CGGCGCAGATATCCGGGGTAG for hβ4 (which introduced also an *Eco*RV restriction site), and the reverse primers 5′-GTCCCTCAGGAATTCAGGCCAC for hβ3 and 5′-CAGGCACGTGAATTCTACGGTC for hβ4 (which introduced also an *Eco*RI restriction site). The amplified fragments were then digested with *Eco*RV and *Eco*RI, and subcloned into the bicistronic vector pIRES-CFP for hβ3 and pIRES-dsRED2 for hβ4, obtaining the plasmids pIRES-CFP-hβ3 and pIRES-dsRED2-hβ4. The nucleotide sequences of the cloned subunits were identical to GenBank accession number NM_018400 for hβ3 and NM_174934 for hβ4.

The plasmid EBO-pcD-CD8 expressing human lymphocyte T-killer CD8 receptor has been described previously (Margolskee et al., 1988). We cotransfected pCDM8-YFPhNa_v1.1-M1841 with EBO-pcD-CD8 and tested the cells after 48 h identifying cotransfected cells by YFP fluorescence and labeling with Dynal anti-CD8 antibody-coated beads (Dynabeads; Invitrogen).

The plasmid pJPA7 encoding rat calmodulin was provided by Dr. John Adelman (Vollum Institute, Portland, OR). The coding sequence and 5′ and 3′ UTRs were amplified by PCR with the following primers: 5′-CTCTCCACA*GATATC*CACTCCCAG (forward, which introduced an *Eco*RV restriction site), and 5′-TAAACAAGT*GGATCC*ATGGCGG (reverse, which introduced a *Bam*HI restriction site). The amplified fragment was subcloned into pIRES-dsRED2 bicistronic vector (Clontech) using *Eco*RV and *Bam*HI restriction sites, and sequenced for control.

The cDNA of G-protein $\beta_2\gamma_3$ subunits was provided by Dr. Melvin Simon (Caltech, Pasadena, CA). The bicistronic plasmids pIRES-YFP-Gβ_2 and pIRES-CFP-Gγ_3, respectively, expressing G-protein β_2 and γ_3 subunits, have been described previously (Mantegazza et al., 2005b).

Cell culture and transfection. TsA-201 cells were cultured in modified Dulbecco's medium and Hams-F12 mix supplemented with 10% fetal bovine serum and transiently transfected by using the $CaPO_4$ method as described previously (Mantegazza et al., 2005b). hNa_v1.1 was cotransfected with the bicistronic plasmids expressing interacting proteins using an hNa_v1.1/interacting protein molar ratio of 2/1. CD8 receptor was cotransfected using the same ratio.

Electrophysiology. Transfected cells were selected visually by their fluorescence using a Zeiss Axiovert 100 microscope (Zeiss, Oberkochen, Germany) equipped with selective epifluorescence filters for YFP (Chroma 41028; Chroma, Rockingham, VT), CFP (Chroma 31044v2), and dsRed (Chroma 31004). If used alone, plasmids that do not express a fluorescent protein were cotransfected with the plasmid pEYFP-C1 using a protein-of-interest/YFP molar ratio of 10/1. Whole-cell patch-clamp recordings were performed at room temperature (22–25°C) using an Axopatch 1D amplifier with pClamp 8.2 software (Molecular Devices, Union City, CA). Pipette resistance was between 1.5 and 2.0 MΩ, and series resistance was between 2.5 and 4.5 MΩ. Maximum recorded current was 5.9 nA. Cell capacitance and series resistance errors were carefully compensated (~85%) before each run of the voltage clamp protocol; maximum accepted voltage clamp error was 2.5 mV. Remaining linear capacity and leakage currents were eliminated by P/N leak subtraction procedure. Current signals were filtered at 10 kHz and sampled at 100 kHz.

The current–voltage (*I–V*) relationships were obtained by applying depolarizing pulses from a −90 mV holding potential. The conductance–voltage (g-V) relationships (activation curves) were calculated from the *I–V* relationships according to $g = I\mathrm{Na}/(V - \mathrm{ENa})$, where *I*Na is the peak Na^+ current measured at potential, *V*, and ENa is the calculated equilibrium potential (69 mV). The normalized activation and inactivation curves were fit to Boltzmann relationships in the form: $y = 1/\{1 + \exp[(V - V1/2)/k]\}$, where *y* is normalized gNa or *I*Na, *V*, the membrane potential, *V*1/2, the voltage of half-maximal activation (*V*a) or inactivation (*V*h), and *k* is a slope factor. The inactivation protocol used a test pulse to 0 mV, preceded by 100-ms-long prepulses at depolarized potentials; holding potential was −90 mV. Recovery from inactivation was studied with a test pulse to 0 mV, preceded by a 100 ms long prepulse to 0 mV and by increasingly longer repolarizations to the holding potential (−90 mV).

Solutions and drugs. The extracellular recording solution contained the following (in mM): 140 NaCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, pH 7.4, with NaOH. The pipette recording solution contained the following (in mM): 195 NMDG, 10 NaCl, 4 $MgCl_2$, 5 EGTA, 10 HEPES, pH 7.2, with H_3PO_4. Drugs were purchased from Sigma (St. Louis, MO). Thapsigargin and curcumin were dissolved in DMSO, phenytoin in methanol. After the transfection, cells were incubated with the drugs at the indicated concentrations and for the indicated times (see Results); the electrophysiological recordings were done after 36–48 h. The final DMSO or methanol concentration was always <1‰.

Data analysis. Fits were achieved using the Levenberg–Marquardt algorithm with Origin 7.5 (OriginLab, Northampton, MA). The statistical analysis were made using Origin or SYSTAT 9 (SPSS, Chicago, IL). The results are given as mean ± SEM, and threshold *p* value for statistical significance was 0.05. Statistical comparisons were performed with the *t* test or ANOVA with Tukey's posttest.

Results

We studied the M1841T GEFS+ mutation of hNa_v1.1 Na^+ channel α subunit transfecting human tsA-201 cells with human DNA clones. We used the shorter (1998 aa) splice variant isoform of the human Na_v1.1 Na^+ channel (Noda et al., 1986; Schaller et al., 1992), which could be the predominant Na_v1.1 variant expressed in brain (Schaller et al., 1992) and that we have used previously for functional studies (Oliveira et al., 2004; Mantegazza et al., 2005a,b). According to the different numeration, the mutation that we studied would be M1852T in the longer isoforms used by (Spampanato et al., 2004). The mutation D1866Y that these authors have studied would be D1855Y in our clone (Fig. 1).

hNa_v1.1-M1841T is a loss of function mutant rescued by the interaction with accessory subunits

In cells transfected with hNa_v1.1-M1841T, Na^+ currents had very small amplitude (Fig. 2*A*) similarly to endogenous Na^+ currents that are often present in untransfected tsA-201 cells (Fig. 2*G*). Recordings from cells transfected with wild-type hNa_v1.1 showed much larger Na^+ currents (Fig. 2*B*), suggesting that hNa_v1.1-M1841T is a loss-of-function mutant, similar to most of SMEI and some GEFS+ Na^+ channel mutants.

Because M1841T is in a region important for interactions with β1 accessory subunit (Spampanato et al., 2004), we tested the effect of β1 in cotransfection experiments and observed a partial rescue of the function of the mutant channel, the current of which reached on average ~50% of the amplitude of the wild-type current (Fig. 2*C*). The effect of β1 Na^+ current amplitude was selective for the mutant channel, in fact cotransfection of β1 with wild-type hNa_v1.1 did not significantly modify the amplitude of the current (Fig. 2*D*).

The effects of β1 cotransfection are evident also analyzing the current–voltage plots obtained applying test pulses to membrane potentials between −60 and +15 mV for hNa_v1.1-M1841T and hNa_v1.1-M1841T cotransfected with β1 (Fig. 2*E*) and for hNa_v1.1 and hNa_v1.1 cotransfected with β1 (Fig. 2*F*). The amplitude of the inward Na^+ current was maximal at approximately

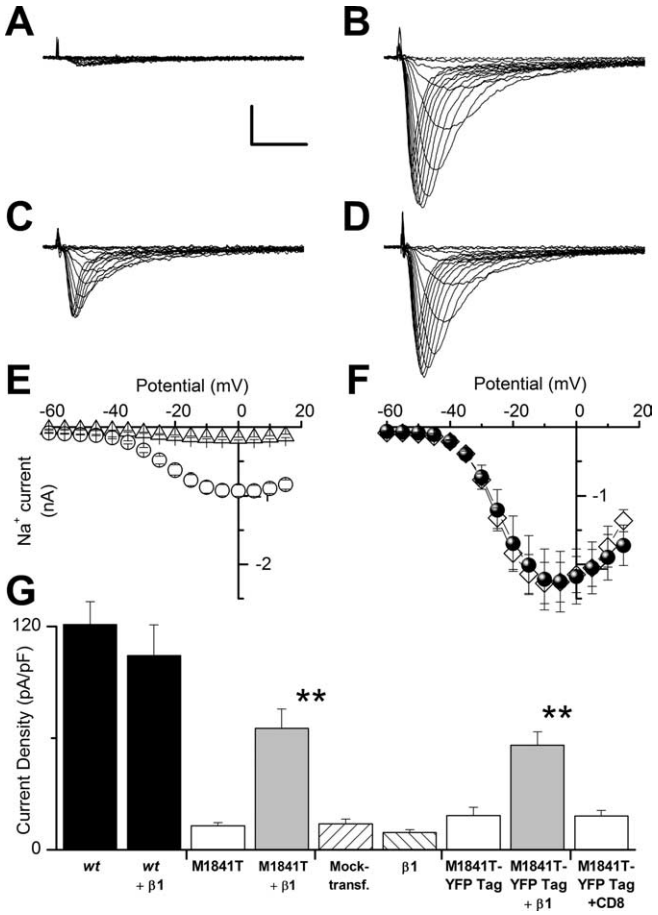

Figure 2. $hNa_v1.1$-M1841T is a loss-of-function mutant rescued by $\beta1$. Representative Na^+ current traces recorded with depolarizing voltage steps between −60 and +15 mV in 5 mV increments, from a holding potential of −90 mV in tsA-201 cells transfected with $hNa_v1.1$-M1841T (***A***), $hNa_v1.1$ (***B***), $hNa_v1.1$-M1841T, and $\beta1$ accessory subunit (***C***) or $hNa_v1.1$ and $\beta1$ (***D***). ***E***, Mean current–voltage plots from cells transfected with $hNa_v1.1$-M1841T (triangles) or cotransfected with $hNa_v1.1$-M1841T and $\beta1$ (circles). Calibration: 1 nA, 1 ms (for all panels). ***F***, Mean current–voltage plots from cells transfected with $hNa_v1.1$ (diamonds) or cotransfected with $hNa_v1.1$ and $\beta1$ (filled circles). ***G***, Bar graph showing mean maximum current density in cells transfected with $hNa_v1.1$ (121.1 ± 12.2 pA/pF; $n = 29$), $hNa_v1.1$ and $\beta1$ (104.5 ± 16.4 pA/pF; $n = 15$), $hNa_v1.1$-M1841T (12.9 ± 1.4 pA/pF; 30), $hNa_v1.1$-M1841T and $\beta1$ (65.4 ± 10.3 pA/pF; $n = 21$), empty pCDM8 and pEYFP-N1 (mock transfection; 13.9 ± 2.5 pA/pF; $n = 6$), $\beta1$ alone (9.2 ± 1.6 pA/pF; $n = 4$), $YFPNa_v1.1$-M1841T fusion protein (18.4 ± 3.3 pA/pF; $n = 7$), $YFPhNa_v1.1$-M1841T fusion protein and $\beta1$ (56.2 ± 7.1 pA/pF; $n = 12$), $YFPhNa_v1.1$-M1841T fusion protein and CD8 receptor (18.2 ± 2.9 pA/pF; $n = 22$). Black, Wild-type (wt) channel in all the conditions; white, $hNa_v1.1$-M1841T without β subunits; gray, $hNa_v1.1$-M1841T with β subunits; stripes, transfections without α subunit. The results are given as mean ± SEM. $^*p < 0.05$; $^{**}p < 0.01$.

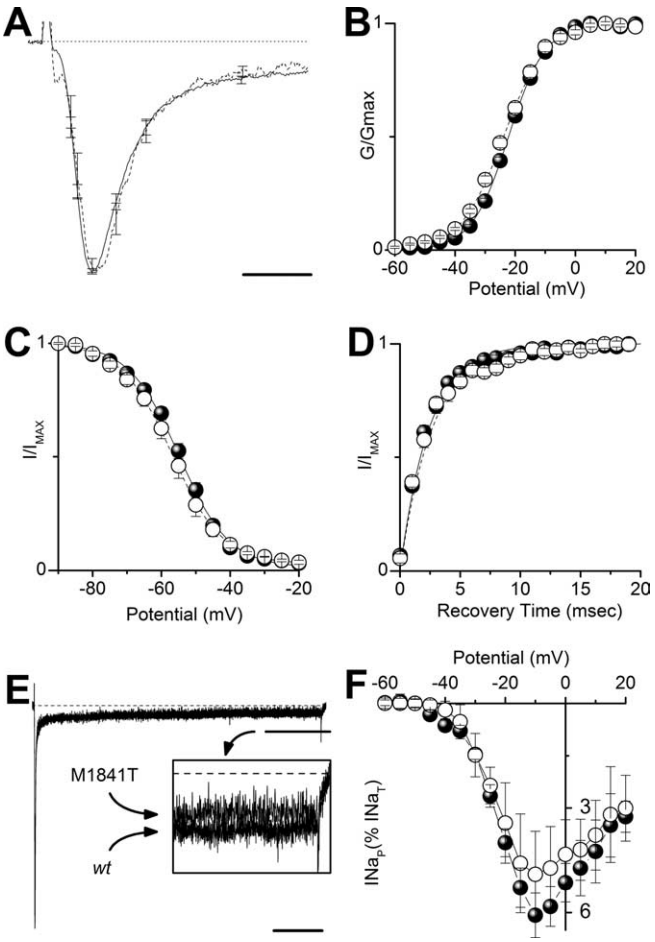

Figure 3. Functional properties of wild-type and mutant channels cotransfected with $\beta1$. ***A***, Mean normalized current traces recorded with a depolarizing step to 0 mV for wild-type (wt) $hNa_v1.1$ (solid line) and $hNa_v1.1$M1841T (dashed line), cotransfected with $\beta1$ subunit; the error bars are the SEM of selected data points. Calibration: 500 μs. ***B***, Mean voltage dependence of activation for $hNa_v1.1$ (filled circles) and $hNa_v1.1$-M1841T (open circles); the lines are Boltzmann relationships, the parameters of which were calculated averaging the parameters of the fits of the single cells: solid line, $hNa_v1.1$ (Va = −22.2 ± 0.5 mV, Ka = 6.2 ± 0.3; $n = 15$); dashed line, $hNa_v1.1$-M1841T (Va = −23.9 ± 0.7, Ka = 7.1 ± 0.4; $n = 15$). ***C***, Mean voltage dependence of inactivation; the lines are Boltzmann relationships with the following mean parameters: $hNa_v1.1$, Vh = −54.9 ± 0.5 mV, Kh = 7.3 ± 0.4 mV ($n = 11$); $hNa_v1.1$-M1841T, Vh = 57.0 ± 0.6 mV, Kh = 7.4 ± 0.4 mV ($n = 11$). ***D***, Mean kinetics of recovery from a 100 ms inactivating pulse to 0 mV; the lines are mean fits of single exponential relationships to the data: $hNa_v1.1$, $\tau_{REC} = 2.5 \pm 0.2$ ms ($n = 12$); $hNa_v1.1$-M1841T, $\tau_{REC} = 2.7 \pm 0.3$ ms ($n = 12$). ***E***, Superimposed wild-type $hNa_v1.1$ and $hNa_v1.1$-M1841T mean normalized current traces (same as in ***A***) shown for the whole duration of the depolarizing step to 0 mV (150 ms). Calibration: 25 ms. The inset shows the final 35 ms of the traces expanded on both axis. ***F***, Mean current–voltage plots for persistent Na^+ current (I_{NaP}). The results are given as mean ± SEM.

−10 mV for $hNa_v1.1$ and −5 mV for $hNa_v1.1+\beta1$, and approximately −5 mV for $hNa_v1.1$-M1841T+$\beta1$. Currents recorded with transfection of just $hNa_v1.1$-M1841T peaked at 0 mV, similar to endogenous Na^+ currents (data not shown).

Analysis of current densities (which gives a more accurate measurement of channel expression) gave similar results. Comparison of maximal current densities in the different experimental conditions (Fig. 2*G*) confirmed that cotransfection of $\beta1$ did not modify $hNa_v1.1$ current density but increased $hNa_v1.1$-M1841T current density by fivefold ($p < 10^{-5}$), at ~50% of wild-type level. When transfected alone, current density of $hNa_v1.1$M1841T was not significantly different compared with that of mock-transfected cells (cotransfected with pEYFP-N1 and empty pCDM8 plasmids), demonstrating that $hNa_v1.1$M1841T is a complete loss-of-function mutant. Moreover, transfection of $\beta1$ had no significant effect on endogenous Na^+ currents, demonstrating that it selectively rescues M1841T mutant.

To more accurately monitor the expression of the α subunit, we studied the effect of the mutation and the rescuing activity of $\beta1$ subunit also expressing wild-type and mutant α subunits tagged with YFP on the N terminus (Fig. 1). Average current densities in these experimental conditions were not significantly different than with untagged α subunits (Fig. 2*G*). Therefore, in the subsequent statistical analysis, we used pooled data, except for the analysis of the gating properties (Fig. 3), which was done using only recordings of untagged channels.

We also tested, as negative control, the effect of cotransfection

with lymphocyte T-killer CD8 receptor (Cole and Gao, 2004), which has a structure similar to β1 (an N-terminal extracellular Ig-like domain, a single transmembrane segment and a C-terminal short cytoplasmatic domain) but does not interact with Na^+ channels. CD8 has been used extensively as reporter gene in cotransfections with Na^+ channel α and β subunits, with no evidence of modification of the functional properties of the channel (Qu et al., 2001; Mantegazza et al., 2005b). Fig. 2*G* shows that CD8 did not induce any functional rescue of $hNa_v1.1$-M1841T, thus the effect of β1 is attributable to a selective interaction between Na^+ channel α and β1 subunits.

Therefore, interaction with β1 can rescue $hNa_v1.1$-M1841T and thus, unlike the mutation D1855Y (Fig. 1) studied by Spampanato et al. (2004), M1841T does not inhibit the interaction between β1 and α subunit.

M1841T does not alter the main functional properties of the channel

We observed no significant differences in the main functional properties of $hNa_v1.1$ and $hNa_v1.1$-M1841T mutant cotransfected with β1 accessory subunit. In fact, kinetics of activation and inactivation of transient Na^+ current (Fig. 3*A*), voltage dependence of activation (Fig. 3*B*) and inactivation (Fig. 3*C*), kinetics of recovery from a 100 ms inactivating step at 0 mV (Fig. 3*D*) were similar for $hNa_v1.1$ and M1841T. Persistent slowly inactivating Na^+ current (INa_P) is important for neuron excitability and epileptogenic mutations can increase its amplitude (Lossin et al., 2002). We recorded INa_P with 150-ms-long depolarizing steps to 0 mV (Fig. 3*E*), and we found no significant difference in the kinetics of the decay or in its amplitude (expressed as percentage of transient Na^+ current). Fig. 3*F* shows mean current–voltage plots obtained applying test pulses to membrane potentials between -60 and $+20$ mV and quantifying INa_P as the mean current remaining between 45 and 55 ms after the beginning of the voltage step (Mantegazza et al., 2005b). INa_P amplitude was maximal at approximately -10 mV both for $hNa_v1.1$ and $hNa_v1.1$M1841T, and there was no significant difference in current amplitude over the range of potentials.

Thus, when rescued by β1, $hNa_v1.1$M1841T shows functional properties that are similar to those of the wild-type subunit. In these conditions, the mutation causes just a decrease in Na^+ current.

$hNa_v1.1$M1841T is trafficking defective

We hypothesized that $hNa_v1.1$M1841T could be a trafficking defective mutant, because β1 was able to rescue the mutant α subunit, and the mutation did not modify the main properties of the channel. In fact, mutations involved in human diseases can cause protein trafficking defects, often because of the incorrect folding of the mutant protein that cannot pass the quality control systems of the endoplasmic reticulum (ER) and is degraded (Cobbold et al., 2003; Sitia and Braakman, 2003). Interacting molecules can, in some cases, rescue trafficking defective mutants (Bernier et al., 2004), as β1 did in our experiments. Incubation at permissive temperature (in general, <30°C) often increases the quantity of functional trafficking defective mutants transported to the plasma membrane (Bernier et al., 2004).

We tested whether $hNa_v1.1$-M1841T is trafficking defective incubating transfected cells at 27°C for 48 h before the recordings. The current density in $hNa_v1.1$-M1841T transfected cells incubated at 27°C was 5.7-fold larger ($p < 10^{-5}$) than in control (Fig. 4*A*), reaching 65% of the value of the wild-type channel. Thus, plasma membrane targeting of the mutant channel is temperature sensitive. Current density of $hNa_v1.1$-M1841T cotransfected with β1 and incubated at 27°C was 12.2-fold larger than for $hNa_v1.1$-M1841T alone ($p < 10^{-5}$), 2.7-fold larger than for $hNa_v1.1$-M1841T cotransfected with β1 ($p = 9 \times 10^{-5}$), and 2.1-fold larger than for $hNa_v1.1$-M1841T alone incubated at 27°C ($p = 3 \times 10^{-4}$). Notably, in these conditions, the current density of the mutant channel was not significantly different than that of the wild-type channel maintained at 37°C, and some cells showed a larger current density (range, 25.6–584.4 pA/pF for $hNa_v1.1$-M1841T+β1 incubated at 27°C, compared with 40.5–236.2 pA/pF for $hNa_v1.1$ alone and 32.9–231.1 pA/pF for $hNa_v1.1+\beta1$ maintained at 37°C).

We also found that expression of wild-type channel is temperature sensitive (Fig. 4*A*). In fact, incubation at 27°C significantly increased current density both of $hNa_v1.1$ expressed alone (1.8-fold larger compared with $hNa_v1.1$ expressed alone at 37°C; $p = 0.008$) and of $hNa_v1.1$ coexpressed with β1 (1.7-fold larger com-

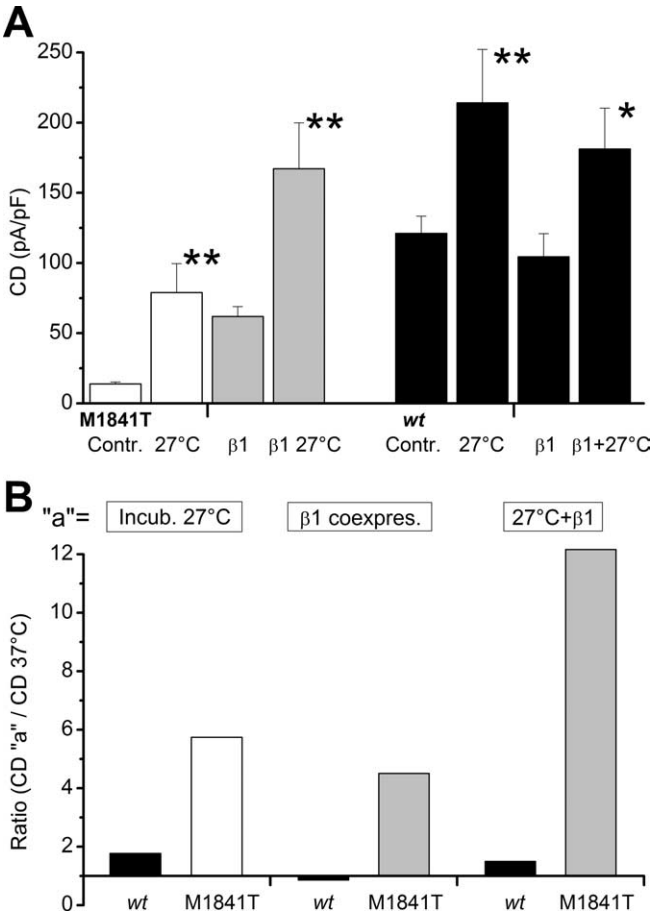

Figure 4. M1841T is trafficking defective. ***A***, Mean maximum current density in cells transfected with $hNa_v1.1$-M1841T (untagged and tagged channels pooled, 13.9 ± 1.3 pA/pF; $n = 36$), $hNa_v1.1$-M1841T incubated at 27°C (78.9 ± 20.7 pA/pF; $n = 8$), $hNa_v1.1$-M1841T and β1 (untagged and tagged α subunits pooled, 61.6 ± 7.0 pA/pF; $n = 33$), $hNa_v1.1$-M1841T and β1 incubated at 27°C (167.2 ± 32.6 pA/pF; $n = 16$), $hNa_v1.1$ (same data as in Fig. 1), $hNa_v1.1$ incubated at 27°C (214.1 ± 38.1 pA/pF; $n = 29$), $hNa_v1.1$ and β1 (same data as in Fig. 1), $hNa_v1.1$ and β1 incubated at 27°C (181.2 ± 29.1 pA/pF; $n = 13$). The results are given as mean ± SEM. *$p < 0.05$; **$p < 0.01$. ***B***, Same data as in ***A*** shown as the ratio between the current density of wild-type (wt) and mutant α subunits in the experimental condition "a" displayed above the graph and the current density in the control condition (Contr.) (α subunit expressed alone with no incubation at 27°C); "a" is incubation at 27°C of cells transfected with α subunit alone (left), coexpression of α and β1 subunits (center), or incubation at 27°C of cells cotransfected with α and β1 subunits (right). Black, Wild-type channel in all the conditions; white, $hNa_v1.1$-M1841T without β subunits; gray, $hNa_v1.1$-M1841T with β subunits.

pared with $hNa_v1.1$ coexpressed with $\beta1$ at 37°C; $p = 0.03$). However, the effect was much smaller than for $hNa_v1.1$-M1841T, as is shown more clearly by the ratio plots in Figure 4*B*. The plots also show more clearly the influence of $\beta1$ and the synergic effect of $\beta1$ and low temperature on the expression of the mutant, effects that were not observed for the wild-type channel.

This indicates that $hNa_v1.1$-M1841T is a trafficking-defective mutant probably retained in cytoplasmatic compartments and degraded when expressed alone, but rescued and delivered to the plasma membrane when coexpressed with $\beta1$ subunit or incubated at permissive temperature. We did confocal laser scanning imaging of cells transfected with YFP-tagged subunits and obtained variable results that are presented in supplemental material (available at www.jneurosci.org).

All the accessory subunits can rescue $hNa_v1.1$-M1841T

We evaluated by cotransfection experiments the effect of $\beta2$, $\beta3$, and $\beta4$ accessory subunits on α subunit functional expression in tsA-201 cells. Consistently with the results obtained with $\beta1$, also the other accessory β subunits did not significantly modify the current density of wild-type $hNa_v1.1$ (Fig. 5*A*). However, we found that all the β subunits have a statistically significant effect on the functional rescue of the mutant channel (Fig. 5*B*). In fact, $\beta2$ subunit increased Na^+ current density of cotransfected $hNa_v1.1$-M1841T by 2.2-fold ($p = 0.008$), $\beta3$ by twofold ($p = 0.005$), and $\beta4$ by 2.9-fold ($p < 10^{-5}$), and the increase is significant also compared with CD8 (Fig. 2*G*): $\beta2$, $p = 0.045$; $\beta3$, $p = 0.03$; $\beta4$, $p = 0.001$. Interestingly, $\beta3$ is structurally very similar to $\beta1$ but has much lower rescuing capacity on $hNa_v1.1$-M1841T.

There are probably several binding sites between α and β subunits. In particular, $\beta1$ can associate with the α subunit both through intracellular and the extracellular domains (Fig. 1) (McCormick et al., 1998, 1999; Qu et al., 1999; Meadows et al., 2001; Spampanato et al., 2004). To determine which domain of $\beta1$ is more important for rescuing $hNa_v1.1$-M1841T, we studied the effect of $\beta1$ subunits with defective extracellular domain or with deleted intracellular domain.

The GEFS+ mutation C121W of $\beta1$ (Fig. 1 and Fig. 5*C*) destroys a disulfide bridge in the extracellular Ig-loop, and the mutant is a loss of function, the capacity of which to modulate α subunit functions and to establish extracellular protein–protein interactions is impaired (Wallace et al., 1998; Meadows et al., 2002). Coexpression of $hNa_v1.1$-M1841T and $\beta1$C121W mutants induced a 2.6-fold increase in current density (Fig. 5*C*) ($p = 10^{-5}$), at 60% of the level of wild-type $\beta1$ subunit (which induced a 4.5-fold increase), and the increase is significant also compared with CD8 (Fig. 2*G*): $p = 0.001$. The $\beta1_{STOP}$ is a truncated protein with a deletion of the whole intracellular domain (the last 34 aa) (Fig. 5*C*), but it is not a complete loss-of-function mutant, because it shows reduced association with $Na_v1.2$ α subunit when expressed in Chinese hamster lung cells (Meadows et al., 2001) but not when expressed in *Xenopus* oocytes, where it is fully functional (McCormick et al., 1998). Coexpression in tsA-201 cells of $hNa_v1.1$-M1841T and $\beta1_{STOP}$ resulted in a 2.3-fold increase in current density (Fig. 5*C*) ($p = 5 \times 10^{-5}$) at 50% of the level of wild-type $\beta1$, and the increase is statistically significant also compared with CD8 (Fig. 2*G*): $p = 0.007$.

Thus, the interaction between the cytoplasmic C-terminal domains of α and $\beta1$ subunits is sufficient for a partial rescue of $hNa_v1.1$-M1841T, but also extracellular interactions play a role and can induce some rescue. Interestingly, according to yeast two hybrid experiments, $\beta2$ does not bind to $Na_v1.1$ cytoplasmatic C-terminal region (Spampanato et al., 2004), but it is able to partially rescue $hNa_v1.1$-M1841T, similarly to $\beta1_{STOP}$. Therefore, also interactions that are distant from the C-terminal region may rescue the trafficking defective α subunit.

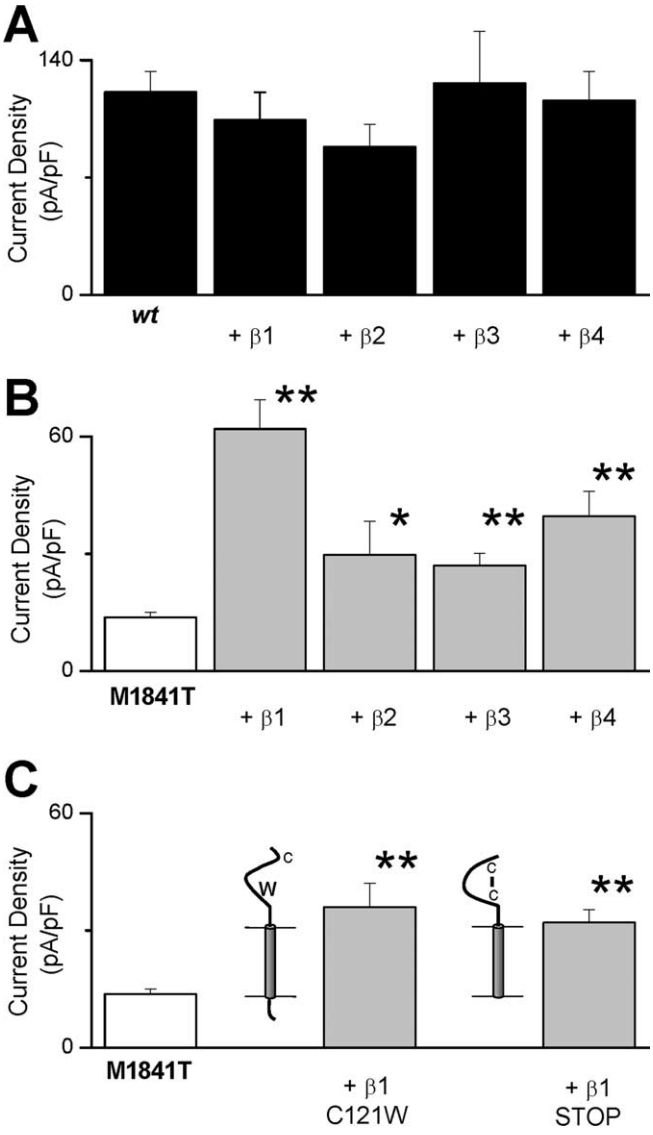

Figure 5. $hNa_v1.1$-M1841T is rescued by all of the accessory subunits. ***A***, Mean maximum current density in tsA-201 cells transfected with wild-type (wt) $hNa_v1.1$ (same as in Fig. 1), or cotransfected with $hNa_v1.1$ and $\beta1$ (same as in Fig. 1), $\beta2$ (88.3 ± 13.4 pA/pF; $n = 12$), $\beta3$ (126.4 ± 30.7 pA/pF; $n = 9$), or $\beta4$ (115.9 ± 17.3 pA/pF; $n = 8$) accessory subunits. ***B***, Cells transfected with $hNa_v1.1$-M1841T alone (same as in Fig. 3) or cotransfected with $hNa_v1.1$-M1841T and $\beta1$ (same as in Fig. 3), $hNa_v1.1$-M1841T and $\beta2$ (29.7 ± 8.6 pA/pF; $n = 13$), $hNa_v1.1$-M1841T and $\beta3$ (26.9 ± 3.1 pA/pF; $n = 15$), or $hNa_v1.1$-M1841T and $\beta4$ (39.6 ± 6.3 pA/pF; $n = 12$); note the scale difference compared with ***A***. ***C***, Cells transfected with $hNa_v1.1$-M1841T alone (same as above) or cotransfected with the GEFS+ C121W $\beta1$ mutant (35.9 ± 6.2 pA/pF; $n = 14$) or $\beta1_{STOP}$ mutant (32.1 ± 3.3 pA/pF; $n = 24$); diagrams are schematic representations of $\beta1$C121W (left) and $\beta1_{STOP}$ (right) mutants. Results are given as mean ± SEM. Black, Wild-type channel in all the conditions; white, $hNa_v1.1$-M1841T without β subunits; gray, $hNa_v1.1$-M1841T with β subunits.*$p < 0.05$; **$p < 0.01$.

Other molecular interactions can rescue $hNa_v1.1$-M1841T

We tested the effectiveness of other interactions in rescuing $hNa_v1.1$-M1841T by coexpression experiments. Na^+ channel α subunits bind to several modulatory proteins, and the binding sites that have been better characterized are in the C-terminal cytoplasmic domain (Abriel and Kass, 2005). We studied the

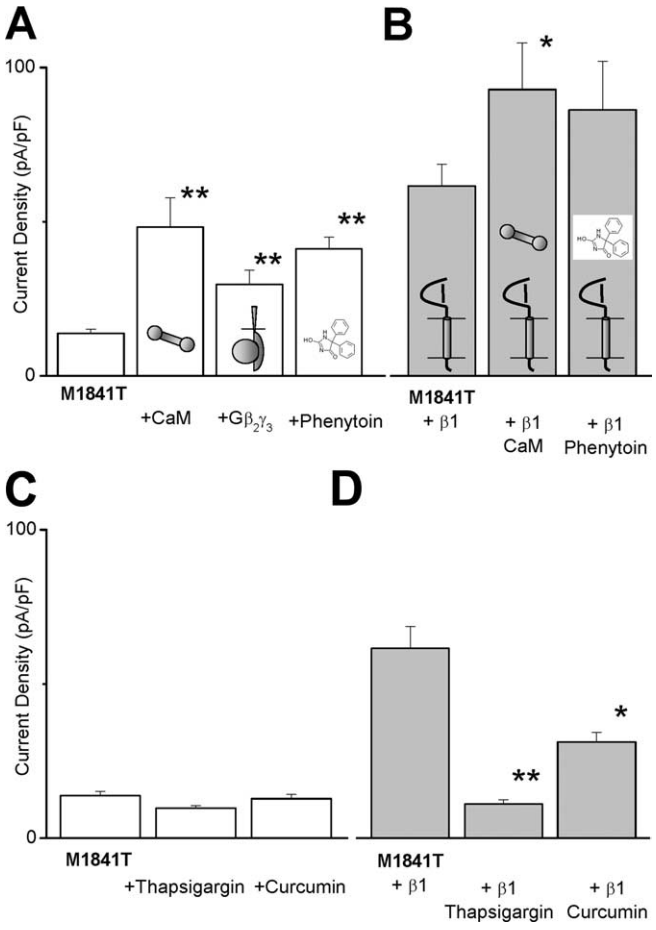

Figure 6. $hNa_v1.1$-M1841T is rescued by molecular interactions but not by intracellular Ca^{2+} stores depletion. ***A***, Mean maximum current density in tsA-201 cells transfected with $hNa_v1.1$-M1841T alone (same as in Fig. 3), cotransfected with $hNa_v1.1$-M1841T and calmodulin (CaM; 48 ± 10 pA/pF; $n = 19$), or with $hNa_v1.1$-M1841T and G-protein $\beta_2\gamma_3$ subunits (29.7 ± 4.6 pA/pF; $n = 24$), transfected with $hNa_v1.1$-M1841T alone and incubated for 24 h with phenytoin 100 μM (41.2 ± 3.8 pA/pF; $n = 20$); cartoons refer to Figure 1. ***B***, Cells cotransfected with $hNa_v1.1$-M1841T and $\beta1$ subunit (same as in Fig. 3), cotransfected with $hNa_v1.1$-M1841T, $\beta1$, and calmodulin (93 ± 15 pA/pF; $n = 14$), cotransfected with $hNa_v1.1$-M1841T and $\beta1$ and incubated with phenytoin (81 ± 15 pA/pF; $n = 16$). ***C***, Cells transfected with $hNa_v1.1$-M1841T alone in control (same as in Fig. 3), incubated for 24 h with 1 μM thapsigargin (9.8 ± 0.8 pA/pF; $n = 5$), or for 6 h with 10 μM curcumine (12.8 ± 1.4 pA/pF; $n = 5$). ***D***, Cells cotransfected with $hNa_v1.1$-M1841T and $\beta1$ subunit in control (same as in Fig. 3), incubated with thapsigargin (11.1 ± 1.3 pA/pF; $n = 10$), or incubated with curcumine (31.2 ± 3.1 pA/pF; $n = 5$). Results are given as mean ± SEM. White, $hNa_v1.1$-M1841T without $\beta1$ subunit; gray, $hNa_v1.1$-M1841T with $\beta1$ subunit. *$p < 0.05$; **$p < 0.01$.

rescuing activity of two modulatory proteins that interact with this domain: calmodulin and G-protein $\beta\gamma$ subunits ($G\beta\gamma$).

A calmodulin IQ binding domain is located 70 aa downstream of M1841T (Fig. 1). Cotransfection of calmodulin with $hNa_v1.1$-M1841T induced a 3.5-fold increase in Na^+ current density (Fig. 6*A*) ($p = 6 \times 10^{-5}$), and the increase was significant also compared with CD8 (Fig. 2*G*) ($p = 10^{-3}$) but did not have significant effects on the current density of the wild-type channel (132 ± 25 pA/pF; $n = 10$) (data not shown). Similarly to $\beta1$ subunit, calmodulin coexpression had a synergic effect with incubation at 27°C. In fact, in these conditions, current density was 131 ± 22 pA/pF ($n = 10$) (data not shown), 2.7-fold larger than $hNa_v1.1$-M1841T coexpressed with calmodulin but maintained at 37°C ($p = 5 \times 10^{-5}$). More importantly, also calmodulin and $\beta1$ had a synergic effect, because coexpression of the two proteins at 37°C resulted in a significant 1.5-fold increase in current density compared with $hNa_v1.1$-M1841T cotransfected with just $\beta1$ (Fig. 6*B*; $p = 0.04$), and it was not significantly different compared with wild-type $hNa_v1.1$.

The motifs implicated in the binding of G-protein $\beta\gamma$ subunits have not been clarified completely yet, but the last 28 aa of the C terminus are important for modulation, and a QxxER motif located 36 aa downstream of M1841T mutation may play a role (Mantegazza et al., 2005b) (Fig. 1). Cotransfection of $G\beta_2\gamma_3$ with $hNa_v1.1$-M1841T induced a 2.2-fold increase in Na^+ current density (Fig. 6*A*) ($p = 5 \times 10^{-4}$), and the increase was significant also compared with CD8 (Fig. 2*G*) ($p = 0.037$) but did not have significant effects on the current density of the wild-type channel (149 ± 27 pA/pF; $n = 10$) (data not shown).

A binding site for intracellular pore blockers of Na^+ channels is in transmembrane segment S6 of domain IV (Catterall, 2000), ~70 aa upstream of M1841T (Fig. 1). We studied the rescuing activity of the antiepileptic Na^+ channel pore blocker phenytoin, incubating transfected tsA-201 cells for 24 h with 100 μM phenytoin before the recordings. Incubation with the drug increased $hNa_v1.1$-M1841T current density by threefold (Fig. 6*A*) ($p < 10^{-5}$), showing that also this interaction can rescue the mutant channel. We also studied the synergic effect of $\beta1$ and phenytoin incubating cells cotransfected with $hNa_v1.1$-M1841T and $\beta1$. In these experimental conditions, there was a large scattering in current densities and no significant effect (Fig. 6*B*).

The rescue activity that we have observed cotransfecting proteins that have been shown to have interactions with Na^+ channel α subunits might in theory be an artifact caused by the overexpression of the proteins, and thus to nonspecific effects on the folding of $hNa_v1.1$-M1841T. However, the results that we obtained cotransfecting CD8 receptor rule out this hypothesis, because we showed that an overexpressed protein with structure similar to Na^+ channel β subunits, but that does not interact with α subunits, cannot rescue the mutant.

Thus, $hNa_v1.1$-M1841T can be partially rescued by interactions with modulatory proteins and also by molecular interactions with drugs.

Drug-induced depletion of ER Ca^{2+} stores does not rescue $hNa_v1.1$-M1841T

It has been shown that depletion of ER Ca^{2+} stores by inhibitors of Ca^{2+} pumps, such as curcumin and thapsigargin, can rescue some trafficking-defective mutants (Cobbold et al., 2003; Egan et al., 2004).

To assess whether these agents could rescue M1841T mutant, we incubated the cells for 24 h with 1 μM thapsigargin or for 6 h with 10 μM curcumin before the recordings. In these conditions, we found no significant increase in current density (Fig. 6*C*). The drugs were dissolved in DMSO, so we tested the effect of incubation with 1% DMSO, and we found no significant effects (91 ± 18 pA/pF; $n = 10$) (data not shown). Interestingly, in cells cotransfected with $hNa_v1.1$-M1841T and $\beta1$, we observed a significant reduction in current density ($p = 3 \times 10^{-4}$ for thapsigargin; $p = 0.05$ for curcumin) (Fig. 6*D*). In particular, thapsigargin completely blocked the rescuing effect of $\beta1$. This inhibitory action may be attributable to a Ca^{2+} dependence of the interactions between α and β subunits in the ER.

Discussion

GEFS+ is a childhood-onset autosomal dominant genetic epilepsy with ~60% penetrance, characterized by fever-induced seizures also at >6 years of age (FS+) associated with various types

of generalized epilepsy (Scheffer and Berkovic, 1997). GEFS+ families show heterogeneous phenotypes, comprising milder febrile seizures and the more severe myoclonic-astatic epilepsy (Scheffer and Berkovic, 1997).

SMEI is a very severe drug-resistant epileptic encephalopathy presenting with frequent seizures, slowed psychomotor development, mental decline, ataxia, and high mortality rate (Dravet et al., 2005), and is attributable to mutations of $Na_v1.1$ often causing complete loss of function (Meisler and Kearney, 2005; Mulley et al., 2005). Most SMEI mutations arose *de novo*, but in few cases, they have been inherited from mildly affected parents (Claes et al., 2001; Nabbout et al., 2003). A family history of epilepsy has been reported for some SMEI cases, and it has been proposed to extend the GEFS+ spectrum including SMEI as the most severe phenotype (Singh et al., 2001).

Thus, GEFS+ spectrum is characterized by a remarkable variability, but it is not clear how this variability is generated. Moreover, similarly to many SMEI mutations, some GEFS+ mutations cause a complete loss of function (Lossin et al., 2003), complicating genotype–phenotype correlation and thus early diagnosis and genetic counseling (Ferraro et al., 2006).

Our data show that the mutation M1841T of $Na_v1.1$ Na^+ channel, identified in a GEFS+ family with a particularly large phenotypic spectrum ranging from mild FS+ to SMEI (Annesi et al., 2003), causes a complete loss of function of $Na_v1.1$, similarly to other described GEFS+ mutations (Lossin et al., 2003). This effect is analogous to that of many SMEI mutations that cause truncations and consequently complete loss of function of Nav1.1, the haploinsufficiency of which generates seizures probably by decreasing excitability of GABAergic neurons, as observed in $Na_v1.1$ knock-out mice (Yu et al., 2006). Therefore, $hNa_v1.1$-M1841T is a loss of function mutant that can cause the SMEI phenotype observed in one member of the GEFS+ family (Annesi et al., 2003).

We have shown that 48 h incubation at 27°C before the experiment rescues $hNa_v1.1$-M1841T. This temperature sensitivity is typical of trafficking-defective mutants (Cobbold et al., 2003). Trafficking defects are usually caused by the inability of the mutant protein to correctly fold and pass ER quality control system, and thus by its subsequent degradation (Cobbold et al., 2003; Sitia and Braakman, 2003). Incubation at permissive temperature typically rescues several of these misprocessed proteins (Denning et al., 1992; Payne et al., 1998; Halaban et al., 2000; Valdivia et al., 2002; Anderson et al., 2006). This has been interpreted as a kinetic effect because of more functional proteins able to correctly fold and escape the quality control of the ER because of the slowed kinetics of folding (Bernier et al., 2004). Trafficking defective mutants are rescued also by molecular interactions, probably through stabilization of a correct folding intermediate (Bernier et al., 2004). We found that several molecular interactions can rescue $hNa_v1.1$-M1841T: coexpression with accessory β subunits, calmodulin or G-protein $\beta2\gamma3$ subunits can increase current density, in some conditions to levels that were not significantly different than those of wild-type $Na_v1.1$, which is consistent with the limited penetrance of GEFS+ mutations. However, relative expression levels *in vivo* may be different than in our experiments; thus, functional rescue in neurons may be not complete even if several interacting proteins are present. We also have shown that interactions and incubation at permissive temperature are synergic, probably because decreased kinetics of folding can improve stabilization of folding intermediates operated by interactions.

β subunits can increase Na^+ current density and membrane insertion of wild-type α subunits in some expression systems (Isom et al., 1992, 1995) but not in others (Morgan et al., 2000; Qu et al., 2001; Yu et al., 2003). Thus, in some conditions, β subunits can act as chaperones also for wild-type α subunits, but in our experimental conditions, they did not have any significant effects.

Therefore, $hNa_v1.1$-M1841T is the first identified trafficking-defective neuronal Na^+ channel mutant involved in a human disease, and it can be rescued by accessory and modulatory proteins.

The protein–protein interactions that we studied may be particularly important in determining phenotypic variability. Modifications in the type or strength of these interactions may modify the rescuing potential of interacting proteins, modulating the effect of the epileptogenic mutation *in vivo* and thus phenotype severity. This hypothesis is supported by the fact that the M145T mutation of $Na_v1.1$, causing pure simple febrile seizures, induces just a moderate loss of function, consistently with the milder phenotype of febrile seizures compared with SMEI (Mantegazza et al., 2005a). Thus, also a partial rescue of $hNa_v1.1$M1841T may result in a milder phenotype.

Mutations or polymorphisms in interacting proteins, or competing interactions, may modify their affinity for the α subunit and thus their rescuing power, and early molecular diagnosis of phenotype severity in GEFS+ families and suspected SMEI cases may be improved by analysis of genes of Na^+ channel interacting proteins. An additional factor that could alter rescuing capacity of interacting proteins is their expression levels. In addition to highlighting the importance of mutations and polymorphisms in their promoters, this possibility opens a complex scenario, because expression patterns in CNS neurons are highly variable. For instance, β subunits show neuron-specific expression in many brain areas (Morgan et al., 2000; Yu et al., 2003). Little is known about interneuron-specific expression levels of Na^+ channel interacting proteins, and this is particularly important because $Na_v1.1$ loss-of-function mutations have a prominent effect on the excitable properties of GABAergic neurons (Yu et al., 2006). Thus, at the cellular level, the effect of the mutation could vary according to the type and expression level of the interacting proteins expressed in a specific subtype of neuron, and they could also vary according to the developmental stage.

Our data show that also the antiepileptic drug phenytoin can partially rescue $hNa_v1.1$-M1841T, similarly to other Na^+ channel blockers that can rescue some Brugada syndrome trafficking-defective mutants of cardiac $Na_v1.5$ Na^+ channel (Valdivia et al., 2002). However, in general, these drugs cannot be used in a clinical setting, because they also block the rescued channels already targeted to the plasma membrane: it would be necessary to develop pharmacological chaperones able to rescue mutant proteins but unable to affect the rescued channels delivered to the plasma membrane. It is important to determine whether other GEFS+ and SMEI mutants are also trafficking defective. A particularly interesting hypothesis is that there may be SMEI loss-of-function mutants that are not rescued by interactions with endogenous proteins but that are responsive to pharmacological chaperones.

It has been hypothesized that molecular interactions promote proper folding of mutant proteins stabilizing the correct conformation of the protein domains containing the mutation (Bernier et al., 2004). We have shown that molecular interactions taking place in the C-terminal cytoplasmic domain of Na^+ channel α subunit, where M1841T is located, can effectively rescue the mutant channel. This domain is functionally important, because it

modulates inactivation properties and contains several binding sites for modulatory proteins (Mantegazza et al., 2001; Abriel and Kass, 2005; Glaaser et al., 2006). M1841T lies in an area that includes a $\beta1/\beta3$ binding site (Spampanato et al., 2004), and we have shown that the interaction between the C-terminal domains of α and mutant $\beta1$-C121W subunits is sufficient to induce ~60% of the rescue observed with wild-type $\beta1$. Consistently, calmodulin, $G\beta\gamma$, and phenytoin are able to partially rescue $hNa_v1.1$-M1841T. Several epileptogenic missense mutations have been found in $Na_v1.1$ C-terminal domain (Mulley et al., 2005; Meisler and Kearney, 2005), and there could be other trafficking defective mutants that may be rescued by the C-terminal interactions. Some pharmacological chaperones also correct folding defects caused by mutations that are distant from their binding site (Rajamani et al., 2002). Consistently, we observed functional rescue cotransfecting $\beta2$ that, according to yeast two-hybrid experiments, does not bind to the C-terminal cytoplasmic domain (Spampanato et al., 2004). Additionally, extracellular $\beta1$ interactions, which we probed with the $\beta1_{STOP}$ mutant, also can induce some rescue in our experimental system. Therefore, long-range interactions are probably able to allosterically promote the correct folding of the mutant C terminus and thus can be effective in rescuing $hNa_v1.1$- M1841T. Moreover, the interactions that we studied may be effective also on mutations that are not located in the C-terminal domain.

Our data imply that the interactions that we studied occur also in the ER and not only in the plasma membrane. Little is known about interactions occurring in the ER between nascent membrane proteins and their modulatory partners. However, ER interactions between $\beta1$ and α subunits have been demonstrated previously (Biskup et al., 2004). Interestingly, our results show that depletion of ER Ca^{2+} stores by incubation with ER Ca^{2+} pump inhibitors does not rescue $hNa_v1.1$-M1841T and blocks the rescuing activity of cotransfected $\beta1$, perhaps because luminal ER Ca^{2+} modulates binding of accessory subunits. Calmodulin and $G\beta\gamma$ may bind nascent Na^+ channel α subunit directly from the cytoplasm. IQ binding domains bind apocalmodulin; thus, the interaction could take place also at resting cytoplasmic Ca^{2+}.

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