Current Trends in Dravet syndrome Research

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Received May 23, 2013; Accepted July 17, 2013; Published July 25, 2013

Citation: Higurashi N, Uchida T, Hirose S, Okano H (2013). Current Trends in Dravet syndrome Research. J Neurol Neurophysiol 4: 152. doi: 10.4172/2155-9562.1000152

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Abstract
Dravet syndrome is devastating infantile-onset epilepsy, often accompanied by severe intellectual disabilities, hyperactive behavior, autistic traits, and ataxia. The discovery in 2001 of the SCN1A mutation as a primary cause of this syndrome has accelerated our understanding of the pathophysiological processes underlying Dravet syndrome. SCN1A encodes the α-subunit of the voltage-gated sodium channel, Na\(_{1.1}\). Recent studies using genetically modified mouse models have indicated that haploinsufficiency of Na\(_{1.1}\) in GABAergic interneurons in the forebrain may be involved in the seizure susceptibility, fever sensitivity of seizures, and autistic traits characteristic of Dravet syndrome. More recently, human cellular models of Dravet syndrome have been established using patient-derived induced pluripotent stem cells (iPSCs) from three groups including ours. By taking advantage of these human disease models, functional vulnerabilities in GABAergic neurons have been revealed. Here, we review recent advances in Dravet syndrome research, particularly focusing on the development of iPSC models, and their future directions.

Keywords: Epilepsy; γ-aminobutyric acid; Disease modeling; Genetics; Induced pluripotent stem cells; Mechanism; Mouse model; SCN1A

Abbreviation: DS: Dravet Syndrome; GABA: γ-amino Butyric Acid; iPSCs: Induced Pluripotent Stem Cells

Introduction
Dravet syndrome (DS) is a devastating epilepsy syndrome, which was first described by Dr. Charlotte Dravet in 1978 [1]. Generalized or unilateral clonic seizures appear in previously healthy infants, and are frequently associated with fever [2]. These seizures tend to evolve into status epilepticus and occur frequently during the early phases of DS. Other seizures, including focal, absence, and myoclonic seizures appear later. Seizures are refractory to many of the currently available treatments, and severe intellectual disabilities develop after seizure onset, often accompanied by hyperactive behavior and autistic traits. Ataxia worsens with age, resulting in significant gait disturbance. Furthermore, 10–20% of the afflicted children experience premature death [3,4]. Thus, the development of new treatment is urgently needed.

Mutations in the SCN1A gene in patients with DS were first reported in 2001 [5]. Subsequent studies supported the hypothesis that the SCN1A defect is the primary cause of DS [6,7]; these findings have triggered a number of studies attempting to uncover the pathogenic mechanisms underlying DS. These studies have employed electrophysiological functional analysis of forcedly expressed Na\(_{1.1}\) mutants [8,9], mouse models based on heterozygotes of an SCN1A knock-out/knock-in mouse [10,11], and more recently, human cellular models using patient-derived induced pluripotent stem cells (iPSCs) [12-14]. First, we briefly review previous research on DS and then describe our recent research on generating an iPSC model for DS.

Genetic alteration in SCN1A
SCN1A encodes the α-subunit of the voltage-gated sodium channel Na\(_{1.1}\) (Figure 1) [15]. Abnormalities in the SCN1A gene were first reported in patients with genetic epilepsy febrile seizures plus (GEFS+) [15], and have been also identified in patients with other clinical phenotypes, including cryptogenic focal/generalized epilepsies [16]. However, DS is the primary phenotype in which a robust correlation with SCN1A defects has been confirmed [17,18]: 70–80% of patients with DS carry SCN1A abnormalities [19,20].

Nearly 700 mutations in the SCN1A gene that are associated with DS have so far been identified [21], and most of them are de novo. These mutations include nonsense, missense, splice site, and frameshift mutations, and are distributed throughout the gene. Specific genotype-phenotype correlations remain unclear, but truncation mutations, harbored by half of the patients with DS, appear to be associated with a more severe phenotype than missense mutations [22]. Exonic or micro chromosomal deletions involving SCN1A or its promoter region have also been identified in patients with DS [23-27]. Other genes potentially associated with DS or similar phenotypes include SCN1B [28], SCN2A [29], GABRG2 [30], and PCDH19 [31,32]. However, mutations in the former three occur rarely, and only a minority of patients with PCDH19 mutations present with the DS phenotype [33,34].

Functional alterations of mutated Na\(_{1.1}\)
Electrophysiological studies examining the functions of mutated Na\(_{1.1}\) channels expressed in HEK293or tsA-201 cells have revealed that many of the DS-associated missense mutations result in an on functional sodium channel [8,9,35,36], and this loss-of-function of the channel may be associated with DS. One study revealed that expressed...
truncated human Na\textsubscript{\(1.1\)} (hNa\textsubscript{\(1.1\)}) did not affect the channel function of co-expressed wild-type human hNa\textsubscript{\(1.1\)}, hNa\textsubscript{\(1.2\)}, hNa\textsubscript{\(1.3\)}, or hNa\textsubscript{\(1.6\)} [37]. It is also true that SCN1A abnormalities in DS frequently include truncation mutations (mainly nonsense mutations) and gene deletions, while there is little evidence showing that the truncated hNa\textsubscript{\(1.1\)} protein is associated with nonsense mutation. Nonsense mutations of SCN1A gene could result in nonsense-mediated mRNA decay. Collectively, it is tempting to speculate that haploinsufficiency may be an underlying mechanism of DS. However, some nonsense mutations were found to induce gain-of-function of Na\textsubscript{\(1.1\)} by way of activating sodium currents because of impairments in fast inactivation mechanisms [9]. It seems likely that the functional basis of SCN1A abnormalities related to DS may be more complex.

**Dysfunction of GABAergic Interneurons in Mouse DS Models**

Yu et al. first reported a mouse DS model based on Scn1a knock-out in 2006 [10], which exhibited spontaneous seizures, temperature-dependent seizure susceptibility [38], and ataxia [39]. They examined Na\textsubscript{\(1.1\)} expression in the hippocampal GABAergic interneurons, and found a reduced sodium current density and impaired action potential generation in those neurons of Scn1a\textsuperscript{−/−} and Scn1a\textsuperscript{−/−} mice compared to wild-type mice, while these were normal in the pyramidal neurons. Reduced sodium current density was also confirmed in the Purkinje neurons of these animals [39]; this reduced current density may be involved in the ataxia associated with DS. Ogihara et al. identified a parvalbumin-positive subgroup of GABAergic interneurons, which directly regulates the excitation of pyramidal neurons, as the primary type of Na\textsubscript{\(1.1\)}-expressing neurons in the neocortex and hippocampus [11]; moreover, intense Na\textsubscript{\(1.1\)} expression was identified in their axon initial segments. The researchers generated Scn1a knock-in mice with the R1407\* mutation, which exhibited spontaneous seizures, autistic traits, and cognitive decline [40], and impaired action potential generation in their neocortical GABAergic interneurons. These heterozygous knock-out/knock-in mice were also prone to premature death [10,11].

Conditional SCN1A knock-out, which was achieved by the Cre-mediated heterozygous deletion of floxed exonic regions, helped elucidate the brain region responsible for some of the major clinical features of DS [41]. Specifically, Dlx1/2-enhancer-driven Cre-deleted Scn1a in the mouse forebrain GABAergic interneurons [42]. These mice exhibited spontaneous seizures, temperature-dependent seizure susceptibility, premature death, and autistic traits[43], as was observed in mice with global SCN1A deletion. Kalume, et al. made an extremely interesting observation regarding the mechanisms of sudden unexpected death during tonic–clonic seizures [44]: the mice that died had increased seizure frequency and ictal bradycardia, which could be ameliorated by atropine, suggesting that an increase in parasympathetic activity occurs. SCN1A knock-out in the heart did not affect heart rate.

In the global knock-in and the conditional knock-out mice, administration of drugs that act to enhance GABAergic transmission, such as clonazepam, clobazam, or stiripentol ameliorated the seizures and behavioral impairments [43,45,46], supporting the notion that dysfunction of GABAergic interneurons is involved in the symptoms described above.

**Human-Based Cellular Models for DS Developed Using Patient-Derived iPSCs**

**Generation of DS patient-derived iPSCs**

Because it remains unclear whether mouse models faithfully reproduce the pathology occurring in the patient’s brain, it is desirable to study patient’s neurons directly. After the development of human iPSCs by Yamanaka et al. in 2007 [47], many neurological diseases have been modeled using patient-derived iPSCs [48-53], and pathogenic alterations have been identified in their differentiated neurons. Until recently, however, no such effort has been reported for epilepsy.

We recently generated two lines of iPSCs (D1-1 and D1-6) from a female patient with a core DS phenotype, who harbors a nonsense mutation in SCN1A (p.R1645*; Figure 1) [12]. Reprogramming factors (Sox2, Klf4, Oct3/4, and c-Myc) were retrovirally transduced into the skin fibroblasts, which were biopsied from her upper arm at the age of 29 years. The generated iPSCs displayed undifferentiated status and pluripotency, harbored the patient’s mutation in their SCN1A gene, and could be efficiently and reliably differentiated into neuronal cells. Neuronal differentiation from the iPSCs was performed using a method established by Okada et al. [54] with a slight modification, in which a month of embryoid body formation is followed by 3–5 weeks of neurosphere formation. Terminal differentiation into mature neurons was achieved by adherent culture of dissociated or undissociated neurosphere cells. For control experiments, we used 201B7, an iPSC line that was developed from a healthy female [47].

**Functional Vulnerabilities in Patient iPSCs-Derived GABAergic Neurons**

Because iPSCs-derived neurons obtained by the method described above are highly heterogeneous with regard to their neuronal subtypes and maturities, it is critical to analyze only one specific type of neuron at a defined level of maturity in order to determine whether the neurons are actually pathologic. As a first step to overcoming these difficulties, we generated a lentiviral reporter for SCN1A expression for electrophysiological assays; this harbors one main SCN1A promoter sequence [26], followed by a 5′-untranslated exon
indicate that the reporter detects NaV1.1-positive GABAergic neurons expression and functional properties sufficiently. These findings neurons appeared only rarely, and we could not evaluate their NaV1.1 positive, respectively). VGlut1-positive (glutamatergic neuron marker) were GABAergic (GAD67 (GABAergic neuron marker) and GABA-

80–90% of the reporter Venus-positive neurons were Na V1.1 positive. (to enhance expression specificity) [55], and Venus cDNA (Figure 2):

Neuronal maturity is also critical for accurate functional assessment. For this purpose, we sought to improve the conditions for neuron selection in electrophysiological assays, and finally set the following requirements: 3–7 weeks (average, 30–45 days) of terminal differentiation period; clear reporter fluorescence; mature morphology (large cell body size and growth of ≥ 4 neurites); larger capacitance (≥ 30 pF); and more negative resting membrane potential (≤ -30 mV). Using the current-clamp technique, the number and the amplitude of action potentials triggered by 500-ms depolarizing current injections were evaluated at various current intensities (input–output relationship).

In the final analyses, functionally immature neurons, which generated <10 action potentials during the stimulation protocol, were further omitted.

Although the capacitances, resting membrane potentials, firing thresholds, and peak voltages of action potentials were identical among the different cell lines, input–output relationships differed when the injection currents intensified; there was a marked attenuation of both the amplitude and the number of evoked action potentials in patient’s neurons compared to control neurons (Figure 3). These findings may indicate a reduced output capacity of the patient-derived neurons, particularly in the GABAergic subgroup, and are reminiscent of findings from murine DS models [10,11].

Hyperexcitability in DS iPSCs-Derived Neurons

Liu et al. also generated iPSCs from two patients with DS and differentiated the cells into neurons by alternate methods [13]. The neurons were divided into two groups according to their morphology—bipolar or pyramidal—and their electrophysiological properties were evaluated. In both groups of neurons, the patient’s-derived lines showed lower firing thresholds, higher sodium current densities, and a higher rate of spontaneously bursting neurons than cell lines derived from controls, suggesting hyperexcitability of DS neurons. Most recently, Jiao et al. also confirmed the hyperexcitability in excitatory neurons derived from DS patient-iPSCs [14]. Although such findings on the hyper excitability were not confirmed in our preliminary experiments (Higurashi et al., unpublished results), they provided a novel gain-of-function hypothesis for the mechanisms underlying seizure susceptibility in DS, which have not been revealed by murine research. Further development of the iPSC research technique will be necessary to elucidate the precise mechanisms involved.

Future Directions of DS Research Using Patient-Derived iPSCs

Disease modeling with patient-derived iPSCs has just begun, particularly in the field of epilepsy. Although the results of these two iPSC studies on DS are not immediately beneficial for patient treatment, they strongly indicate the utility of the new platform for human epilepsy research. In the next step, refinement of the culture technique and a more cell-type-specific analysis for various types of neurons (e.g. glutamatergic, parvalbumin-positive GABAergic, calretinin-positive GABAergic, and somatostatin-positive GABAergic, etc) will be necessary. The continued use of mouse models to confirm our findings

Figure 2: Diagram of the SCN1A reporter and neuron selection strategy for electrophysiological analyses: In the construct diagram, the yellow portion indicates the 5′-untranslated exonic sequence, and light blue indicates the 5′-untranslated sequence of the first coding exon. This construct was transduced into the dissociated neurospheres by using lentiviral vectors. After 3–7 weeks of terminal differentiation, putatively mature neurons that satisfied several requirements (see main text) were selected for electrophysiological analysis. Functionally immature neurons with in adequate action potential generation were excluded from analysis. APs, action potentials; RMP, resting membrane potential.

Figure 3: Representative traces of action potential trains and input–output relationships: (adapted from Reference [12] with modifications). (A) The 500-ms current injection period is indicated below. The traces presented are those evoked by a 100-pA current injection. Toward the end of the current injection, D1-1 neurons showed more prominent attenuation of action potential amplitude compared to 201B7 neurons, as indicated by the dotted arrows. The frequency of action potentials was also lower in D1-1. Scale bars indicate 20 mV and 100 ms. (B) Mean of the total number of action potentials evoked during the 500-ms stimulation period vs. injection current intensity (input–output relationship). In all cell lines, the number of action potentials increased with intensifying currents. However, when current intensities exceeded 50 pA, the rate of action potential increase plateaued in both patient lines, which was not the case in control cells.
from iPSCs will facilitate the elucidation of key pathophysiological mechanisms and critical therapeutic targets in human patients with DS.

Acknowledgements

Original investigation described in the present review article was supported by the Project for the Realization of Regenerative Medicine from the Japanese Ministry of Education, Culture, Sports, Science and Technology (MEXT) to H.O.; The Program for Intractable Disease Research Utilizing Disease-specific IPS Cells from Japan Science and Technology Agency (JST) to H.O.; a Grant-in-aid for Scientific Research (A) (21249062) for the Research on Measures for Intractable Diseases (No. H22-Nanji-Ippan-49) from the Ministry of Health, Labor and Welfare (MHLW) to S.H.; Grant-in-aid for the Research on Measures for Intractable Diseases (No. H22-Nanj-Ippan-49) from the Ministry of Health, Labor and Welfare (MHLW) to S.H.; Research grants from The Japan Foundation for Pediatric Research (10-003), and a Grant-in-aid for the Research on Measures for Intractable Diseases (No. H22-Nanj-Ippan-49) from the Ministry of Health, Labor and Welfare (MHLW) to S.H.;

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