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Why Does Fever Trigger Febrile Seizures? GABA$_A$ Receptor $\gamma_2$ Subunit Mutations Associated with Idiopathic Generalized Epilepsies Have Temperature-Dependent Trafficking Deficiencies

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With a worldwide incidence as high as 6.7% of children, febrile seizures are one of the most common reasons for seeking pediatric care, but the mechanisms underlying generation of febrile seizures are poorly understood. Febrile seizures have been suspected to have a genetic basis, and recently, mutations in GABA$_A$ receptor and sodium channel genes have been identified that are associated with febrile seizures and generalized seizures with febrile seizures plus pedigrees. Pentameric GABA$_A$ receptors mediate the majority of fast synaptic inhibition in the brain and are composed of combinations of $\alpha$(1–6), $\beta$(1–3), and $\gamma$(1–3) subunits. In $\alpha\beta\gamma_2$ GABA$_A$ receptors, the $\gamma_2$ subunit is critical for receptor trafficking, clustering, and synaptic maintenance, and mutations in the $\gamma_2$ subunit have been monogenically associated with autosomal dominant transmission of febrile seizures. Here, we report that whereas trafficking of wild-type $\alpha\beta\beta_2\gamma_2$ receptors was slightly temperature dependent, trafficking of mutant $\alpha\beta\beta_2\gamma_2$ receptors containing $\gamma_2$ subunit mutations [ gyro (R43Q), gyro (Q351X)], associated with febrile seizures was highly temperature dependent. In contrast, trafficking of mutant $\alpha\beta\beta_2\gamma_2$ receptors containing an $\alpha_1$ subunit mutation [$\alpha_1(A322D)$] not associated with febrile seizures was not highly temperature dependent. Brief increases in temperature from 37 to 40°C rapidly (<10 min) impaired trafficking and/or accelerated endocytosis of heterozygous mutant $\alpha\beta\beta_2\gamma_2$ receptors containing $\gamma_2$ subunit mutants associated with febrile seizures but not of wild-type $\alpha\beta\beta_2\gamma_2$ receptors or heterozygous mutant $\alpha_1(A322D)\beta_2\gamma_2$ receptors, suggesting that febrile seizures may be produced by a temperature-induced dynamic reduction of susceptible mutant surface GABA$_A$ receptors in response to fever.

Key words: GABA$_A$ receptors; temperature; febrile seizures; $\gamma_2$ subunit; trafficking: gyro (R43Q); gyro (Q351X); $\alpha_1(A322D)$

Introduction

Febrile seizures are one of the most common childhood neurological disorders, with a worldwide incidence of 1–14% (Hauser, 1994). It has been suggested that the height of the fever and the rapidity of the elevation of temperature are both involved in triggering a seizure, and thus typical treatment of a febrile seizure is to reduce temperature by antipyretics and passive cooling. Although most are self-limited, complex prolonged febrile seizures have been proposed to lead to hippocampal mesiotemporal sclerosis and complex partial epilepsy, and thus understanding the mechanisms of febrile seizures has substantial clinical importance.

The specific causes of febrile seizures and the role of fever in provoking febrile seizures are unclear. Previous studies have suggested that interleukin-1$\beta$, a pyrogenic proinflammatory cytokine, and hyperpolarization-activated cyclic nucleotide-gated channel channels are involved in the generation of febrile seizures or enhanced seizure susceptibility in animals, whereas neuropeptide Y could prevent febrile seizures by increasing seizure threshold (Bender et al., 2003; Dube et al., 2005a,b). In addition, it is believed that febrile seizures have a major genetic component with dominant inheritance in some families, but complex inheritance is probably operative in the majority of cases (Rich et al., 1987). Multiple loci have been proposed for febrile seizures (chromosomes 8q13-q21, 19p, 2q23–24, 6q22–24, and 5q14-q15) (Baulac et al., 2004). Recently, several mutations in the GABA$_A$ receptor $\gamma_2$ subunit gene were reported to be associated with febrile seizures [$\gamma_2$(R43Q), $\gamma_2$(K289M), and $\gamma_2$(Q351X), $\gamma_2$(IVS6 + 2T -> G)], although there was often an extended phenotypic spectrum in these pedigrees (Baulac et al., 2001; Wallace et al., 2001; Harkin et al., 2002). All patients with febrile seizures or generalized seizures with febrile seizures plus (GEFS$+$) in these pedigrees carried the $\gamma_2$ subunit mutation, strongly suggesting a correlation between febrile seizures and impaired GABA$_A$ receptor $\gamma_2$ subunit function. Quite interestingly, in a pedigree with a mutation of the GABA$_A$ receptor $\alpha_1$ subunit, $\alpha_1(A322D)$, all affected family members had the homogenous phenotype of juvenile myoclonic epilepsy (Cossette et al., 2002) without a history of febrile seizures, suggesting that the pheno-
type resulting from this mutation of the GABA_A receptor α1 subunits may not be temperature related.

The above γ2 subunit missense and truncation mutations are located in different locations, suggesting that each mutation may have a different functional consequence that may produce disinhibition and nonfebrile seizures. However, what is the basis for the common febrile seizure phenotype? The γ2 subunit is critical for receptor trafficking (Keller et al., 2004; Rathenberg et al., 2004), clustering (Essrich et al., 1998), and synaptic maintenance (Schweizer et al., 2003), suggesting that mutant receptors might have temperature-dependent effects on surface receptor stability. Therefore, we determined the role of elevated temperature on the function of α1β2γ2S receptors with or without γ2S subunit mutations identified in febrile seizure or GEFS+ pedigrees.

Materials and Methods

Expression vectors with GABA_A receptor subunits. The cDNAs encoding human α1, β2, and γ2S GABA_A receptor subunit subtypes were subcloned into the expression vector pcDNA3.1 (+) and the cDNAs encoding rat α1, β2, and γ2L subunits were subcloned into the expression vector pCMVNeo. Enhanced yellow fluorescent protein (EYFP) or enhanced cyan fluorescent protein (ECFP) was inserted between amino acids 4 and 5 of the mature human α1 and γ2S subunit cDNAs. The eclipic pHluorin [a pH-sensitive green fluorescent protein (GFP) variant]-tagged rat γ2L GABA_A receptor subunit was kindly provided by Dr. Stephen J. Moss (University of Pennsylvania, Philadelphia, PA). All point mutations in both fluorescence-tagged and untagged human γ2S and γ2L and rat γ2L subunit constructs were made using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and were confirmed by DNA sequencing.

Hippocampal neuron culture. Hippocampi were dissected from the brains of embryonic day 19 (E19) Sprague Dawley rat pups. Dissociation of cells and culturing procedures have been described previously (Chong et al., 2003). The neurons were plated on 35 mm dishes or coverslips at a density of 10^5 cells/ml and first maintained in DMEM (Invitrogen, Carlsbad, CA) supplemented with 6% fetal bovine serum for the first 3 d and then maintained with Neurobasal and B-27 supplement. The experiments were initiated on days 5–7 in culture. Neurons were transfected with rat α1, β2, and pHluorin-tagged γ2L wild-type subunit cDNAs (cDNA ratio of 1:1:1 for wild type) and γ2L(Q351X) mutant subunit cDNAs (cDNA ratio of 1:0.5:0.5 for heterozygous) for 6 d with Fugene reagents per the suggestions of the manufacturer before imaging.

Electrophysiology. Lifted whole-cell recordings were performed as reported previously (Kang and Macdonald, 2004). Briefly, human embryonic kidney 293T (HEK293T) cells were cotransfected with 4 μg of each subunit plasmid and 2 μg of the pHook-1 cDNA (Invitrogen) using a modified calcium phosphate precipitation method and selected 24 h after transfection by magnetic hapten-coated beads. Whole cells were voltage clamped at −50 mV.

Live cell confocal microscopy and fluorescence quantification. Live cell confocal microscopy and data quantification were performed as described previously with minor modifications (Kang and Macdonald, 2004). The temperature-controlled confocal microscopy was performed using CTI-Control 3700 digital plus TempControl 37-2 digital system. COS-7 cells were plated on poly-d-lysine-coated, glass-bottom imaging dishes at the density of 1–2 × 10^5 cells and cotransfected with 1 μg of each human subunit plasmid with either calcium phosphate precipitation or Lipofectamine Plus reagents according to the suggestions of the manufacturer. Fluorescence-tagged cells were imaged with a fluorescein laser (excitation, 488 nm; emission, 530-nm filter) and a rhodamine laser (excitation, 568 nm; emission, 590-nm filter) using a 100×, 1.4 NA oil immersion objective. The images were collected and analyzed with MetaMorph 7.0 (Molecular Devices, Sunnyvale, CA). The pipette solution was 150 mM KCl, 10 mM NaCl, 10 mM HEPES, 1 mM CaCl_2, 1 mM MgCl_2, 0.5 mM EGTA, and 5 mM glucose, pH 7.3. Data were never subjected to any post hoc corrections.

Data analysis. The data were analyzed by statistical software (GraphPad Prism 4.0; GraphPad Software, San Diego, CA). Western blot data were quantified by densitometry with ImageJ software (http://imagej.nih.gov/ij). The data were compared using one-way analysis of variance (ANOVA) followed by post hoc tests. The numbers of neurons analyzed are indicated in the figure legends. The results are expressed as mean ± SEM (n = 4; *p < 0.05, **p < 0.01, ***p < 0.001 vs wild-type subunit at 37°C; ††p < 0.01 vs wild type at 40°C; ‡‡p < 0.05 vs the same mutation at 37°C; two-tailed unpaired Student’s t test).
A Wildtype or heterozygous α1β2γ2S receptors incubated for 30 min at 40°C

|          | ECFP | EYFP | FM4-64 | Co localization |
|----------|------|------|--------|-----------------|
| wt       | wt   | wt   | mem   | co              |
| mut      | mut  | mut  | mem   | co              |
| γ2(R43Q) | γ2(R43Q) | γ2(R43Q) | γ2(R43Q) | co |
| γ2(K289M)| γ2(K289M) | γ2(K289M) | γ2(K289M) | co |
| α1(A322D)| α1(A322D) | α1(A322D) | α1(A322D) | co |

Bar: 15 μm

Figure 2. Elevated temperature rapidly increased intracellular retention of mutant γ2S subunit-containing GABA_A receptors. A, Confocal microscopy images of fluorescence-tagged wild-type and mutant γ2S subunit-containing α1β2γ2S receptors and α1(A322D) subunit-containing α1β2γ2S receptors in COS-7 cells after a 30 min incubation at 40°C. wt, ECFP-tagged wild-type subunits; mut, EYFP-tagged mutant subunits; mem, membrane marker FM4-64; co, colocalization of all ECFP, EYFP, and FM4-64 channels. With heterozygous expression, mutant and wild-type γ2S(R43Q), γ2S(K289M), and γ2S(Q351X) subunits and mutant α1(A322D), but not wild-type α1, subunits were localized intracellularly in compartments that had the morphology of the ER and colocalized with the ECFP-ER marker (data not shown). The loss of surface receptor illustrated here was an extreme example to illustrate the point. Other cells showed less extensive loss of surface receptor. B, Total membrane surface receptor fluorescence pixel intensity values of heterozygous α1β2γ2S(R43Q), α1β2γ2S(K289M), and α1β2γ2S(Q351X) receptors were reduced after incubation at 37°C compared with wild-type receptors (filled bars) and were further reduced after a 30 min incubation at 40°C (open bars). In all groups, data represent the mean ± SEM (n = 19–23 cells from 5 transfections; *p < 0.05 vs wild type at 37°C; †p < 0.05 vs wild type at 40°C; ††p < 0.01 vs wild type at 37°C; ‡p < 0.05, §§p < 0.01 vs the same mutation at 37°C; two-tailed unpaired Student’s t-test).
Results
Heterozygous γ2S subunit-containing α1β2γ2S receptors displayed reduced surface receptor expression with temperature elevation

Because all pedigrees with γ2 subunit mutations are associated with febrile seizures and previous studies indicated that one of the mechanisms underlying the afebrile seizures associated with these mutations is reduced receptor surface expression and endoplasmic reticulum (ER) retention (Harkin et al., 2002; Kang and Macdonald, 2004; Sancar and Czajkowski, 2004; Hales et al., 2005), it is possible that elevated temperature reduces receptor surface expression. To explore this, we expressed wild-type α1β2γ2S and heterozygous α1β2γ2S(R43Q), α1β2γ2S(K289M), and α1β2γ2S(Q351X) receptors in HEK293T cells and varied temperature between 37 and 40°C.

We used biotinylation and Western blotting with anti-GFP antibody of coexpressed α1, β2, and EYFP-tagged γ2S subunits to determine surface expression of wild-type and heterozygous mutant receptors incubated at 37°C (Fig. 1A). We found that γ2S subunit surface expression was significantly reduced for all heterozygous receptors relative to wild-type receptors (Fig. 1B, filled bars). After incubation of wild-type receptors at 40°C for 1 h, there was no significant change in γ2S subunit surface expression relative to that at 37°C (Fig. 1A, B). However, after incubation of heterozygous receptors at 40°C for 1 h, surface expression of each mutant γ2S subunit was significantly reduced relative to wild-type γ2S subunit incubated at 40°C for 1 h or to mutant γ2S subunits at 37°C (Fig. 1A, B). After a 2 h incubation at 40°C, wild-type receptors also showed reduced surface expression, but total protein was not changed compared with control dishes maintained at 37°C (data not shown).

Because a previous study suggested that γ2 subunits could traffic to the cell surface alone (Connolly et al., 1999), we also detected α1 subunit surface expression at 37°C after expression of wild-type and heterozygous receptors to confirm that there was a temperature-dependent alteration of surface expression of heterozygous α1β2γ2S receptors, not just γ2 subunits (Fig. 1A). We found that α1 subunit surface expression was also reduced for each mutant receptor relative to receptors containing wild-type γ2S subunits (Fig. 1C, filled bars), suggesting that surface expression of pentameric mutant α1β2γ2 receptors was reduced instead of γ2 subunits alone. After incubation of heterozygous receptors at 40°C for 1 h, surface expression of α1 subunits was significantly reduced relative to surface expression in wild-type receptors or in mutant receptors at 37°C (Fig. 1A, C).

To control for a nonspecific effect of elevated temperature on receptor surface expression, heterozygous α1(A322D)β2γ2S receptors were expressed also, because the α1(A322D) mutation is not associated with febrile seizures. In contrast to the results obtained with heterozygous expression of receptors containing mutant γ2S subunits, after heterozygous expression of α1(A322D)β2γ2 receptors, α1 subunit surface reduction was the same at 37°C and after incubation at 40°C for 1 h (data not shown).
Heterozygous mutant γ2S subunit-containing, but not mutant α1(A322D) subunit-containing, α1β2γ2S receptors displayed decreased surface and increased intracellular localization with temperature elevation

Consistent with the reduction of receptor surface expression demonstrated using immunoblotting, we also found that after a 30 min incubation at 40°C, heterozygous α1β2γ2S(R43Q), α1β2γ2S(K289M), and α1β2γ2S(Q351X) receptors had reduced membrane surface and increased intracellular expression in COS-7 cells (Fig. 2). Wild-type γ2S subunits tagged with ECFP (blue) and EYFP (green) and the plasma membrane marker FM4-64 (red) were primarily colocalized (white) on the membrane surface (Fig. 2A, top row) and expressed a small amount of intracellular receptor (aqua) with incubation at 37°C or after a 30 min incubation at 40°C (Fig. 2A). For heterozygous expression, wild-type and mutant γ2S subunits were tagged with ECFP and EYFP, respectively. Membrane surface fluorescence intensities of all heterozygous mutant γ2S subunits were significantly reduced compared with wild-type γ2S subunits (Fig. 2B, filled bars). After a 30 min incubation at 40°C, there was reduced membrane surface expression of receptor (loss of white), and both mutant and coexpressed wild-type γ2S subunits were localized intracellularly (cyan) (Fig. 2A, middle three rows). Consistent with this, membrane surface fluorescence of each mutant subunit was decreased after a 30 min incubation at 40°C compared with incubation at 37°C and to wild-type subunits at 37 or 40°C (Fig. 2B).

In contrast to the results obtained with the mutant γ2S subunits, with heterozygous expression, wild-type α1-ECFP subunits coregistered with the cell membrane marker FM4-64 (purple) and were also distributed intracellularly (Gallagher et al., 2005), but mutant α1(A322D)-EYFP subunits did not coregister with wild-type subunits and surface membrane (absence of white) and were mainly localized intracellularly with wild-type receptors (cyan) (Fig. 2A, bottom row). These results are consistent with the report that at room temperature or at 37°C with heterozygous expression of α1(A322D)β2γ2S receptors, there was intracellular ER retention and ER associated degradation of mutant subunits before receptor assembly (Gallagher et al., 2005) and also suggests that attachment of EYFP or ECFP to the γ2S subunit did not alter its temperature-dependent trafficking or folding.

Decrease of surface receptor and increase of intracellular receptor with elevated temperature was rapid in heterozygous mutant γ2 subunit-containing α1β2γ2 receptors in heterologous cells

Because all of the heterozygous α1β2γ2S(R43Q), α1β2γ2S(K289M), and α1β2γ2S(Q351X) receptors developed extensive intracellular localization with a 30 min incubation at elevated temperature (40°C), we determined how soon the intracellular localization occurred after temperature elevation. Using heterozygous α1β2γ2S(K289M) receptors, we detected a rapid change in membrane surface and intracellular receptor localization. COS-7 cells were cotransfected with heterozygous α1β2γ2S(K289M) receptors with the wild-type γ2S subunits tagged with ECFP and the mutant γ2S subunit tagged with EYFP (Fig. 3) and maintained at 37°C. The culture dish was transferred to a microscope stage that was heated to 40°C. After 5 min on the heated stage, the receptors displayed a merged aqua color both on the surface and intracellularly (Fig. 3, 5 min, bottom, red box). Over the next 6 min at 40°C, the intensity of the surface receptors rapidly diminished, and the intensity and amount of intracellular receptor rapidly increased (Fig. 3, 7 min and 11 min, bottom, red boxes).

Figure 4. Rapid reduction of heterozygous α1β2γ2/α1β2γ2(Q351X) receptors with elevated temperature on the membrane surface of hippocampal neurons. A, Heterozygous α1β2γ2L-phluorin/α1β2γ2L(Q351X)-phluorin receptor on the surface of rat hippocampal neurons was reduced rapidly by temperature elevation to 40°C. As illustrated in the left panels, neurons were cotransfected with heterozygous α1β2γ2L-phluorin/α1β2γ2L(Q351X)-phluorin receptors, and receptors were imaged as puncta on the surface of neurons. With incubation at 40°C, the fluorescent puncta were reduced, with less or fading of fluorescence on the cell surface (red arrows). TI, Transmitted image; wt, wild-type α1β2γ2L-phluorin receptors; mut, heterozygous mutant α1β2γ2L-phluorin/α1β2γ2L(Q351X)-phluorin receptors. B, Membrane surface fluorescence clusters of heterozygous mutant α1β2γ2L-phluorin/α1β2γ2L(Q351X)-phluorin receptors were reduced after incubation at 40°C at different times compared with wild-type receptors measured over equivalent areas and were further reduced after a 20 min incubation at 40°C compared with the receptor fluorescence puncta in the same regions. In all groups, data represent the mean ± SEM (n = 10–14 neurons from 4 transfections; p < 0.05 vs wild type at the same time points; †p < 0.05 vs the same mutation after incubation at 40°C for 3 min; two-tailed unpaired Student’s t test).

Decrease of surface heterozygous mutant γ2 subunit-containing α1β2γ2 receptors was rapid at elevated temperature in hippocampal neurons

Because neurons may have different trafficking machinery, the effects of elevated temperature on surface expression of wild-type and mutant receptors must be determined. To explore the receptor surface dynamics in neurons, pHLuorin-tagged rat wild-type γ2L and mutant γ2L(Q351X) subunits were coexpressed with rat α1 and β2 subunits in hippocampal neurons for 6 d. pHLuorin should only effectively generate fluorescence when at the cell surface and should produce no or minimal fluorescence at acidic pH levels (pH < 6.5) characteristic of vesicular compartments. After 6 d, γ2L subunit-coupled fluorescence was visible and appeared as puncta on neurons expressing both wild-type and mutant receptors (Fig. 4A) (see supplemental Figs. 1 and 2 for enlarged views, arrows, available at www.jneurosci.org as supplemental material). Because pHLuorin only fluoresces at the surface, the
puncta on neurons were assumed to be surface receptors. Neurons transfected with heterozygous mutant receptors had fewer fluorescent puncta compared with wild-type receptors from the same areas (Fig. 4B), indicating there were fewer receptors trafficked to the surface in neurons. The fluorescent puncta with wild-type receptors showed minimal reduction in fluorescence after 40 min at 40°C, whereas fluorescent puncta with heterozygous mutant receptors showed substantial reduction in fluorescence within 20 min at 40°C. After incubation at 40°C for 30 min, there was more loss of puncta expressing mutant than wild-type receptors by comparing the total number of puncta in the same regions of chosen neurons (Fig. 4B). This loss was not likely caused by the experimental manipulations such as photobleaching, because there was no appreciable loss of puncta after incubation at 37°C over the same time course (data not shown). The rapid reduction of surface receptor expression and increased intracellular localization in both heterologous cells and neurons suggested that receptor trafficking deficiency and/or accelerated endocytosis was very dynamic and rapid.

Wild-type α1β2γ2S receptor currents were reduced reversibly by elevated temperature

We next determined the temperature dependence of wild-type α1β2γ2S receptor currents. Wild-type receptor peak amplitude was significantly reduced after incubation at 40°C for 2.5 h (Fig. 5A, B) but was not reduced after incubation at 40°C for 30 min (data not shown) to 1 h (Fig. 5C, D). After incubation at 40°C for 2.5 h, only minimal current was evoked by a saturating GABA concentration (1 mM) (Fig. 5A, B). However, during recovery at 25°C, peak current amplitude of the same cells increased 30-fold in 45 min and was then stable for at least 2 h, suggesting that at 25°C, ~45 min was required for full recovery of α1β2γ2S receptors from an elevated temperature-induced reduction in surface receptors. The results also demonstrate that the reduction in surface wild-type α1β2γ2S receptors produced by decreased trafficking or the accelerated endocytosis at elevated temperature was reversible.

Mutant γ2S subunit-containing, but not α1(A322D) subunit-containing, α1β2γ2S receptor currents were reduced additionally by elevated temperature

To determine the temperature sensitivity of heterozygous α1β2γ2S receptors containing γ2S subunit mutations, we recorded from cells expressing each mutant receptor at room temperature (25°C) after incubation at 37°C or within 30 min after a 1 h incubation at 40°C (Fig. 5C). Peak α1β2γ2S(R43Q), α1β2γ2S(K289M), and α1β2γ2S(Q351X) currents were substantially reduced relative to wild-type currents after incubation at 37°C (Fig. 5D, filled bars). Peak heterozygous α1β2γ2S(R43Q), α1β2γ2S(K289M), and α1β2γ2S(Q351X) currents were reduced substantially after 1 h at 40°C (Fig. 5C) compared with wild-type currents after incubation at 37°C or a 1 h incubation at 40°C or compared with heterozygous currents after incubation at 37°C (Fig. 5D).

Heterozygous α1(A322D)β2γ2S receptor currents were significantly reduced compared with wild-type currents recorded at 25°C after incubation at 37°C (Fig. 5D, wt and A322D, filled bars). However, in contrast to the results obtained with receptors containing mutant γ2S subunits, currents obtained from heterozygous α1(A322D)β2γ2S receptors after a 1 h incubation at 40°C were not further decreased relative to currents obtained after incubation at 37°C (Fig. 5D, A322D, filled and open bars).

Discussion

Variations in temperature have effects on most cellular events, and several neurological disorders are provoked by elevated temperature, including febrile seizures and febrile episodic ataxia (calcium channels, CACN1A) (Subramony et al., 2003). Temperature changes have been shown to affect plasma membrane states (Thompson et al., 1985) and synaptic transmission (Volgushev et al., 2000). For example, synaptic vesicle recycling has been shown to be temperature dependent. The size of recycling vesicles is twice as large, and the speeds of both endocytosis and exocytosis are faster at physiological temperature than at room temperature (Micheva and Smith, 2005). Although the dynamic temperature dependence of turnover of GABAA receptors is unclear, there is evidence that inhibitory synaptic strength can be modulated within 10 min through recruiting more functional GABAA receptor to the postsynaptic plasma membrane (Wan et al., 1997).

The basis for the common febrile seizure phenotype resulting from mutations in GABAA receptor γ2(R43Q), γ2(K289M), and γ2(Q351X) is unclear. However, it is likely that the reduced trafficking or accelerated endocytosis in GABAA receptors at elevated temperatures is responsible for the increase in neuronal excitability and the associated behavioral abnormalities. Further studies are needed to determine the molecular mechanisms underlying these temperature-dependent changes in GABAA receptor trafficking and endocytosis.
γ2 (Q531X) subunits has been unknown. Previous studies indicated that each of the γ2 subunit epilepsy mutations produced different alterations in receptor function that would lead to disinhibition and thus afebrile seizures. The γ2 subunit mutation, γ2 (R43Q), associated with autosomal dominant childhood absence seizures and febrile seizures is located in the N terminus and was suggested to impair diazepam sensitivity (Wallace et al., 2001) or to alter receptor kinetics or to reduce peak amplitude (Bianchi et al., 2002) of α1 β2 γ2 receptor currents caused by receptor ER retention and degradation leading to impaired receptor surface expression (Kang and Macdonald, 2004; Sancar and Czaikowski, 2004; Hales et al., 2005). The autosomal dominant γ2 subunit mutation, γ2 (K289M), associated with GEFS+ is located in the extracellular TM2-TM3 loop, and it was reported that the mutation caused reduced α1 β2 γ2 receptor peak current in oocytes (Baulac et al., 2001). However, we reported that the mutation caused accelerated channel deactivation and reduced single-channel open time (Bianchi et al., 2002) with normal peak amplitude when recorded at room temperature (25° C), suggesting a gating defect. In another small autosomal dominant GEFS+ pedigree, a γ2 subunit mutation, γ2 (Q351X), introduces a premature stop codon with the loss of 78 C-terminal amino acids. It was reported that homozygous expression of the γ2 (Q351X) subunit truncation totally abolished α1 β2 γ2 currents, and use of a fluorescent epitope-tagged γ2 (Q351X) subunit revealed ER retention of homozygous α1 β2 γ2 (Q351X) receptors.

Our results demonstrated that with heterozygous expression, each of these γ2S subunit mutation-containing receptors had reduced γ2S subunit protein surface expression at 37° C and additional reduced surface expression with elevated temperature, suggesting a reduced complement of α1 β2 γ2S receptors on the cell surface. In addition, we demonstrated that all three heterozygous receptors had reduced α1 subunit protein surface expression at 37° C and additionally reduced surface expression with elevated temperature. From these results, we conclude that pentameric α1 β2 γ2S receptors were reduced on the surface, and that there was no compensatory increase in cell surface α1 β2 receptors when the mutation-containing receptors were challenged with increased temperature.

In cystic fibrosis transmembrane conductance regulator (CFTR), intracellular mutant protein trafficking has been studied extensively. The most common cystic fibrosis mutation in the CFTR gene (ΔF508) results in a protein that fails to mature and converses with temperature but before receptor assembly, resulting in ER-associated degradation (Gallagher et al., 2005). Thus, mutant α1 subunit-containing α1 β2 γ2S receptors are not assembled, and membrane α1 β2 γ2S receptors almost exclusively contain wild-type α1 subunits and are not subject to temperature-dependent effects. In contrast, we demonstrated that although trafficking of the mutant γ2 subunit-containing α1 β2 γ2S receptors to the surface is deficient, both mutant and wild-type receptors are present on the surface (Macdonald et al., 2004). In addition, we have evidence that heterozygous wild-type and mutant α1 β2 γ2S (Q351X) receptors have trafficking interactions, suggesting that the temperature-sensitive mutant γ2 subunit-containing receptors interact with wild-type receptors to alter trafficking/endocytosis of both wild-type and mutant receptors.

Our data also suggest that wild-type α1 β2 γ2S receptors have impaired trafficking during prolonged temperature elevation. After incubation for 2.5 h at 40° C, peak whole-cell current amplitudes from cells expressing wild-type receptors increased 30-fold over 45 min at room temperature and were then stable for the next 2 h, suggesting that ~45 min was required for full recovery of α1 β2 γ2S receptors from an elevated temperature-induced trafficking deficiency. Our data also suggested that the trafficking deficiency of wild-type α1 β2 γ2S receptors at elevated temperature was reversible.

GABA<sub>α</sub> receptors containing mutant γ2 subunits associated with febrile seizures and GEFS+ have impaired αβ2 γ2 channel function, thus lowering the threshold for nonfebrile seizures. We have shown that wild-type α1 β2 γ2S receptor trafficking is also vulnerable to high temperature but to a lesser degree. Because the γ2 subunit is critical for receptor trafficking, clustering, and synaptic maintenance, any mutations causing misfolding and im-
paired assembly and ER retention, increased degradation, or rapid endocytosis would result in reduced surface expression and inhibitory synaptic strength in neurons that would be worsened by fever resulting in febrile seizures. Our finding that surface expression of GABA<sub>α</sub> receptors containing mutant γ2S subunits, but not containing the α1(322D) subunit mutation, is vulnerable to elevated temperature and that all three GABA<sub>α</sub> receptor γ2S subunit mutations showed rapidly compromised receptor trafficking or accelerated endocytosis when challenged with elevated temperature may explain why febrile seizures are provoked by fever in individuals harboring the γ2S, but not the α1(322D), subunit mutations.

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