Homozygous *SCN1B* variants causing early infantile epileptic encephalopathy 52 affect voltage-gated sodium channel function.

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1. Supplementary materials and methods

Exome sequencing analysis

Genomic DNA was extracted from peripheral blood leukocytes using the QIAamp DNA blood midi kit (Qiagen, Hilden, Germany) and analyzed as previously described.[1] GERP score was calculated as described in[2].

Two-electrode voltage-clamp recording from Xenopus oocytes

Human (h) $hNa_V1.1$ (NM_001165963.1), $hNa_V1.2$ (NM_021007.2), $hNa_V1.6$ (NM_014191.3), and h*β1* (NM_001037.5) were expressed in *Xenopus laevis* oocytes (Nasco®, USA) as previously described.[3] To check for undesired rearrangement events, the DNA sequence of all constructs[3] was confirmed by automated DNA sequencing before further usage. cRNA was synthesized using T7 mMESSAGE-mMACHINE® kit (ThermoFisher Scientific, USA) after the DNA had been linearized with the appropriate restriction enzymes. hNa_V channel constructs were expressed in *Xenopus* oocytes together with h*β1*-subunit (1:5 molar ratio) and electrophysiological recordings (OC-725C, Warner Instruments, USA) were taken 1−3 days post cRNA injection. Oocytes were maintained at 17°C in Barth's medium (88 mM NaCl, 1 mM KCl, 5 mM HEPES, 2.4 mM $NaHCO_3$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, and 50 µg/mL gentamycin (pH 7.4) with NaOH) and studied using the two-electrode voltage-clamp recording technique (OC-725C, Warner Instruments, USA) with a 150 µl recording chamber. All data were filtered at 4 kHz and digitized at 20 kHz using pClamp10 software (Molecular Devices, USA). Microelectrode resistances were 0.5−1 MΩ when the devices were filled with 3 M KCl. The external recording solution contained 100 mM NaCl, 5 mM HEPES, 1 mM $MgCl_2$, and 1.8 mM $CaCl_2$ (pH 7.6) with NaOH. All experiments were performed at room temperature (RT) (~21 °C). Leak and background conductances, identified by blocking Na_v channels with tetrodotoxin (TTX), were subtracted for all of the Na_V channel-mediated currents. Voltage−activation relationships were obtained by measuring peak currents and calculating conductance (G), and a Boltzmann function was fit to the data according to the equation $G/G_{max} = [1 + e^{-zF(V-V1/2)/RT}]^{-1}$, where G/G_{max} is the normalized conductance, z is the equivalent charge, $V_{1/2}$ is the half-activation voltage, F is Faraday's constant, R is the gas constant, and T is the absolute temperature in kelvin. The fast inactivation time constants and RFI measurements were fit using a single exponential fit. Off-line data analysis was performed using Clampfit10 (Molecular Devices, USA), Excel (Microsoft Office, USA), Prism 4 (GraphPad, USA) and Origin 8 (OriginLab, USA).

Qualitative biochemical assessment of hβ1 expression in Xenopus laevis oocytes

Oocytes expressing wild-type h*β1*, and the described mutants were washed with saline solution and incubated overnight with 0.5 mg/ml Sulfo-NHS-LC-biotin (Pierce, USA). Pooled oocytes (50) were lysed in 10 µl/oocyte buffer H (1% Triton X-100, 100 mM NaCl, 20 mM Tris-HCl, pH 7.4) plus protease inhibitor cocktail (Sigma-Aldrich, USA) and shaken for 60 min at 4°C after which they were centrifuged at 16,200x*g* for 3 min. The pellet was discarded and the supernatant (SN) transferred to a fresh 1.5 ml Eppendorf® tube. 200 µl of hydrophilic streptavidin magnetic beads (New England Biolabs, USA) were then added and the sample shaken gently at 4°C overnight. Beads were washed twice with buffer H and resuspended in 40 µl buffer H, after which the biotinylated protein was dissociated through the addition of

1X loading buffer plus reducing agent (10% 2-ME, 50 mM DTT final conc.) and boiled at 95°C for 5 min to generate the surface protein fraction. 35 µl of all samples were loaded on a Novex™ WedgeWell™ 10% Tris-Glycine Mini-Gel (Thermo Fisher Scientific, USA) with Tris-Glycine running buffer and analyzed by Western analysis. Nitrocellulose membranes were probed overnight at 4°C with 1:1000 rabbit anti-*SCN1B* antibody as primary (Cell Signaling Technologies, USA) and for 60 min at room temperature with 1:5000 goat anti-rabbit HRP-conjugated antibody as secondary (Abcam, United Kingdom). Membranes were incubated for 5 min with Clarity Max Western ECL Substrate (Bio-Rad Laboratories, USA) before imaging.

Statistical analysis

Significance of all normalized G-V and steady-state inactivation (I-V) relationships was analyzed using two-way analysis of variance (ANOVA) with post-hoc Bonferroni correction. Individual time point values for fast inactivation time constants (τ) and recovery from fast inactivation (RFI) were analyzed using two-way Student's *t*-test. For data presented in Supplemental Tables, Student's *t*-test (unpaired) against h*β1*-subunit was used. Values in all cases reflect the mean and error bars reflect standard error of the mean (S.E.M.); $p<0.05$ (*), 0.01 (**) or 0.001 ($^{\#}$). All statistical analysis was carried out using Excel (Microsoft, USA) and Prism 4 (GraphPad, USA).

2. Supplementary clinical information

Family A

This is a consanguineous family of Pakistani ancestry. Family history was negative for epileptic disorders but was remarkable because of four miscarriages, occurring before the birth of the two affected siblings. Patient 1 is a 9-year-old male regularly achieving the developmental milestones in the first 6 months of life. At the age of 6 months, he started to suffer from myoclonic and generalized tonic-clonic seizures (GTCS). These seizures were more common during sleep at night and were refractory to carbamazepine and clonazepam. EEG showed a diffuse slowing of background associated with multifocal epileptiform abnormalities. Subsequently, a developmental stagnation followed by a true psychomotor regression was observed at the age of 3 years. The child lacked speech and social interaction. Physical examination revealed generalized spasticity and hyperreflexia. Patient 2 is a 5-year-old female with a similar clinical course. After a regular psychomotor development, she started to suffer from recurrent GTCS and myoclonic seizures, followed by developmental stagnation and regression. EEG was consistent with diffuse encephalopathy. Seizures remained intractable despite the use of several antiepileptic drugs (AEDs) such as carbamazepine and clonazepam. Physical examination revealed intellectual disability, generalized spasticity, and hyperreflexia.

Family B

This is a consanguineous family of Pakistani ancestry with a negative family history for epileptic disorders. Patient 3 is a 10-year-old female. She was diagnosed with psychomotor delay at the age of 5 years, when she started to suffer from generalized tonic-clonic seizures. Each tonic-clonic seizure lasted about 3-5 minutes. At the age of 9, she started to suffer from clonic jerks during sleep and to wake up screaming during the seizure. These episodes lasted around 1 minute. Seizures poorly responded to levetiracetam, valproic acid, and clobazam. The maximum seizure-free period was 1 month. Currently, the patient has intellectual disability with a severe speech delay (she can only say 3-4 words), but is able to walk with some physical restrictions. Patient 4 is a 9-year-old male. He had a history of psychomotor delay in the first months of life and started to suffer from febrile seizures at the age of 5 months. At the same age, he also had afebrile tonic and tonic-clonic seizures. Despite the employment of several AEDs, seizures were refractory and poorly responded to carbamazepine and valproic acid. Currently, he has intellectual disability with a severe speech impairment (he is able to say 2-3 meaningful words) and he can walk only if supported. Patient 5 is a 5-year-old male with a diagnosis of developmental delay in the first months of life. At the age of 6 months, he started to suffer from febrile seizures and later afebrile GTCS. Seizures were only partially controlled with levetiracetam, valproic acid, and clonazepam. She is currently ambulatory, with

preserved cognitive functions but absent speech. In all patients (patients 3-5), EEG revealed multifocal epileptic discharges.

Family C

This is a consanguineous family of Pakistani ancestry. Family history was negative for epileptic disorders. The first-born was a healthy female, whereas the second- (patient 6) and third-born (patient 7) were both affected. Both patient 6 and 7 were diagnosed with psychomotor delay in the first months of life and started to suffer from recurrent seizures by the age of 6 months. Seizures resulted refractory to several AEDs. Both patients experienced a developmental stagnation followed by psychomotor regression. They prematurely died due to clinical complications associated with refractory epilepsy at the age of 2 years (patient 6) and 7 months (patient 7).

Family D

This is a consanguineous family of Pakistani ancestry with a negative family history for epileptic disorders. The first-born was an apparently healthy female prematurely deceased due to unknown reason. The second-born is a healthy male. Patient 8 started to suffer from clonic seizures during the neonatal period (20 days of life). Subsequently, febrile seizures and multifocal tonic-clonic seizures (MFTCS) with post-ictal drowsiness were observed, with each MFTCS episode lasting from 2-3 minutes to 2 hours and requiring intravenous administration of diazepam. She also developed myoclonic seizures. Seizure were refractory to several AEDs, including phenobarbitone, levetiracetam, valproic acid, and topiramate. She had severe cognitive impairment, being nonverbal and able to walk only if supported. She died at the age of 5 years after a refractory status epilepticus. Patient 9 suffered from clonic and myoclonic seizures by the age of 4 months. Seizures poorly responded to phenobarbitone, levetiracetam, and clonazepam. She had severe psychomotor delay and deceased at 1 year of age due to uncontrolled seizures.

3. Supplementary References

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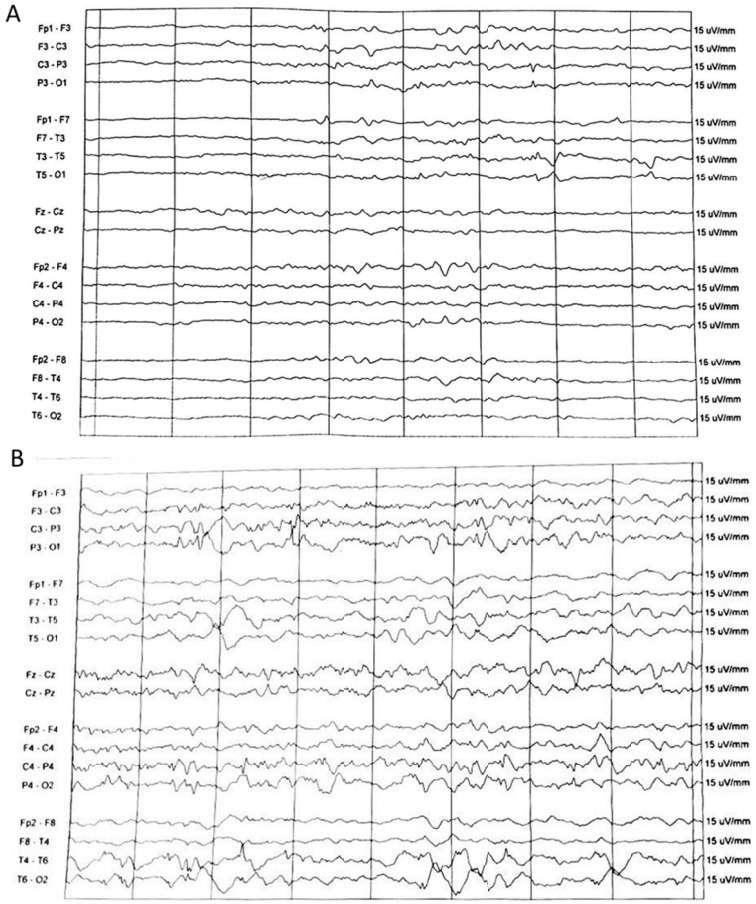
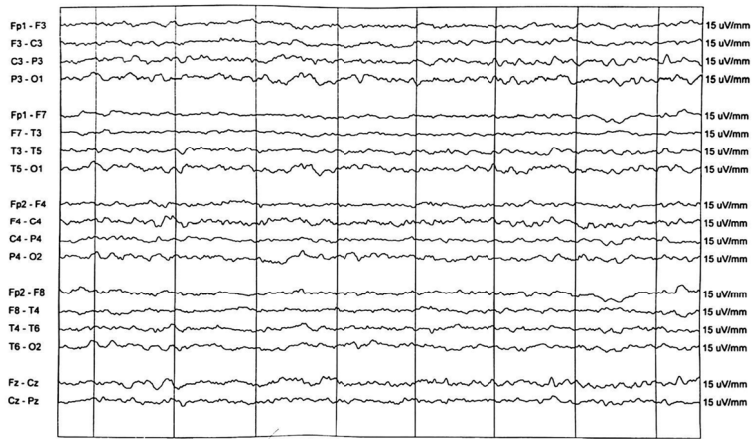
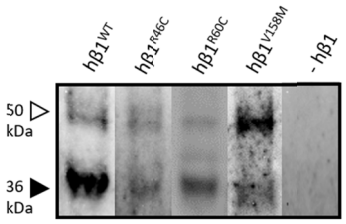
4. Supplementary Figures



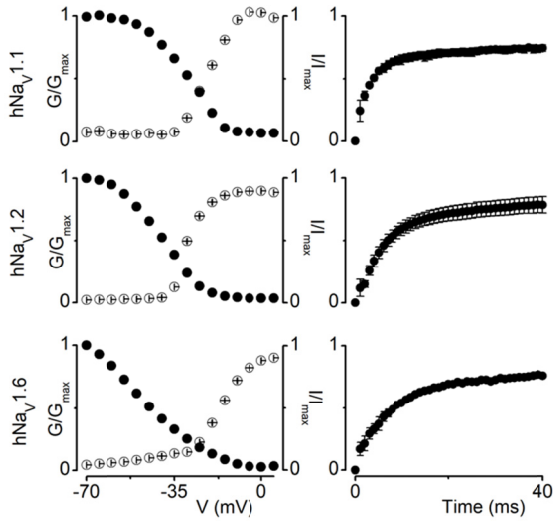
Supplementary Figure 1. EEG recordings in patients 3 (A) and 5 (B) showing intermixed low-voltage cerebral activity and modified suppression-burst pattern.



Supplementary Figure 2. EEG recordings in patient 8 showing globally slowed cerebral activity and theta polymorphic activity intermixed with slow-waves over the posterior regions.



Supplementary Figure 3. Cell membrane insertion of WT h*β1* and h*β1* mutants using Western blot analysis by probing for *β1* protein combined with primary amine biotinylation of surface proteins. Arrowheads indicate masses of detected protein. Observed bands indicate *β1* protein, possibly in different glycosylated states as observed previously. (12, 13) Bands were absent in uninjected oocytes illustrating specificity.



Supplementary Figure 4. Left, normalized G-V (open circles) and channel availability (filled circles) relationships elicited by 50 ms depolarizations from -90 mV. Right, normalized RFI measured over a 40ms timeframe using a double-pulse protocol to the maximum current of the corresponding G-V curve. Error bars reflect S.E.M. with n = 5-6.

5. Supplementary Tables

Supplementary Table 1. Summary of electroclinical features of patients with EIEE52

Abbreviations: AEM, abnormal eye movements; AP, aspiration pneumonia; AS, absence seizure; CS, clonic seizures; DS, dyscognitive seizures; FFA, Fenfluramine; FS, febrile seizures; GTCS, generalized tonic-clonic seizures; Hom, homozygous; HS, hemiclonic seizures; MFTCS, multifocal tonic-clonic seizures; mo, months; MS, myoclonic seizures; NA, not available; SE, status epilepticus; TS, tonic seizures; y, years.

	Homozygous *SCN1B* variant (NM_001037.5)	Developmental delay before seizure onset	Epileptic phenotype (onset)	Status epilepticus (age)	EEG features	Developmental stagnation/regression after seizure onset	Refractory epilepsy	Neurological features	Premature death (age, cause)
Pt 1 (Family A)	c.136C>T; p.(R46C)	-	MS and GTCS (6 mo)	-	Slow background, multifocal epileptic abnormalities	+	+	Generalized spasticity, hyperreflexia	-
Pt 2 (Family A)	c.136C>T; p.(R46C)	-	MS and GTCS (6 mo)	-	Slow background, multifocal epileptic abnormalities	+	+	Generalized spasticity, hyperreflexia	-
Pt 3 (Family B)	c.472G>A; p.(V158M)	+	GTCS (5 mo)	-	Multifocal epileptic discharges	+	+		-
Pt 4 (Family B)	c.472G>A; p.(V158M)	+	FS, TS, GTCS (5 mo)	-	Multifocal epileptic discharges	+	+		-
Pt 5 (Family B)	c.472G>A; p.(V158M)	+	GTCS (6 mo)	-	Multifocal epileptic discharges	+	+		-
Pt 6 (Family C)	c.472G>A; p.(V158M)	+	<6 mo	-	-	+	+	Microcephaly	+ (2 y, NA)
Pt 7 (Family C)	c.472G>A; p.(V158M)	+	<6 mo	-	-	+	+	Microcephaly	+ (7 mo, NA)
Pt 8 (Family D)	c.178C>T; p.(R60C)	-	MFTCS (20 days)	-	-	-	+		+ (5 y, SE)
Pt 9 (Family D)	c.178C>T; p.(R60C)	-	CS, MS (4 mo)	-	-	-	+		+ (1 y, NA)
Patino et al., 2009	c.373C>T; p.(R125C)	-	GTCS, MS, FS (3 mo)	-	Rolandic discharges	+	+	Hypotonia, tetrapyramidal syndrome	+ (13 mo, AP)
Ogiwara et al., 2012	c.316A>T; p.(I106F)	-	HS, GTCS-FS, MS, AS, DS (6 mo)	+ (13 mo)	Generalized multifocal spike and slow waves in isolation or in bursts	+	+	Ataxia, pyramidal signs	-
Ramadan et al., 2017 (Family 1, V-4)	c.449-2A>G; p.(?)	+	NA	+ (8 mo)	Bursts of high voltage spikes, paroxysmal delta activity	NA	+	-	+ (9 mo, NA)
Ramadan et al., 2017 (Family 1, V-6)	c.449-2A>G; p.(?)	NA	GTCS, AS, AEM, singultus (2 mo)	-	Generalized asynchronous slowing	+	+	Axial hypotonia, appendicular spasticity	+ (9 mo, NA)
Ramadan et al., 2017 (Family 2, V-1)	c.355T>G; p.(Y119D)	NA	NA (2 mo)	-	NA	+	+	Axial hypotonia, spastic tetraplegia, microcephaly	-
Ramadan et al., 2017 (Family 2, V-2)	c.355T>G; p.(Y119D)	NA	NA (1 mo)	-	Posterior slowing, bursts of spikes-and-waves discharges	+	+	Axial hypotonia, spastic tetraplegia, microcephaly	+ (8 y, NA)
Ramadan et al., 2017 (Family 3, IV-1)	c.449-2A>G; p.(?)	NA	MS, GTCS (<1 mo)	-	Slow background, frequent multifocal spikes	+	+	-	+ (3.5 y, esophageal varices hemorrhage)
Ramadan et al., 2017 (Family 3, IV-4)	c.449-2A>G; p.(?)	NA	MS, GTCS (<1 mo)	-	Slow background, frequent multifocal spikes	+	+	-	+ (6 y, NA)
Ramadan et al., 2017 (Family 3, IV-7)	c.449-2A>G; p.(?)	NA	MS, GTCS (<1 mo)	-	Slow background, frequent multifocal spikes	+	+	-	+ (13 y, NA)
Aeby et al., 2019	c.253C>T; p.(R85C)	+	MS (2 mo)	+ (3 mo)	Frequent bilateral central and temporal spikes	+	+, response to FFA as add-on	Diffuse hypotonia	-
Mitta et al., 2020 (11)	c.77C>T; p.(S26L)	-	MS,GTCS, FS, Focal seizures (6 mo)	-	NA	+	+	NA	-

Supplementary Table 2. *In silico* analysis of all reported *SCN1B* variants.
Abbreviations: ACMG, American College of Medical Genetics and Genomics; CADD, Combined Annotation Dependent Depletion; EIEE, early infantile epileptic encephalopathy; GEFS+, generalized epilepsy with febrile seizures plus; GERP, Genomic Evolutionary Rate Profiling;; het, heterozygous; N/A, not applicable; PM, pathogenic moderate; PP, pathogenic supporting; PS, pathogenic strong; PVS, pathogenic very strong. ‡ In-house database of 15,500 control exomes. The GME, Greater Middle East Variome Project, and Iranome yielded no results.

***SCN1B* variant [NM_001037.5]**	**g. (hg38)**	**Origin**	**gnomAD (v2.1.1)**	**gnomAD (v3)**	**Ensembl**	**ClinVar (GEFS+/ EIEE52)**	**SIFT**	**Mutation Taster**	**GERP score**	**CADD score (hg38, v1.6)**	**ACMG class**
c.136C>T; p.(R46C)	chr19:35032623C>T	This study (Family A)	0.0003978% (1het)	-	rs371646049	-	Damaging (0)	Disease causing (1)	3.82	28.7	Pathogenic (PS3, PM1-2, PP1, PP3-4)
c.472G>A; p.(V158M)	chr19:35039140G>A	This study (Families B, C)	-	0.0006977% (1 het)	rs138450474	Uncertain significance (SCV000223616.10)	Damaging (0.015)	Disease causing (0.99)	4.09	18.8	Pathogenic (PS3, PM2, PM5, PP1, PP3-4)
c.178C>T; p.(R60C)	chr19:35032665C>T	This study (Family D)	-	0.001395 % (2 het)	-	-	Damaging (0)	Disease causing (1)	3.82	29.5	Likely pathogenic (PM1-2, PP1, PP3-4)
c.373C>T; p.(R125C)	chr19:35033664C>T	Patino et al., 2009	0.003186 % (1 het)	0.0006980% (1 het)	rs1135401736	Pathogenic (RCV000417191)	Damaging (0)	Disease causing (1)	4.22	31	Pathogenic (PS3, PM1-2, PM5, PP3, PP5)
c.316A>T; p.(I106F)	chr19:35033607A>T	Ogiwara et al., 2012	-	-	rs931949929	Pathogenic (RCV000417192)	Damaging (0.002)	Disease causing (0.99)	4.54	27	Likely pathogenic (PM1-2, PP3, PP5)
c.449-2A>G; p.(?)	chr19:35039115A>G	Ramadan et al., 2017 (Families 1, 3)	-	-	-	Pathogenic (RCV000856658)	N/A	Disease causing (1)	4.09	22	Pathogenic (PVS1, PM2, PP1, PP3-4)
c.355T>G; p.(Y119D)	chr19:35033646T>G	Ramadan et al., 2017 (Family 2)	-	-	-	Pathogenic (RCV000856659)	Damaging (0)	Disease causing (0.99)	4.22	29.5	Likely pathogenic (PM1-2, PP1, PP3-5)
c.253C>T; p.(R85C)	chr19:35033544C>T	Aeby et al., 2019	-	-	rs786205830	Likely pathogenic (RCV000500892)	Damaging (0.003)	Disease causing (1)	4.54	27.7	Pathogenic (PS3, PM1-2, PM5, PP1, PP3-4)
c.77C>T; p.(S26L)	chr19:35032564C>T	Mitta et al., 2020	0.0003984% (1 het)	0.0006979% (1 het)	rs768653839	-	Damaging (0)	Disease causing (1)	3.82	28.5	Likely pathogenic (PM1-2, PP3-4)

Supplementary Table 3. Effects of h*β1* subunit variants on human Na_V channel isoforms.
Statistical results of the Student's *t*-test are indicated with p-value <0.05 (*), <0.01 (**) and <0.001 (#).

		$hNa_v1.1$	$hNa_v1.2$	$hNa_v1.6$
h$\beta1^{WT}$	activation ($V_{1/2}$) (mV)	-24,4 ± 0.3	-26,2 ± 0.8	-25,2 ± 0.7
	inactivation ($V_{1/2}$) (mV)	-37,9 ± 0.8	-41,1 ± 0.6	-53,9 ± 1.2
	recovery (T) (ms)	3,9 ± 0.2	4,6 ± 0.2	4,2 ± 0.2
	tau (ms)	4,5 ± 2.9	2 ± 0.5	3,5 ± 0.3
h$\beta1^{R46C}$	activation ($V_{1/2}$) (mV)	-21.5 ± 0.3	-17,4 ± 0.3**	-20,5 ± 0.4**
	inactivation ($V_{1/2}$) (mV)	-35,2 ± 0.6*	-35,2 ± 1.1**	-52,9 ± 2.8
	recovery (T) (ms)	4,8 ± 0.6	3,5 ± 0.2	4,5 ± 0.1
	tau (ms)	2,8 ± 0.3	2,8 ± 0.4	2,5 ± 0.5
h$\beta1^{R60C}$	activation ($V_{1/2}$) (mV)	-26.7 ± 0.6	-21.6 ± 0.7*	- 17.0 ± 1.1**
	inactivation ($V_{1/2}$) (mV)	-41.5 ± 0.4	-30.6 ± 1.3#	-50.7 ± 0.4
	recovery (T) (ms)	2.5 ± 0.1	4.7 ± 0.2	5.1 ± 0.1
	tau (ms)	5.5 ± 0.8	0.7 ± 0.2*	6.2 ± 0.5
h$\beta1^{V158M}$	activation ($V_{1/2}$) (mV)	-22,8 ± 0.4	-22,1 ± 0.7	-19,6 ± 0.8#
	inactivation ($V_{1/2}$) (mV)	-34.8 ± 0.6*	-38,8 ± 0.8	-51.6 ± 0.4*
	recovery (T) (ms)	7,6 ± 0.6**	3,5 ± 0.1	4,2 ± 0.2
	tau (ms)	3,2 ± 0.4	6,0 ± 5.4	1,7 ± 0.1
No h*β1*	activation ($V_{1/2}$) (mV)	-22.3 ± 1.4	-30.0 ± 0.7	-16.8 ± 0.7
	inactivation ($V_{1/2}$) (mV)	-30.6 ± 1.2	-40.4 ± 0.7	-49.3 ± 1.8
	recovery (T) (ms)	2,3 ± 0.2	4,9 ± 0.2	5 ± 0.3