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Primidone improves symptoms in *TRPM3*-linked DEE-SWAS

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Abstract

Developmental and epileptic encephalopathy (DEE) with continuous spike-and-wave activation in sleep (CSWS) or DEE-SWAS is an age-dependent disease, often accompanied by a decline in cognitive abilities. Early successful treatment of CSWS is associated with a better cognitive outcome. We retrospectively analyzed the clinical, electrophysiological, radiological, and genetic data of children with DEE-SWAS associated with melastatin-related transient receptor type 3 gene (*TRPM3*) missense variants.

We report two unrelated children with pharmaco-resistant DEE-SWAS and developmental delay/regression and different heterozygous *de novo* missense variants in the *TRPM3* gene (NM_001366145.2; c.3397T>C/p.Ser1133Pro, c.2004G>A/p.Val1002Met). The variant p.Val1002Met (previously known as p.Val990Met or p.Val837Met) and p.Ser1133Pro were recently shown to result in a gain-of-function (GoF) effect. Based on this fact, previous drug resistance, and the experimentally demonstrated inhibitory effect of primidone on TRPM3, we initiated an individualized therapy with this drug. In both children, developmental regression was stopped, psychomotor development improved, and CSWS was no longer detectable.

To our knowledge, this is the first report of a treatment with primidone in *TRPM3*-associated CSWS. Our results highlight the importance of early genetic diagnosis in patients with epilepsy and the possibility of precision medicine, which should be considered in future individuals with a TRPM3-linked DEE-SWAS.

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Introduction

Developmental and epileptic encephalopathy with continuous spike-and-wave complexes during sleep (CSWS) or DEE-SWAS is an age-dependent phenomenon often associated with developmental delay or regression.[1,2] The network dysfunction, expressed in the characteristic EEG pattern with continuous and diffuse epileptiform discharges occupying a significant proportion of slow wave sleep, is thought to disrupt normal neuropsychological development.[2] Despite the dramatic impact of CSWS on development in many children, a cause is identified in only about 66% of affected individuals.[1,2] A structural cause can be identified in 45%, particularly perinatally acquired thalamic lesions and malformations of cortical development.[3] Moreover, variants in genes such as *KCNQ2*, *KCNA2*, *GRIN2A*, *MECP2*, and *SLC9A6* are monogenic causes, found in about 20% of patients with CSWS.[2-5] Treatment is unsuccessful in approximately half of the patients,[3] with clobazam and corticosteroids instead of conventional anti-seizure medication being more commonly used.[3,6]

We report successful treatment with primidone in two children with pharmaco-resistant DEE-SWAS, with diagnosis of CSWS due to developmental regression, in whom we identified heterozygous *de novo* missense variants in the transient receptor potential melastatin channel type 3 gene (*TRPM3*, HGNC: 17882, c.3397T>C/p.Ser1133Pro, NM_001366145.2: c.3004G>A/p.Val1002Met, formally known as NM_020952.6: c.2509G>A/p.Val837Met[7] and NM_001007471.2: c.2968G>A/p.Val990[8]). TRPM3 encodes a calcium-permeable cation channel activated by heat and chemical ligands such as the neurosteroid pregnenolone sulphate (PS).[9,10] TRPM3 channel activity in vitro and in vivo can be blocked by the anticonvulsant primidone (IC50 = 0.6 ± 0.15 µM). [11] TRPM3 is known to act as a temperature sensor in sensory neurons from dorsal

root ganglion and trigeminal ganglion neurons, but is also expressed in various other brain areas.[7,12,13]

Following the first report of pathogenic *TRPM3* variants in eight individuals with moderate-to-severe DEE in 2019, nine further individuals with *TRPM3* variants with and without epilepsy phenotypes have been reported.[14-17] Very recently, another report was published showing seven additional de novo variants of TRPM3.[13] Various disease-associated variants including the p.Val1002Met variant identified in an affected individual in this study (patient 2) render TRPM3 overactive, resulting in increased Ca^{2+} and Na^{+} influx into the cell, both in basal conditions and upon stimulation with PS or heat.[8,12] In addition, also the p.Ser1133Pro variant resulted in an elevated basal activity and an increased Ca^{2+}-influx after PS stimulation.[13] Recently, the anti-seizure medication primidone was reported to suppress TRPM3 activity in HEK293 cells overexpressing mouse TRPM3 (HEK_{mTRPM3} cells) and isolated nociceptive rat dorsal root ganglia neurons *in vitro*, and also *in vivo* in a PS-induced chemical pain mouse model.[11] Primidone is metabolized to phenobarbital and phenylethylmalonilamide acid (PEMA), and all three metabolites have their individual effects.[18] Since primidone inhibits TRPM3 activity at clinically relevant concentrations and has sufficient levels in the cerebrospinal fluid,[19] we investigated whether the drug would be effective in the two children with drug-resistant DEE-SWAS due to *TRPM3* variants as an off-label use.

Methods

Patient information

We retrospectively analyzed the effect of an off-label therapy of patients with DEE-SWAS and *TRPM3* variants with primidone, performed upon written informed consent by the parents. We extracted seizure- and treatment-specific data from electronic and paper-based medical records using a standardized data collection sheet. The retrospective analysis of epilepsy patient data was approved by the local ethics committee (approval no. EA2/084/18). Written informed consent was obtained from the parents of both patients.

Fluorescence Imaging and Electrophysiology

For patch-clamp experiments, cells were transfected with 2 µg of channel cDNA using TransIT-293 transfection reagent (MirusBio) and analyzed 36-48 hours after transfection. When indicated, to mimic heterozygous conditions, a mixture of wild-type and TRPM3 variant cDNA was used (ratio 1:1).

Primidone, phenobarbital and phenylethylmalonic acid (PEMA) were present in black 384- well assay plates (Greiner) with buffer containing (in mM): 138 NaCl, 5.4 KCl, 4 CaCl2, 2 MgCl2, 10 glucose, and 10 Hepes; pH 7.2 with NaOH. HEK293 cells stably expressing rat TRPM3 were loaded with 2 µM Fluo-4 AM (Invitrogen) for 30 min, detached using trypsine, collected in calcium-free medium (in mM) (138 NaCl, 5.4 KCl, 2 MgCl2, 10 glucose, and 10 Hepes; pH 7.2 with NaOH), and added to the assay plate at ± 2,000 cells per well. The final CaCl2 concentration in the assay solution was 2.4 mM. Fluorescence (excitation at 485 nm; emission at 535 nm) was measured with an Envision fluorescence reader before and after the addition of 50 µM PS. All compounds were tested at different doses of 0.00128, 0.0064, 0.032, 0.16, 0.8, 4 and 20 µM. Channel inhibition was calculated compared to a non-PS stimulated control versus a

condition stimulated with PS (50 µM) with vehicle. The ability of the compounds of the invention to inhibit this activity was determined as: Percentage inhibition = [1-((RFU determined for sample with test compound present – RFU determined for sample with positive control inhibitor) divided by (RFU determined in the presence of vehicle – RFU determined for sample with positive control inhibitor))] * 100.

Whole-cell membrane currents were measured with an EPC-10 patch-clamp amplifier and PatchMasterPro Software (HEKA Elektronik). Current measurements were performed at a sampling rate of 20 kHz, and currents were digitally filtered at 2.9 kHz. For whole-cell recordings, the standard extracellular solution contained (in mM) 150 NaCl, 1 MgCl2, and 10 Hepes (pH 7.4 with NaOH), and the standard internal solution contained (in mM) 100 CsAsp, 45 CsCl, 10 EGTA, 10 Hepes, and 1 MgCl2 (pH 7.2 with CsOH). HEK293 cells were transiently transfected with either WT human TRPM3 or a 1:1 mixture of WT and the S1133P variant to mimic the expression profile in cells of heterozygous patients. To evaluate the channel activity at basal level and in the presence of PS (40 µM), a voltage step protocol was applied in which voltage steps of +40 mV were applied starting from -160 mV towards +160 mV with a holding potential at 0 mV.

To determine the IC50 of primidone on WT-TRPM3 and WT+S1133P expressing cells, ionic currents were measured using the whole-cell patch-clamp technique applying a voltage ramp protocol from -150 mV to +150 mV every 2 seconds. PS (40 µM) was used to activate the channel. Different concentrations of primidone (0.01, 0.1, 1, 10 and 100 µM) were subsequently added to obtain concentration-inhibition curves, that were fitted using a Hill equation to obtain IC50 values.

Chemicals

Pregnenolone sulphate (PS) and primidone were obtained from Sigma-Aldrich, PEMA was purchased from Merck-Millipore and phenobarbital was obtained via internal collaboration with CD3-CISTIM. Pregnenolone sulphate (PS) and primidone were dissolved in the bath solutions from a 100 mM stock diluted in DMSO.

Results

Recent reports have identified two de novo *TRPM3* variants, p.(V1002M) and p.(S1133P), as gain-of-function channels in the heterozygous condition, showing an increased intracellular calcium, $[Ca^{2+}]_i$, level at basal conditions and elevated $[Ca^{2+}]_I$ influx after stimulation by PS. [8,12,13] Additional whole-cell patch-clamp experiments further confirmed the elevated basal activity of S1133P mutant in the heterozygous condition (WT+S1133P) (**Figure 1**). Therefore, whole-cell currents were measured in response to a voltage step protocol ranging from -160 mV to +160 mV, both at baseline and upon stimulation with PS (40 µM). HEK293 cells expressing WT+S1133P, showed robust outwardly rectifying currents that were significantly larger compared to WT-hTRPM3 expressing cells at basal level and upon stimulation with PS (**Figure 1**). These data further confirmed the heterozygous condition WT+S1133P as a gain of function channel with increased basal activity and elevated current amplitudes upon stimulation by PS.

Primidone is known as a potent inhibitor of TRPM3,[11] however, no knowledge is available on the inhibitory potency of its metabolites, phenobarbital and phenylethylmalonic acid (PEMA). Therefore, the potency of primidone and its metabolites was further investigated to inhibit the PS-induced TRPM3 activity in HEK293 cells stably expressing rat TRPM3. The concentration for half maximal inhibition (IC_{50}) was calculated for each compound and resulted in primidone (IC_{50} = 1.2 µM) as the most potent TRPM3 inhibitor compared to phenobarbital (IC_{50} = 4.4 µM). Remarkably, preincubation of high dose of PEMA (40 µM) did not induce any block of the PS-induced $[Ca^{2+}]_I$ influx (IC_{50} > 40 µM) (**Figure 2**).

Next, the inhibitory potency of primidone was calculated towards PS-induced channel activity in the disease-associated variant p.Ser1133Pro via whole-cell patch-clamp experiments. In cells co-expressing WT+S1133P, mimicking the heterozygous

condition in patient cells, primidone caused a concentration-dependent inhibition of the outward currents (IC_{50} = 2.7 ± 0.2 µM), that was similar to the WT channel (IC_{50} = 2.9 ± 0.8 µM) (**Figure 3**).

Furthermore, we report two patients with DEE-SWAS and thus with developmental delay and CSWS caused by variants in the *TRPM3* gene (**Table 1**).

The **first patient**, a 4.3-year-old girl, was born as the 3rd child of healthy, unrelated parents of Western European descent after an uneventful pregnancy and delivery. She had delayed developmental milestones (sitting at 12 months, walking at 20 months, first words at 15 month, 20-30 words spoken at 2.5 years-of-age) (**Supplemental Figure 2**). An antibody-mediated thrombocytopenia was diagnosed at the age of 20 months and required prednisolone treatment to stabilize platelet counts prior to an adenectomy. Following this steroid treatment at 2.5 years-of-age, the parents noticed a significant speech improvement, with >100 words spoken in short sentences. Speech production, however, declined, and eventually stopped, concurrent with cognitive regression 4 weeks after steroid application. An EEG revealed a CSWS pattern (**Supplemental Figure 1, 2**), while the result of a cranial MRI was normal. Treatments with sultiame (4.4 mg/kg/d from 2.8 to 2.9 years), lamotrigine (5 mg/kg/d from 2.9 to 3.2 years), cannabidiol (23 mg/kg/d, 3.1 to 3.4 years), clobazam (0.4 mg/kg/d from 2.6 to 3.5 years), ketogenic diet (3.1 to 3.3 years), and nine further oral/intravenous steroid pulses with prednisolone (n = 4) or dexamethasone (n = 5) were unsuccessful (**Supplemental Figure 2)**. Whole exome sequencing (WES) followed by Sanger sequencing revealed the *de novo* variant c.3397T>C (p.Ser1133Pro) in *TRPM3* (NM_001366145.2; NP_001353074.1). The variant affects a highly conserved amino acid (PhyloP100way score = 8.9) located in transmembrane 6 of TRPM3. The variant is absent from population databases (gnomAD) and predicted to be damaging by various tools (MutationTaster, SIFT, PolyPhen2, CADD score: 27.2). Functional

characterization indicates that the variant leads to a gain of channel function (**Figure 1**).[13]

In order to counteract the disease-causing gain of TRPM3 function in the patient, we initiated an off-label treatment with primidone at the age of 3.5 years due to a continuing cognitive regression, exhaustion of multiple above-described treatments, and the request of the parents to offer a novel treatment regime. At an initial oral dose of 2.5 mg/kg/d (given in two doses), the patient developed severe restlessness, auto-aggressive behavior, and itchiness. The dose was then decreased to 1 mg/kg/d, and subsequently increased slowly by 0.3 mg/kg/d each week to a maximum dose of 9 mg/kg/d, which was well tolerated. The latest primidone and phenobarbital blood levels were both within the therapeutic drug level ranges after 14 months (primidone: 6.9 mg/l (aim: 5-15 mg/l), phenobarbital: 14.7 mg/l (aim: 10-40 mg/l). Three months following treatment initiation, CSWS was no longer present on EEG, and only minimal epileptic discharges were detected once every minute. The EEG was normal 9 months after treatment initiation (**Supplemental Figure 1, 2**). No formal neuropsychological evaluation was performed, before treatment initiation, but Bayley Scales of Infant and Toddler Development III at 4.7 years (15 months after treatment initiation) showed an severe developmental delay with an age equivalent score of 22 months. One year and 3 months after treatment initiation the child's concentration continues to improve, and the patient has started to speak single words again.

The **second patient**, a 6-year-old boy, was born as the first child of healthy, unrelated parents of US-American, Caucasian descent after an uneventful pregnancy and delivery. He had axial hypotonia at birth and delayed developmental milestones (walking 4 years, first words 3 years). The result of a cranial MRI was normal. At the age of 3 years, he had daily seizures with behavioral arrest and head nodding, and

occasional bilateral tonic-clonic seizures. Sleep-EEG displayed CSWS (**Supplemental Figure 1. 2**). Multiple treatments including levetiracetam (50 mg/kg/d, since 3 years-of-age), topiramate (2.6 mg/kg/d, since 3.2 years-of-age), clobazam (1 mg/kg/d, since 4 years-of-age), ethosuximide (33 mg/kg/d, from 5 to 5.3 years) and ketogenic diet (from 5.5 to 6.years) were unable to improve the developmental delay and CSWS (**Supplemental Figure 2)**. WES revealed the recurrent pathogenic *TRPM3* variant c.3004G>A (p.Val1002Met) (formally known as NM_020952.6: c.2509G>A/p.Val837Met[7] and NM_001007471.2: c.2968G>A/p.Val990[8]), which leads to a severe gain of channel function.[7,8] Previous studies had already indicated potent inhibition of this variant by primidone. [8,12]. After the first presentation at our center with 6 years-of-age, we initiated an off-label treatment with primidone with an initial dose of 1 mg/kg/d, given in two doses, which was slowly increased by 0.3 mg/kg/d each week to a maximum dose of 6 mg/kg/d. At last contact eight months after treatment initiation no side-effects were reported, CSWS or epileptic discharges were no longer detectable in the EEG, and the daily seizures with behavioral arrest and head nodding had stopped. Although no standardized cognitive testing was performed, the parents reported improved motor skills, concentration, alertness, and language comprehension at last contact (6.8 years-of-age).

Discussion

We report on two patients with treatment-resistant DEE-SWAS and developmental delay/regression caused by *de novo* missense variants in *TRPM3*, in whom gene function-targeted treatment with the TRPM3 inhibitor primidone was initiated. Both patients continued to show development improvement at 15 (patient 1) and 8 (patient 2) months into treatment, and their EEG had normalized. These findings support that primidone is a treatment option in patients with *TRPM3*-related disease.

The positive effect seen in the two affected children is in line with experimental data highlighting an inhibitory effect of primidone on TRPM3 [11,12] (**Figure 2** and **3**) and the notion that gain of TRPM3 function is disease-causing. Nevertheless, it should be noted that primidone is not selective for TRPM3 and has additional targets such as the $GABA_A$ receptors that may contribute to its anti-epileptic effects in our patients. However, primidone was four times more potent compared to phenobarbital to inhibit the PS induced calcium influx, while PEMA did not show any inhibition of TRPM3 activity (**Figure 2**). Furthermore, one study report EEG and clinical worsening under phenobarbital treatment in patients with CSWS not linked to TRPM3.[20] Therefore, this report supports the use of primidone exclusively in patients with gain-of-function *TRPM3* variants. Additionally, our study is limited due to the small cohort size, absence of formal neuropsychological testing to evaluate cognitive outcome, and heterogeneous pre- and co-treatment with anti-seizure medications.

TRPM3-related disorders are characterized by developmental delay, intellectual disability, and muscular hypotonia.[14,16,12] Epilepsy has been reported for a subset of patients: in 11/17 patients reported in the literature epilepsy is present in infancy or childhood (onset 9 months to 7 years) with various seizures types including absence seizures (n = 4), generalized tonic-clonic seizures (n = 3), epileptic spasms in infancy (n = 2), and CSWS (n = 1).[7] However, sleep EEGs to diagnose or exclude CSWS in

light of developmental delay are lacking in most of these reports.[7] Some children seem to have a low seizure burden with only two reports of drug-resistant epilepsy so far, but many patient descriptions lack information on seizure control.[14,15] Our report of a successful treatment in two patients of *TRPM3*-associated CSWS with primidone calls for a systematic analysis of the *TRPM3*-related epilepsy phenotype including detailed seizure type description and systematic sleep EEGs in all individuals with *TRPM3* variants. These results further highlight the importance of early genetic diagnosis in patients with epilepsy and the possibility of the growing number of precision medicines, which should be considered in all epilepsy patients.

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References:

1. Arzimanoglou A, Cross HJ. Cognitive impairment and behavioral disorders in Encephalopathy related to Status Epilepticus during slow Sleep: diagnostic assessment and outcome. *Epileptic Disord.* 2019;21(S1):71-75.
2. Dorris L, O'Regan M, Wilson M, Zuberi SM. Progressive intellectual impairment in children with Encephalopathy related to Status Epilepticus during slow Sleep. *Epileptic Disord.* 2019;21(S1):88-96.
3. Arican P, Gencpinar P, Olgac Dundar N, Tekgul H. Electrical Status Epilepticus During Slow-wave Sleep (ESES): Current Perspectives. *J Pediatr Neurosci.* 2021;16(2):91-96.
4. Veggiotti P, Pera MC, Olivotto S, De Giorgis V. How to Manage Electrical Status Epilepticus in Sleep. *J Clin Neurophysiol.* 2016;33(1):3-9.
5. Gong P, Xue J, Jiao X, Zhang Y, Yang Z. Genetic Etiologies in Developmental and/or Epileptic Encephalopathy With Electrical Status Epilepticus During Sleep: Cohort Study. *Front Genet.* 2021;12:607965.
6. Veggiotti P, Pera MC, Teutonico F, Brazzo D, Balottin U, Tassinari CA. Therapy of encephalopathy with status epilepticus during sleep (ESES/CSWS syndrome): an update. *Epileptic Disord.* 2012;14(1):1-11.
7. Dyment DA, Terhal PA, Rustad CF, et al. De novo substitutions of TRPM3 cause intellectual disability and epilepsy. *Eur J Hum Genet.* 2019;27(10):1611-1618.
8. Van Hoeymissen E, Held K, Nogueira Freitas AC, Janssens A, Voets T, Vriens J. Gain of channel function and modified gating properties in TRPM3 mutants causing intellectual disability and epilepsy. *Elife.* 2020;9.
9. Vriens J, Owsianik G, Hofmann T, et al. TRPM3 is a nociceptor channel involved in the detection of noxious heat. *Neuron.* 2011;70(3):482-494.
10. Wagner TF, Loch S, Lambert S, et al. Transient receptor potential M3 channels are ionotropic steroid receptors in pancreatic beta cells. *Nat Cell Biol.* 2008;10(12):1421-1430.
11. Krugel U, Straub I, Beckmann H, Schaefer M. Primidone inhibits TRPM3 and attenuates thermal nociception in vivo. *Pain.* 2017;158(5):856-867.
12. Zhao S, Yudin Y, Rohacs T. Disease-associated mutations in the human TRPM3 render the channel overactive via two distinct mechanisms. *Elife.* 2020;9.
13. Burglen L, Van Hoeymissen E, Qebibo L, et al. Gain-of-function variants in the ion channel gene TRPM3 underlie a spectrum of neurodevelopmental disorders. *eLife.* 2023.
14. Kang Q, Yang L, Liao H, et al. A Chinese patient with developmental and epileptic encephalopathies (DEE) carrying a TRPM3 gene mutation: a paediatric case report. *BMC Pediatr.* 2021;21(1):256.
15. Lines MA, Goldenberg P, Wong A, et al. Phenotypic spectrum of the recurrent TRPM3 p.(Val837Met) substitution in seven individuals with global developmental delay and hypotonia. *Am J Med Genet A.* 2022.
16. de Sainte Agathe JM, Van-Gils J, Lasseaux E, et al. Confirmation and Expansion of the Phenotype Associated with the Recurrent p.Val837Met Variant in TRPM3. *Eur J Med Genet.* 2020;63(8):103942.
17. Gauthier LW, Chatron N, Cabet S, et al. Description of a novel patient with the TRPM3 recurrent p.Val837Met variant. *Eur J Med Genet.* 2021;64(11):104320.
18. Lenkapothula N, Cascella M. Primidone. In: *StatPearls.* Treasure Island (FL)2022.

19. Schmidt D, Kupferberg J. Diphenylhydantoin, phenobarbital, and primidone in saliva, plasma, and cerebrospinal fluid. *Epilepsia.* 1975;16(5):735-741.
20. Arhan E, Serdaroglu A, Aydin K, Hirfanoglu T, Soysal AS. Epileptic encephalopathy with electrical status epilepticus: an electroclinical study of 59 patients. *Seizure.* 2015;26:86-93.

Figures and Tables

Table 1. Phenotype of patients. ID: intellectual disability.

Patient	**P1**	**P2**
Origin	German	US-American
Sex (age; years)	female (4.3)	male (6)
TRPM3 variant (NM_020952.4)	c.3397T>C	c.2004G>A
Inheritance	*de novo*	*de novo*
Onset	birth	birth
Pregnancy/birth	normal	normal
Motor development	delayed	delayed
Cognition Speech	Moderate ID no sentences, 3 words	Moderate ID sentences with 3-4 words
Regression	yes	no
Behavioral problems	uncooperative	no
sleep	disrupted	disrupted
Muscle strength Muscle tone	normal hypotonia	normal hypertonia distal lower extremities
Walking	yes	yes, broad based
Muscle reflexes	normal	normal
Movement disorder	no	yes
Sensory abnormalities	no	no
Orthopedic abnormalities/ contractures	no	no
Cranial MRI	normal	normal
Eye abnormalities	strabism	no
seizures	no	yes

Figure 1. WT+S1133P transfected HEK293 cells showed increased baseline and PS-induced current densities. (A) Representative whole-cell TRPM3 current densities (pA/pF) recorded at baseline (left) and during the application of the agonist pregnenolone sulphate (PS) (40μM, right) during voltage steps ranging from -160 mV to +160 mV, separated by steps of +40 mV for wild-type (WT), and WT+ S1133P transiently transfected HEK293T cells. (**B**-**E**) Scatter plot of current densities (pA/pF) for WT, and WT+SP transfected HEK293T cells (N = 5 for WT and N = 5 for WT+SP conditions) without the application of an agonist at +160 mV (**B**) and -160 mV (**D**) and during the application of PS at +160 mV (**C**) and -160 mV (**E**). Data are represented

as mean ± SEM and individual cells are represented as a dot (B-E). p-Values are indicated on the graphs using an ANOVA.

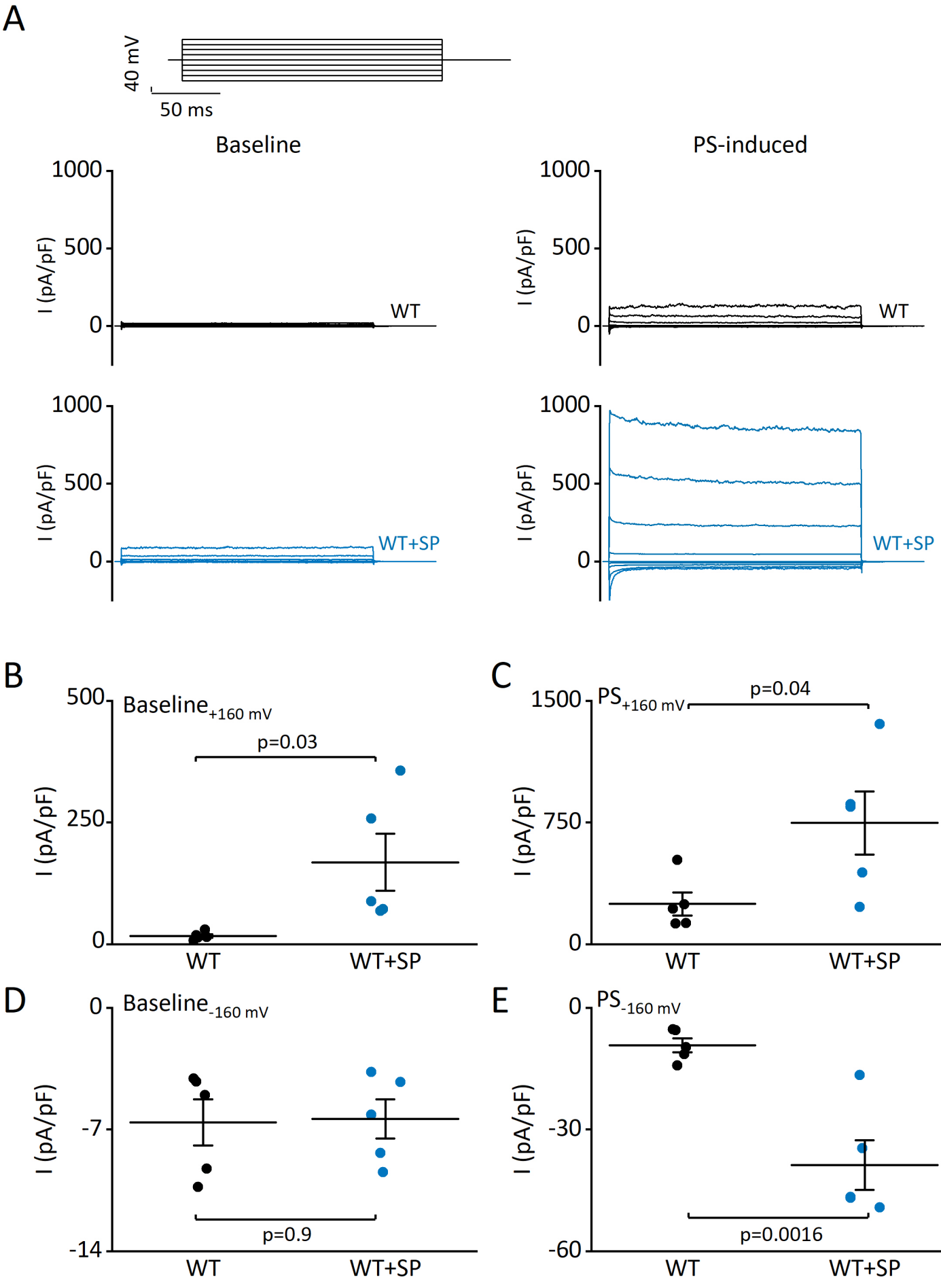
Figure 2. Dose-response relationship of primidone, and its metabolites phenobarbital and PEMA on PS-induced TRPM3 activity. Intracellular calcium concentrations were monitored using Fluo-4 AM in HEK293 cells stably expressing rat TRPM3 after the addition PS (50 µM). Cells were incubated with different doses (0.00128, 0.0064, 0.032, 0.16, 0.8, 4 and 20 µM) of primidone (black), phenobarbital (blue) or phenylethylmalonic acid (PEMA, red) prior to PS stimulation. The ability of the compounds of the invention to inhibit PS-induced activity was determined as percentage inhibition, and given as mean ± SEM (n = 2 independent experiments). Half maximal inhibition values were obtained by fitting a non-linear 4 parameter Hill equation.

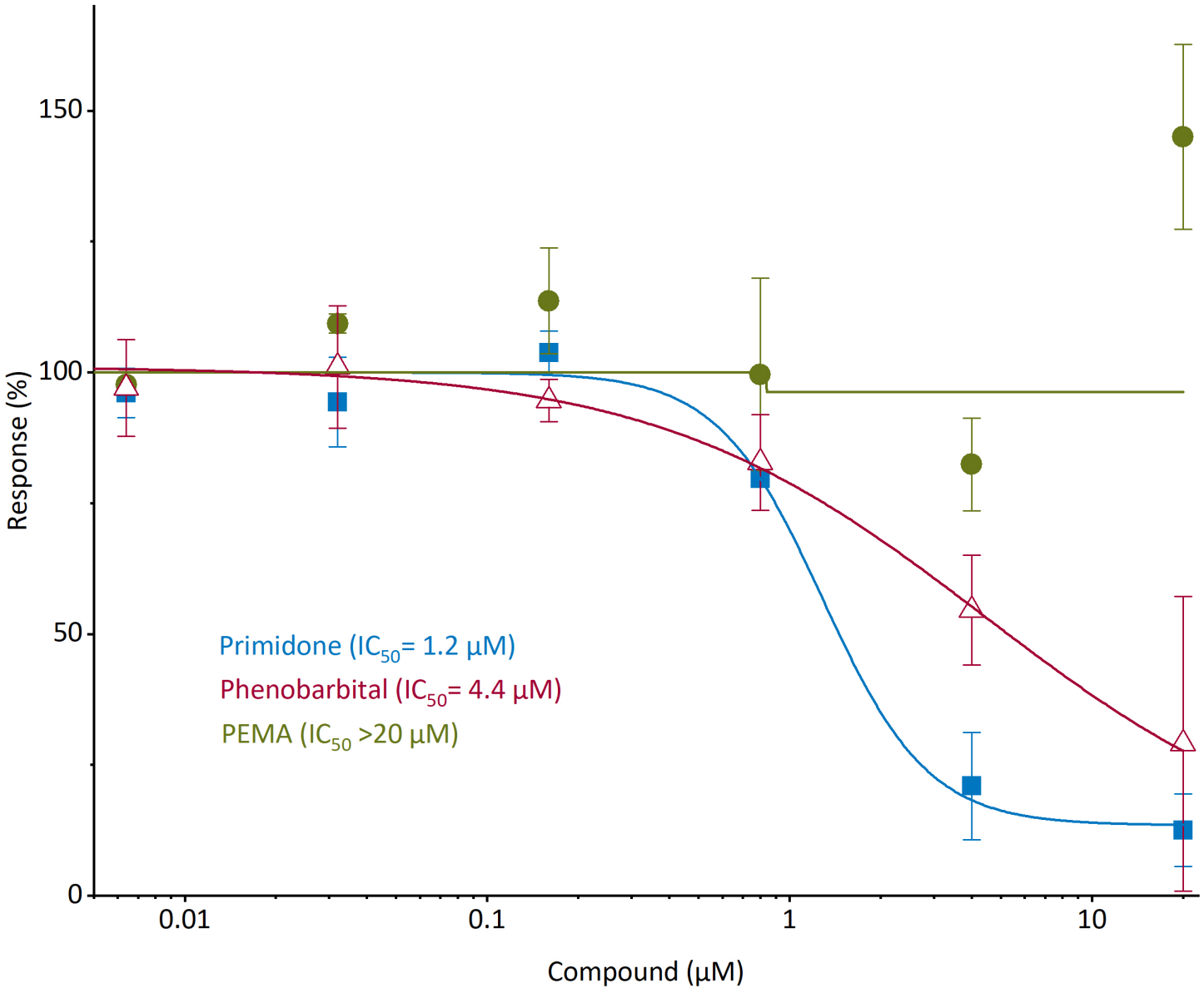
Figure 3. Electrophysiological characterization of the primidone-induced inhibition. (A, D) Representative whole-cell currents from a HEK293T cell overexpressing wild-type (WT) humanTRPM3 (A), and HEK293T cells co-transfected with WT TRPM3 + S1133P constructs (D) after application of pregnenolone sulphate (PS; 40 µM) and increasing concentrations of primidone (0.01 µM, 0.1 µM, 1 µM, 10 µM, and 100 µM). Time course of whole-cell patch clamp recording at holding of +150 mV (closed circles) and -150 mV (open circles). (**B, E**) Current density (*I*)-voltage (*V*) relation at time points indicated in panel (A) and (D) respectively. (**C, F**) Concentration-dependent inhibition of PS-induced outward current at +150 mV by primidone. Concentration-response curves for HEK293T cells overexpressing WT TRPM3 (C; IC50 = 2.9 ± 0.8 µM; n = 6 cells), and WT TRPM3 + S1133P (F; IC50 = 2.7 ± 0.2 µM

n = 8 cells). Half maximal inhibition values were obtained by fitting a non-linear 4 parameter Hill equation to each experiment. Mean values ± SEM were shown.

Supplemental Figure 1. (A) Representative EEG of patient 1 and 2 before and during treatment with primidone**.** EEG of patient 1 shows generalized epileptic discharges during daytime with strong activation in sleep over 80% in line with continuous spike-and-wave complexes during sleep (CSWS) with 3.9 years three weeks prior treatment. Three months after primidone (maximum dose 4,6 mg/kg/d) was started, the epileptic discharges decreased immensely with no CSWS at the age of 4.5 years. **(B)** EEG of patient 2 in wakefullness and during sleep with CSWS one month before therapy.

Supplemental Figure 2. (A) Timeline of developmental milestones, treatment, and EEG of patient 1 and **(B)** patient 2. Abbreviations: CSWS: continuous spike-and-wave during slow-wave sleep; CBD: cannabidiol; CLB: clobazam; D: dexamethasone; E: ethosuximide; y/o: years of age; keto: ketogenic diet; LEV: levetiracetam; LTG: lamotrigine; P: prednisolone; STM: sultiame; TPM: topiramate.





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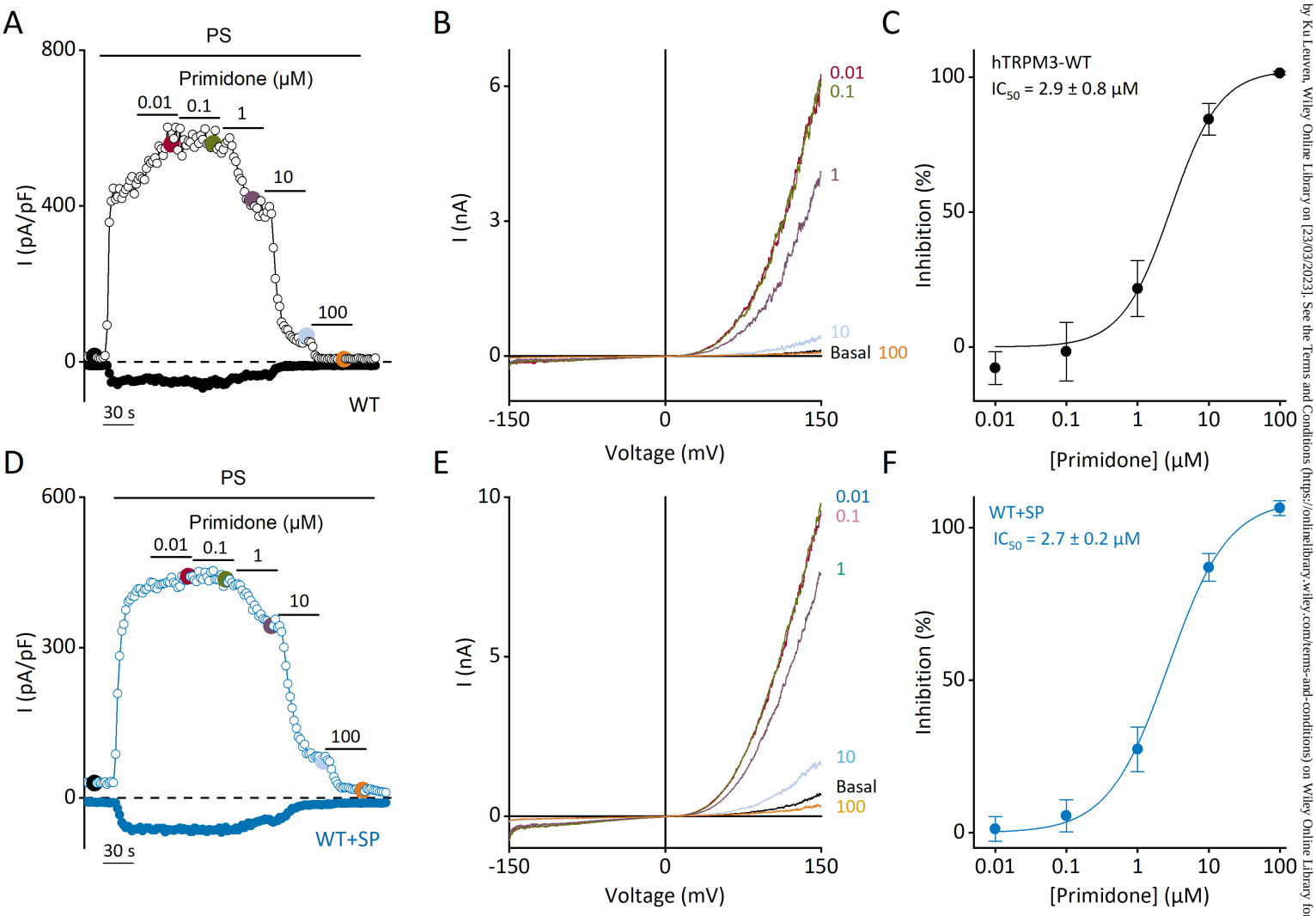

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