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Correlations in timing of sodium channel expression, epilepsy, and sudden death in Dravet syndrome

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Dravet syndrome (DS) is an intractable genetic epilepsy caused by loss-of-function mutations in *SCN1A*, the gene encoding brain sodium channel $Na_v1.1$. DS is associated with increased frequency of sudden unexpected death in humans and in a mouse genetic model of this disease. Here we correlate the time course of declining expression of the murine embryonic sodium channel $Na_v1.3$ and the rise in expression of the adult sodium channel $Na_v1.1$ with susceptibility to epileptic seizures and increased incidence of sudden death in DS mice. Parallel studies with unaffected human brain tissue demonstrate similar decline in $Na_v1.3$ and increase in $Na_v1.1$ with age. In light of these results, we introduce the hypothesis that the natural loss $Na_v1.3$ channel expression in brain development, coupled with the failure of increase in functional $Na_v1.1$ channels in DS, defines a tipping point that leads to disinhibition of neural circuits, intractable seizures, co-morbidities, and premature death in this disease.

Introduction

Dravet Syndrome (DS) is a progressive, infantile onset epileptic encephalopathy caused by heterozygous loss of function of *SCN1A*, the gene encoding the brain voltage sensitive sodium channel $Na_v1.1$. DS begins with febrile seizures in infancy and progresses with development to include refractory generalized tonic-clonic (GTC) seizures and other complex seizure types. Individuals with DS develop additional comorbid conditions including cognitive impairment, ataxia, psychomotor regression, and increased risk of premature death.[1] Sudden unexpected death in epilepsy (SUDEP) is the leading cause of death in individuals with refractory epilepsy and constitutes a constant threat to patients with DS and their families.[2,3] Risk of SUDEP is correlated with higher frequency of GTC seizures.[4]

Mice bearing a heterozygous loss-of-function DS mutation recapitulate the sensitivity to temperature-induced seizures, progressive increase in seizure severity, cognitive impairment, comorbidities, and premature death of this disease.[5-8] This DS mutation causes specific loss of Na^+ currents and impaired excitability of GABAergic interneurons.[8] SUDEP in these mice is caused by profound bradycardia due to increased parasympathetic output to the heart, immediately following a severe GTC seizure.[9] Paralleling human SUDEP, all spontaneous deaths in DS mice occurred immediately following a GTC seizure, and the frequency of seizures and premature deaths rose sharply after spontaneous seizures began.[9] Targeted deletion of $Na_v1.1$ channels in forebrain GABAergic interneurons using the Cre-Lox method recapitulates the susceptibility to seizures, co-morbidities, and premature death of this disease,[10] indicating that this specific deletion of $Na_v1.1$ channels is sufficient to cause seizures, severe bradycardia, and premature death analogous to DS mice.[9]

Individuals with DS develop normally from birth until the time of initial seizure presentation at 5 to 11 mo.[1] Similarly, DS mice are indistinguishable from their wild-type littermates at birth and develop normally through the third week of life. During the fourth postnatal week, DS mice become susceptible to thermally induced and spontaneous seizures as well as premature death.[7,8] Susceptibility to thermally induced seizures, spontaneous seizures, and SUDEP begins near the time of weaning in both mouse (P21) and human (6-9 mo), suggesting that disease onset occurs at a similar stage of development in both species. This timing of disease onset and progression in human and mouse DS suggests a common underlying molecular event may trigger disease onset.

$Na_v1.1$ is a member of a family of voltage-sensitive sodium channels expressed primarily in the brain, including $Na_v1.1$, $Na_v1.2$, $Na_v1.3$, and $Na_v1.6$.[11] In rat, $Na_v1.1$, $Na_v1.2$, and $Na_v1.6$ channel expression increases continually from birth, while $Na_v1.3$ channel expression is highest just prior to birth and declines in

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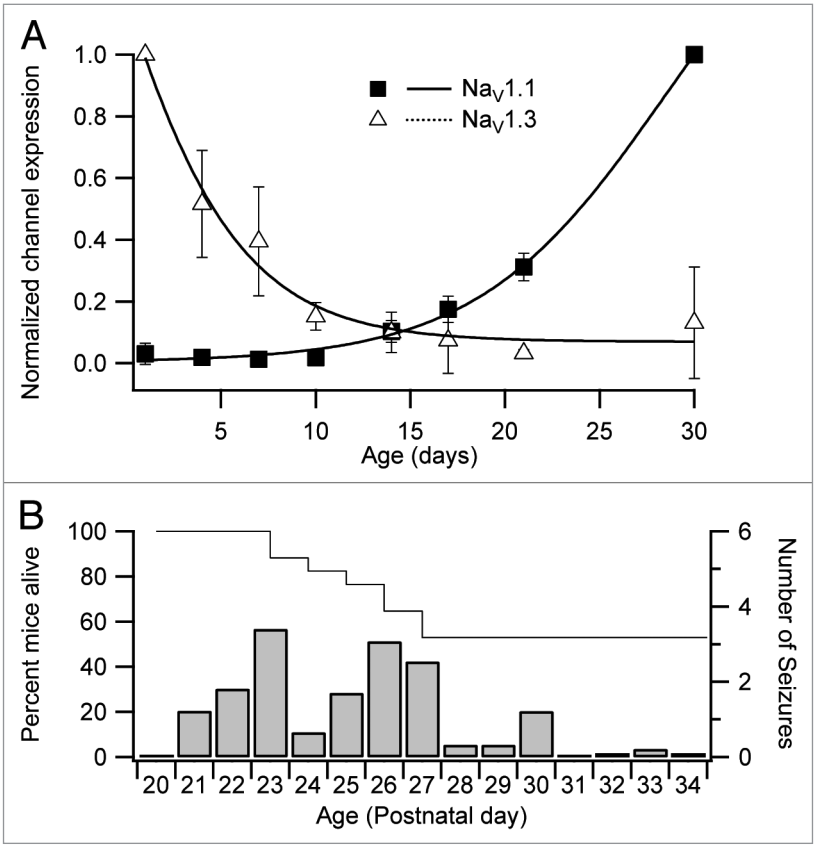

Figure 1. Expression of sodium channel proteins in developing mouse cerebral cortex. (**A**) Sodium channel expression in membrane proteins from normal C57Bl/6 mouse cortex. Samples are expressed as a fraction of max expression after normalizing to β-actin. Data points represented as mean ± SEM, closed squares, $Na_v1.1$; open triangles, $Na_v1.3$. (**B**) Time course of spontaneous seizures (right axis) and premature death (left axis) in DS mice. Adapted from Kalume et al.[9] with permission.

early postnatal life.[12-15] $Na_v1.1$ and $Na_v1.3$ are both found primarily in the cell soma and proximal dendrites, but have complementary temporal expression patterns.[16-18] In humans, $Na_v1.3$ protein remains detectable in adult brain,[19] but the developmental time course of expression of these channels remains unknown. In the experiments described here, we have determined the levels of $Na_v1.1$ and $Na_v1.3$ proteins in developing mouse and human brain and correlated these measurements with the onset of seizures and premature death in the 2 species.

Results and Discussion

Time course of expression of $Na_v1.1$ and $Na_v1.3$ channels in mouse brain

Previous studies of rat brain showed $Na_v1.3$ mRNA and protein is expressed at high levels in embryonic life and declines in the first 3 weeks after birth, whereas $Na_v1.1$ channel expression is first observed in early postnatal development and reaches its maximum expression level by 4 weeks of age.[13,14] Immunoblotting of membrane proteins isolated from wild-type C57Bl/6 mouse cerebral cortex showed an expression pattern similar to that seen in rat (**Fig. 1A**). In comparison with rat, the decline in $Na_v1.3$ channel protein was more rapid, reaching its lowest levels at postnatal day 21 (P21), and the rise in $Na_v1.1$ channels occurred later and the level of $Na_v1.1$ was still increasing at P30 (**Fig. 1A**). Susceptibility to thermally induced seizures begins at P20 and there is an increase in the frequency of spontaneous seizures and death between P20 to P25 (**Fig. 1B**).[7,9] By this time the level of $Na_v1.3$ channel protein has declined to baseline, and the level of $Na_v1.1$ channel protein is rapidly rising. In DS mice, the rise in functional $Na_v1.1$ channel protein would be substantially reduced by the loss-of-function mutations in 1 allele of the *Scn1a* gene.

Time course of expression of $Na_v1.1$ and $Na_v1.3$ channels in human brain

Children diagnosed with DS develop normally until the time of seizure onset, which occurs between 5 and 11 mo, with an average age of onset of 5.7 mo (**Table 1**).[1] After initial seizure onset, DS progresses over 1 to 2 years to multiple forms of spontaneous seizures, including generalized tonic-clonic seizures, resistance to drug therapy, developmental delay, and permanent mental and physical deficiencies.[20-24] Following the onset of increased seizure incidence, the risk of SUDEP is also elevated with the majority of unexpected deaths occurring during the first 3 years of life.[25]

In order to compare sodium channel expression patterns during development of human brain, membrane proteins were isolated from non-epileptic human postmortem cerebral cortex between 1.5 and 30 mo. Immunoblotting with subtype-specific

Table 1. Age of seizure onset in DS patients

SCN1A mutation	Number of cases	Age of onset (months)	Citation
+	32	5.6 ± 2.1	Ragona, et al. 2010[20]
-	5	6.2 ± 2.4	
+	20	5.6 ± 2.1	Ragona, et al.[21]
-	6	5.8 ± 2.6	
+	29	5.7 ± 2.4	Lim, et al. 2011[22]
+	18	5.6 ± 2.3	Petrelli, et al. 2011[23]
-	7	7.3 ± 4.2	
+	7	5.7 ± 1.9	Chieffo, 2011[24]
-	5	5.0 ± 1.1	
Average onset:		5.7 ± 2.3	

+, indicates confirmed *SCN1A* mutation; – , denotes no *SCN1A* mutation detected. Compilation of age of initial seizure onset in patients diagnosed with Dravet Syndrome. Studies were chosen that identified mutations present in *SCN1A* in addition to behavioral characteristics. Mutation positive (+) and mutation negative (-) were tabulated separately and reported.[20-24]

antibodies showed $Na_v1.1$ protein expression parallels rodent expression, with low levels of $Na_v1.1$ at birth, steadily increasing to its highest level by 20 mo (**Fig. 2**). Human $Na_v1.3$ protein expression also parallels the rodent pattern and is detected at its highest levels at birth, decreasing to its lowest level at 6 mo of age (**Fig. 2**). The time at which the decline of $Na_v1.3$ protein levels and the rise of $Na_v1.1$ expression cross (approximately 5-6 mo) correlates closely with the mean time of seizure onset in patients with DS (approximately 6 mo; **Table 1**). The complementary time courses of expression $Na_v1.1$ and $Na_v1.3$ coupled with their similar localization in neuronal cell bodies suggest that these channels may perform similar functions and that $Na_v1.3$ may be protective against loss of $Na_v1.1$ channels at early ages.

Complementary Expression of $Na_v1.1$ and $Na_v1.3$ Define a Tipping Point in DS

The results of our experiments demonstrate that the decline of $Na_v1.3$ and the rise of $Na_v1.1$ expression in human cerebral cortex occur in the same time frame as the initial presentation of seizures in patients diagnosed with DS, both at around 6 mo (**Fig. 2**). Similarly, the timing of the decline of $Na_v1.3$ and the rise of $Na_v1.1$ expression in rodents occur in the third and fourth week of life (**Fig. 1A**),[13] coincident with susceptibility of our DS mouse model to thermally induced seizures, spontaneous seizures, and SUDEP.[7-9] These data in human, rat, and mouse suggest that the timing of DS onset is a result of the failure of $Na_v1.1$ channels to fully replace the normal developmental loss of embryonic $Na_v1.3$ channels.

In previous work,[8] we used immuncytochemical methods to assess possible up-regulation of $Na_v1.3$ channels in $Na_v1.1$+/- and $Na_v1.1$-/- mice at P14, when the level of $Na_v1.3$ has decreased to approximately 10% of maximum in cerebral cortex of WT mice (**Fig. 1**). We found no up-regulation of $Na_v1.3$ channels in hippocampal interneurons of $Na_v1.1$+/- mice, our DS model. There was significant up-regulation of $Na_v1.3$ channels in hippocampal interneurons of $Na_v1.1$-/- mice, but the level was insufficient to rescue either the sodium currents or the epileptic phenotype of these mice.[8] We found no up-regulation of $Na_v1.2$, $Na_v1.3$, or $Na_v1.6$ channels in excitatory or inhibitory neurons in

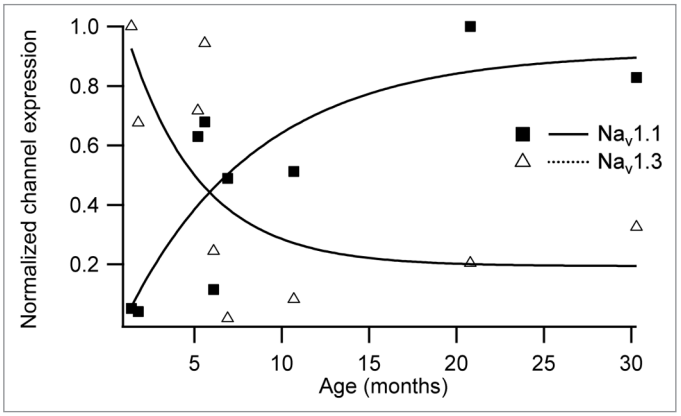

Figure 2. Expression of sodium channel proteins in developing human cerebral cortex. Channel expression in membrane proteins from normal postmortem human cortical tissue. Samples are represented as a fraction of maximum expression after normalizing to β-tubulin; closed squares, $Na_v1.1$; open triangles, $Na_v1.3$.

immunocytochemical analyses of cerebral cortex, thalamus, or cerebellum of $Na_v1.1$-/- mice. Thus, surprisingly, there is little compensatory up-regulation of $Na_v1.3$ or other sodium channels in mouse forebrain, even in the face of complete deletion of $Na_v1.1$ channels.

In light of the complementary time courses of expression of $Na_v1.1$ and $Na_v1.3$ channels, the loss of $Na_v1.3$ channel expression may be one of the precipitating factors that initiates disease onset in DS, and $Na_v1.3$ may also function as a genetic modifier that contributes to the striking genetic background effects in DS in mice and humans. The time of crossover of declining levels of $Na_v1.3$ channels and rising levels of $Na_v1.1$ channels (**Figs. 1** and **2**) may define the tipping point for development of DS, as the mutations characteristic of this disease prevent the normal increase in functional $Na_v1.1$ channels in forebrain GABAergic inhibitory neurons leading to disinhibition of neural circuits, epilepsy, neurodevelopmental co-morbidities, and SUDEP.

Methods

Immunoblotting

Normal postmortem human brain samples were obtained from the NICHD Brain and Tissue Band for Developmental Disorders at the University of Maryland. Ages analyzed were 1.4, 1.8, 5.2, 5.6, 6, 6.8, 10.7, 20.8, 30.3 mo. Immunoblotting of human tissue was done at Seattle Children's Research Institute as previously described in detail.[26] Briefly, membrane proteins were isolated from 100-200 μg of cortical tissue cleaved from the underlying white matter, transferred to PVDF membranes and probed for $Na_v1.1$ (1/200, rabbit anti-$Na_v1.1$, Alomone labs), $Na_v1.3$ (1/200, rabbit anti-$Na_v1.3$, Alomone labs) and β-tubulin (1/10,000, mouse anti-tubulin, Novus Biologicals). Infrared fluorescence was used for signal detection and quantitation (rabbit anti-Alexa Fluor 680, Molecular Probes; IRDye 800 goat antimouse IgG, LI-COR; Odyssey Infrared Imagins System, LI-COR Biosciences). Background was subtracted, and levels of $Na_v1.1$ α subunit protein are represented as relative expression to β-tubulin.

Mouse cortical tissues were dissected from WT C57Bl/6 animals (Jackson Laboratories) at postnatal days (P) 1, 4, 7, 10, 14, 17, 21, and 30 to represent a wide range of developmental ages. Following membrane protein isolation as previously described.[26] Pelleted membrane proteins were resuspended in 1x RIA buffer (25 mM Tris, 150 mM NaCl, 1 mM EDTA, 2% Triton X-100, pH 7.4) and centrifuged at 20,000xg for 20 min, and the supernatant containing the membrane proteins was collected for analysis. Complete Protease Inhibitor set (Roche) was included in all solutions. Protein concentration was determined by BCA assay (Pierce) and transferred to nitrocellulose membranes and immunoblotted as previously described[27] with antibodies specific for $Na_v1.1$ (1/200, anti-rabbit $Na_v1.1$, Alomone labs), $Na_v1.3$ (1/100, Sp32iii)[18] and β-actin (1/10,000, anti-mouse β actine, Abcam Inc.). Samples were background subtracted and are represented as relative expression to β-actin normalized to the maximum expression for each channel ± SEM, n = 3 for each time point represented.

Animal care and analysis

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Washington.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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