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SCN3A deficiency associated with increased seizure susceptibility

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ABSTRACT

Mutations in voltage-gated sodium channels expressed highly in the brain (*SCN1A*, *SCN2A*, *SCN3A*, and *SCN8A*) are responsible for an increasing number of epilepsy syndromes. In particular, mutations in the *SCN3A* gene, encoding the pore-forming $Na_v1.3$ α subunit, have been identified in patients with focal epilepsy. Biophysical characterization of epilepsy-associated *SCN3A* variants suggests that both gain- and loss-of-function *SCN3A* mutations may lead to increased seizure susceptibility. In this report, we identified a novel *SCN3A* variant (L247P) by whole exome sequencing of a child with focal epilepsy, developmental delay, and autonomic nervous system dysfunction. Voltage clamp analysis showed no detectable sodium current in a heterologous expression system expressing the SCN3A-L247P variant. Furthermore, cell surface biotinylation demonstrated a reduction in the amount of SCN3A-L247P at the cell surface, suggesting the SCN3A-L247P variant is a trafficking-deficient mutant. To further explore the possible clinical consequences of reduced *SCN3A* activity, we investigated the effect of a hypomorphic *Scn3a* allele ($Scn3a^{Hyp}$) on seizure susceptibility and behavior using a gene trap mouse line. Heterozygous *Scn3a* mutant mice ($Scn3a^{+/Hyp}$) did not exhibit spontaneous seizures nor were they susceptible to hyperthermia-induced seizures. However, they displayed increased susceptibility to electroconvulsive (6 Hz) and chemiconvulsive (flurothyl and kainic acid) induced seizures. $Scn3a^{+/Hyp}$ mice also exhibited deficits in locomotor activity and motor learning. Taken together, these results provide evidence that loss-of-function of *SCN3A* caused by reduced protein expression or deficient trafficking to the plasma membrane may contribute to increased seizure susceptibility.

1. Introduction

Voltage-gated sodium channels (VGSCs) are responsible for the initiation and propagation of action potentials in excitable cells such as neurons. In the mammalian brain, the most highly expressed VGSC α subunits are *SCN1A*, *SCN2A*, *SCN3A*, and *SCN8A*, which encode $Na_v1.1$, $Na_v1.2$, $Na_v1.3$, and $Na_v1.6$ respectively. Mutations in these VGSCs are responsible for an increasing number of epilepsy syndromes (Escayg et al., 2000; Estacion et al., 2014; Fung et al., 2015; Howell et al., 2015; Schwarz et al., 2016; Sugawara et al., 2001; Surovy et al., 2016). Mutations in *SCN1A* have been established as the main cause of Dravet syndrome and have also been identified in some families with generalized epilepsy with febrile seizures plus (GEFS+) (Escayg and Goldin, 2010; Scheffer et al., 2009; Volkers et al., 2011). *SCN2A* mutations have been identified in patients with benign familial neonatal-infantile seizures, and both *SCN2A* and *SCN8A* mutations have been identified in some cases of severe epileptic encephalopathies (de Kovel et al., 2014; Estacion et al., 2014; Hackenberg et al., 2014; Heron et al., 2002; Sugawara et al., 2001; Vaher et al., 2014; Veeramah et al., 2012).

To date, only a few *SCN3A* mutations have been identified in patients with focal epilepsy. Electrophysiological analysis of these mutations in either transfected rat hippocampal pyramidal neurons (Estacion et al., 2010; Holland et al., 2008) or tsA201 cells (Chen et al., 2015; Vanoye et al., 2014) revealed increased persistent sodium current or elevated ramp currents. In contrast, there is one report of an *SCN3A* mutation, N302S, identified through a genetic screen of GEFS+ patients, that resulted in depolarizing shifts in voltage-dependent activation and inactivation, as well as slower recovery from slow inactivation, thereby predicting a reduction in channel activity (Chen et al., 2015). These

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results suggest that both gain- and loss-of-function *SCN3A* mutations may lead to epilepsy.

Epilepsy syndromes, including those caused by sodium channel mutations, are often accompanied by neuropsychiatric comorbidities, such as anxiety, autism spectrum disorders, and intellectual disability (Baasch et al., 2014; de Kovel et al., 2014; Han et al., 2012). Consistent with this, developmental delay or behavioral abnormalities have been reported in patients with *SCN3A* mutations. For example, one patient presented with speech delay and attention-deficit/hyperactivity disorder (Vanoye et al., 2014). Another patient was diagnosed with nonverbal learning disability, and the patient carrying the N302S mutation was diagnosed with intellectual disability (Chen et al., 2014; Vanoye et al., 2014).

In this study, we report a novel *SCN3A* mutation (L247P) associated with childhood focal epilepsy and global developmental delay. Using a combination of electrophysiology and cell surface biotinylation experiments, we demonstrate that SCN3A-L247P encodes a trafficking deficient channel. To more broadly investigate the possible clinical consequences of reduced *SCN3A* activity, we characterized the seizure and behavioral phenotypes of the available $Scn3a^{Gt(OST52130)Lex}$ mouse line, which expresses a hypomorphic *Scn3a* allele ($Scn3a^{Hyp}$). Heterozygous mutants ($Scn3a^{+/Hyp}$) displayed increased susceptibility to induced seizures, but did not exhibit spontaneous seizures. Additionally, $Scn3a^{+/Hyp}$ mice showed deficits in locomotor activity and motor learning. These observations demonstrate that reduced *SCN3A* function via reduced trafficking or expression can result in increased seizure susceptibility.

2. Material and methods

2.1. Clinical presentation

Parental consent for release of de-identified medical information was obtained. An 18 month-old female patient was evaluated due to concerns for global developmental delay, central hypotonia, right-sided renal pelviectasis, and microcephaly. She was the second child of a 39-year-old mother and 50-year-old father, both of whom denied consanguinity. The pregnancy was uncomplicated, amniocentesis was declined, and the child was born at full term. At birth, her weight was 2950 g (10%), her length was 50 cm (20%), and her head circumference was 33 cm (10%). Her Apgar scores were 9 and 9. At two days of age she had an episode of cyanosis, apnea, tonic posturing and bradycardia. EEG recording at that time was normal.

Beginning during the first week of life, two distinct types of episodes were observed, and occasionally coincided. The first type consists of a "scared look" followed by paroxysmal events, which include apnea, eyes rolling back, and limbs stiffening. These episodes occur approximately once a month and were subsequently identified as focal seizures. The second type of episode includes skin flushing and sweating and occurs several times a day for 30-second intervals. This clinical presentation is consistent with Harlequin syndrome, which is characterized by unilateral facial erythema with contralateral pallor that is strikingly demarcated at the midline. Although the color change is limited to the head, it can initiate on one side and shift to the other side within the same episode. This coincides with contralateral pupil dilation and ipsilateral ptosis. Video EEG at 14 months of age revealed right frontal-temporal electrographic seizures compatible with focal epilepsy. Various anticonvulsant medications were tried with limited success. The EEG abnormalities were not associated with the autonomic changes; therefore, it was concluded that the episodes of autonomic dysregulation are not epileptic by nature. The patient also developed progressive microcephaly, although her brain and cervical spine MRIs were normal. A routine cardiology examination, which included EKG, echocardiogram, and Holter monitor, showed no abnormalities. On telemetry, however, several episodes of sinus and junctional bradycardia were noted and thought to correspond with vagal activity and the Harlequin flushing. The child was also diagnosed with global developmental delay. She did not sit without support until 16 months after birth and was still unable to walk on her last evaluation at two years of age. Her speech development was also delayed. Additionally, her height, weight and head circumference were below the third percentile for two year olds. She also had trunkal hypotonia and mid-face hypoplasia.

Metabolic screening was negative. Familial Dysautonomia was ruled out by observation of normal fungiform tongue papillae and normal axon flare on intradermal histamine testing. Chromosomal microarray analysis was normal. Clinical whole-exome sequencing revealed a single, novel, de novo heterozygous sequence change in the gene *SCN3A*, L247P. In silico analysis concluded that this change was "likely pathogenic". Other changes reported were deemed as "benign" or were rare population variants inherited from an unaffected parent, suggesting they were not pathogenic (Supplemental Table 1). Results were confirmed by Sanger sequencing.

2.2. Plasmids and cell transfection

Electrophysiology and biochemistry experiments were conducted using tsA201 cells (HEK-293 stably transfected with SV40 large T antigen) grown at 37 °C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and penicillin (50 U/ml)-streptomycin (50 μg/ml). Only cells from passage number <13 were used. A plasmid encoding the major splice isoform of the human *SCN3A* with exon 5 adult (5A) and exon 12v1 (646 bp) splice variants was used (Wang et al., 2010). Full-length $Na_v1.3$ was propagated in STBL2 cells at 30 °C (Invitrogen), and the open reading frame of all plasmid preparations was fully sequenced prior to transfection. The L247P variant was introduced by site-directed mutagenesis. Plasmids encoding the human Na_v channel accessory subunits β1 or β2 in vectors containing the marker genes CD8 (pCD8-IRES-β1) or GFP (pGFP-IRES-β2) were also used.

For electrophysiology experiments, expression of $Na_v1.3$, β1, and β2 subunits was achieved by transient transfection (2 μg of total cDNA: *SCN3A*, β1, β2 mass ratio was 10:1:1) using Superfect Transfection Reagent (QIAGEN, Valencia, CA, USA). Cells were incubated as described above for 48 h after transfection before use in electrophysiology experiments. For low temperature rescue experiments, cells were incubated for 24 h at 37 °C followed by 24 h at 28 °C prior to electrophysiology. Transfected cells were dissociated by brief exposure to trypsin/EDTA, resuspended in supplemented DMEM medium, plated on glass coverslips, and allowed to recover for ~2 h at 37 °C or 28 °C in 5% CO_2. Polystyrene microbeads pre-coated with anti-CD8 antibody (Dynabeads M-450 CD 8, Dynal, Great Neck, NY, USA) were added and only cells positive for both CD8 antigen (i.e., β1 expression) and GFP fluorescence (i.e., β2 expression) were studied.

For cell surface biotinylation experiments, expression of $Na_v1.3$, β1 and β2 subunits was achieved by transient transfection (2 μg of total cDNA: *SCN3A*, β1, β2 mass ratio was 10:1:1) using Lipofectamine 2000 (Life Technologies, Grand Island, NY, USA). Before use in these experiments, cells were incubated as described above for 48 h after transfection.

2.3. Electrophysiology

Coverslips were placed into a recording chamber on the stage of an inverted epifluorescence microscope (IX 50, Olympus, Center Valley, PA, USA) and allowed to equilibrate for 10 min in bath solution prior to starting experiments. Bath solution contained (in mM): 145 NaCl, 4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 10 HEPES (N-(2-hydroxyethyl) piperazine-N′-2-ethanosulphonic acid), pH 7.35, 310 mOsm/kg. The composition of the pipette solution was (in mM): 10 NaF, 110 CsF, 20 CsCl, 2 EGTA (ethyleneglycol-bis-(β-aminoethylether), 10 HEPES, pH 7.35, 310 mOsm/kg. Osmolarity and pH values were adjusted with sucrose and NaOH, respectively. A 2% agar-bridge with composition similar to