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Altered Sleep Regulation in a Mouse Model of SCN1A-Derived Genetic Epilepsy with Febrile Seizures Plus (GEFS+)

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Summary

Purpose—Mutations in the voltage-gated sodium channel SCN1A are responsible for a number of epilepsy disorders, including genetic epilepsy with febrile seizures plus (GEFS+) and Dravet syndrome. In addition to seizures, patients with SCN1A mutations often experience sleep abnormalities, suggesting that SCN1A may also play a role in the neuronal pathways involved in the regulation of sleep. However, to date, a role for SCN1A in the regulation of sleep architecture has not been directly examined. To fill this gap, we tested the hypothesis that SCN1A contributes to the regulation of sleep architecture, and by extension, that SCN1A dysfunction contributes to the sleep abnormalities observed in patients with SCN1A mutations.

Methods—Using immunohistochemistry we first examined the expression of Scn1a in regions of the mouse brain that are known to be involved in seizure generation and sleep regulation. Next, we performed detailed analysis of sleep and wake electroencephalographic (EEG) patterns during 48 continuous hours of baseline recordings in a knock-in mouse line that expresses the human SCN1A GEFS+ mutation R1648H (RH mutants). We also characterized the sleep-wake pattern following 6 hours of sleep deprivation.

Key Findings—Immunohistochemistry revealed broad expression of Scn1a in the neocortex, hippocampus, hypothalamus, thalamic reticular nuclei, dorsal raphe nuclei, pedunculopontine and laterodorsal tegmental nuclei. Co-localization between Scn1a immunoreactivity and critical cell types within these regions was also observed. EEG analysis under baseline conditions revealed increased wakefulness and reduced non-rapid eye movement (NREM) and rapid eye movement (REM) sleep amounts during the dark phase in the RH mutants, suggesting a sleep deficit. Nevertheless, the mutants exhibited levels of NREM and REM sleep that were generally similar to WT littermates during the recovery period following 6-hours of sleep deprivation.

Significance—These results establish a direct role for SCN1A in the regulation of sleep and suggest that patients with SCN1A mutations may experience chronic alterations in sleep,

‡The first two authors contributed equally to the study.

Disclosure of Conflict of Interest
The mouse model of GEFS+ described in this manuscript has been licensed to Allergan by Andrew Escayg. The terms of this arrangement have been reviewed and approved by Emory University in accordance with its conflict of interest policy. The remaining authors have no conflicts of interest. We confirm that we have read the Journal’s position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.
potentially leading to negative outcomes over time. In addition, the expression of Scn1a in specific cells types/brain regions that are known to play critical roles in seizure generation and sleep now provides a mechanistic basis for the clinical features (seizures and sleep abnormalities) associated with human SCN1A mutations.

Keywords
sleep; epilepsy; Scn1a; sodium channel; sleep deprivation

Introduction

The voltage-gated sodium channel (VGSC) genes SCN1A, SCN2A, SCN3A, and SCN8A are expressed primarily in the mammalian central nervous system (CNS) and encode the transmembrane proteins Na\textsubscript{v}1.1, Na\textsubscript{v}1.2, Na\textsubscript{v}1.3, and Na\textsubscript{v}1.6, respectively. VGSCs are responsible for the initiation and propagation of transient depolarizing currents and play an important role in electrical signaling between cells. SCN1A mutations are linked to a number of human epilepsy disorders, including genetic (generalized) epilepsy with febrile seizures plus (GEFS+) (Escayg et al., 2000), Dravet syndrome (severe myoclonic epilepsy of infancy) (Claes et al., 2001), and intractable childhood epilepsy with generalized tonic-clonic seizures (Fujisawa, 2006; Mulley et al., 2005). These disorders share some clinical characteristics, but differ in severity. SCN1A mutations are also associated with familial hemiplegic migraine (FHM) (Castro et al., 2009) and sudden unexpected death in epilepsy (SUDEP) (Hindocha et al., 2008).

In addition to epilepsy, some individuals with SCN1A mutations display neuropsychiatric abnormalities, such as affective and anxiety disorders (Mahoney et al., 2009). A relationship between altered SCN1A function and sleep disturbances has also been noted. For instance, parents have observed that children with Dravet syndrome experience difficulty falling asleep and staying asleep (Nolan et al., 2006), and electroencephalographic (EEG) recordings of adolescents with Dravet syndrome reveal frontal slow spikes during wakefulness and sleep (Nabbout et al., 2008). In addition, two brothers with the SCN1A mutation S1713N exhibited diffused slow spike and wave complexes during sleep and abnormal paralysis of the muscle mentalis during NREM sleep (Kimura et al., 2005).

One large GEFS+ family with 13 affected members harbored the SCN1A mutation R1648H (Escayg et al., 2000). Affected family members had variable clinical presentations, with some individuals experiencing either febrile seizures or afebrile epilepsy subtypes, while others presented with both febrile and afebrile seizures. We recently generated a mouse model of GEFS+ by knocking in the R1648H mutation into the orthologous mouse Scn1a gene (Martin et al., 2010). Heterozygous Scn1a\textsuperscript{R1648H/+} mice (RH mutants) exhibit infrequent spontaneous generalized seizures and lower thresholds to flurothyl- and hyperthermia-induced seizures, whereas homozygous RH mutants exhibit frequent seizures and premature lethality. Electrophysiological analysis of dissociated cortical GABAergic interneurons from RH mutants detected slower recovery from inactivation and reduced action potential firing, suggesting that seizure phenotypes in these mice are the result of decreased GABAergic inhibition (Martin et al., 2010).

To better understand the role of SCN1A in sleep physiology, we examined the expression of Na\textsubscript{v}1.1 channels in the mouse brain in regions that are associated with sleep and epilepsy. We also compared the sleep architecture of RH mutants with wild-type (WT) littermates at baseline and following sleep deprivation (SD). The results reported in this study suggest a novel role for altered SCN1A function in the regulation of sleep.
Methods

Animals and Genotyping

The Scn1a-R1648H mutants and wildtype littermates used in the study were derived from two rounds of backcrossing to C57BL/6J (N2 generation) as previously described (Martin et al., 2010). Mice were housed in ventilated cages under uniform conditions in a pathogen-free mouse facility on a 12-h light and 12-h dark cycle (lights on at 7 am and lights off at 7 pm). Food and water were available ad libitum. All experiments were approved by the Emory University IACUC committee. Genotyping for the R1648H (RH) mutation was performed as previously described (Martin et al., 2010).

Immunohistochemistry

Under isoflurane anesthesia, 3 C57BL/6J mice (3 months old) were transcardially perfused with phosphate-buffered saline (1X), followed by paraformaldehyde (4%). Brains were post-fixed in paraformaldehyde (4%) and cryopreserved in 30% sucrose. Forty-five-micrometer sections were cut on a cryostat (Leica, Germany). Coronal sections, 400 µm apart, from bregma −0.46 mm to −3.00 mm, were prepared ensuring that individual cell profiles were not counted in more than one section. Free-floating sections were washed in TBS/Triton X and blocked in 2% avidin and then 2% biotin. Monolabeled slices were incubated in either rabbit polyclonal anti-Scn1a (1:50, Millipore), followed by biotinylated anti-rabbit IgG (1:300, Vector Laboratories) and then fluorescein avidin D (1:300, Vector Laboratories) and Hoechst stain (1:1000, Acros), or mouse monoclonal anti-Na$_v$1.1 (1:200, NeuroMab) followed by Alexa Fluor 555 goat anti-mouse IgG (1:1000, Invitrogen) and Hoechst stain (1:1000, Acros). Double-labeled slices were incubated with either rabbit polyclonal anti-Scn1a (1:50, Millipore) or mouse monoclonal anti-Na$_v$1.1 (1:200, NeuroMab) and one of the following cell-type markers: mouse monoclonal anti-glutamate decarboxylase (1:100, Millipore), rat monoclonal anti-5-HT (1:100, Millipore), mouse monoclonal anti-tyrosine hydroxylase (1:10000, Millipore), rabbit polyclonal anti-ChAT (1:500 Millipore), and mouse monoclonal anti-orexin A (1:50, Santa Cruz Biotechnology Inc.). After washing, sections were incubated in secondary antibodies. Secondary antibodies included biotinylated anti-rabbit IgG (1:300, Vector Laboratories) with fluorescein avidin D (1:300, Vector Laboratories), Alexa Fluor 555 goat anti-mouse IgG (1:1000, Invitrogen), and Alexa Fluor 488 donkey anti-rat (1:500). The sections were then washed and mounted. Images were collected using a Leica DM6000 B upright fluorescence microscope and Simple PCI software. Images were also collected using the Zeiss LSM 510 NLO META system with a confocal Zeiss Axiovert 100M inverted microscope. Cell counts were performed on images captured under 40X or 63X magnification. Regions of interest were identified using a mouse atlas (Paxinos & Franklin, 2004), in conjunction with cell-type markers. Possible differences in cell volume or differential shrinking that may have occurred during processing were not assessed.

Electroencephalogram (EEG) acquisition

Under isoflurane anesthesia, 5 male RH mutants and 7 male WT littermates (3–4 months old) were surgically implanted with EEG and electromyography (EMG) electrodes for polysomnographic recordings (Embla Medical, Reykjavik, Iceland), as described previously by Papale and collaborators (Papale et al., 2009; Papale et al., 2010). Four sterile screw electrodes (Vintage Machines Supplies, OH, USA) were placed subdurally in the following coordinates: one pair of screws was placed on the right hemisphere (2 mm anterior-posterior (AP) and 1.2 mm lateral to the midline (LM); −1.5 mm AP and 1.2 mm LM), and another pair of screws was placed on the left hemisphere (0.5 mm AP and 2.2 LM; −3.5 mm AP and 2.2 mm LM). Fine-wire electrodes were inserted into the neck muscle for EMG acquisition. Post-surgical pain was managed with the administration of ibuprofen (0.1 mg/kg) in the

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drinking water for 3 days. Following 14 days of recovery from the surgery and 2 days of habituation to the EEG cable, the animals were continuously recorded for 48 h (sleep baseline). After the baseline period, the animals were sleep deprived during the first 6 h of the light phase (7 am–1 pm). Sleep deprivation (SD) was achieved by gentle handling that involved the introduction of objects into the cage and tapping the cages whenever the animals appeared drowsy. The animals were never disturbed during feeding or drinking. Immediately following SD, the animals were continuously recorded for 18 h (sleep rebound period).

Sleep scoring and data analysis

The EEG data were manually scored in 10-s intervals. The time spent in each state of vigilance during the 12-hour light and 12-hour dark phases of the EEG recordings from two consecutive days was averaged. Wakefulness was defined as a low-voltage and high-frequency EEG signal with elevated and variable EMG. Non-rapid eye movement (NREM) sleep was characterized by an EEG signal that increased in amplitude and decreased in frequency, with the clear presence of high-amplitude delta waves (0.5–4 Hz) and an EMG signal that displayed low regular muscular tone. Rapid eye movement (REM) sleep was identified by the presence of regular theta waves (5–7 Hz) with a lack of muscle tone and phasic bursts of varying duration and amplitude. For power spectral analysis, EEG waveforms were subjected to fast Fourier transformation. The delta (0.5–4 Hz) frequencies during each 10-s interval of NREM sleep were determined. EEG power was normalized to facilitate comparison. For each animal, NREM delta power at baseline was quantified in 2-hour intervals and normalized to the 24-hour average of the mean amplitude of the same day. NREM delta power values for sleep rebound were normalized to values obtained at corresponding times in the baseline recording.

Statistics

The data are reported as mean±standard error of the mean (SEM). Homogeneity of variance was assessed by the Levene test and normal distribution of the data by the Shapiro-Wilk test. Repeated measures ANOVA (rANOVA) was used to detect differences between genotypes (mutant versus WT) when the light and dark phases were examined in 12-hour periods, as well as for each 2-hour interval. rANOVA was also used to detect differences within and between genotypes between sleep baseline and sleep rebound following SD. Post hoc comparisons were performed by Tukey’s test.

Results

Scn1a is expressed in brain regions involved in sleep regulation and seizure generation

Immunohistochemistry (IHC) was performed on three C57BL/6J mice (three months old) to evaluate the expression of Na$_v$1.1 channels in different brain regions and cell types associated with sleep and epilepsy (Table 1, Fig. 1, Fig. 2). GABA neurotransmission in brain regions like the hippocampus, thalamic reticular nucleus (TRN), and cortex is known to play an important role in epilepsy and sleep. IHC analysis revealed extensive co-localization between glutamic acid decarboxylase (GAD, a GABAergic marker) and Na$_v$1.1 channels in the hippocampus (97%), TRN (95%), and cortex (98%). GABAergic neurons throughout the hypothalamus (HT) play a critical role in sleep onset (Szymusiak et al., 2007) and exhibited 97% co-localization with Na$_v$1.1-immunoreactive cells (Table 1, Fig. 1).

The lateral hypothalamus, where wake-active orexin-containing neurons are located, is important for promoting and maintaining wakefulness (Hassani et al., 2010). IHC analysis revealed that 19% of the orexin-A–positive cells were also immunoreactive for Na$_v$1.1 (Table 1, Fig. 2). Orexinergic neurons also drive wakefulness by sending projections to...
serotonergic and noradrenergic neurons (Siegel, 2004). Serotonergic cells located in the dorsal raphe nucleus (DRN) and median raphe nucleus (MnR), as well as the noradrenergic cells from the locus coeruleus (LC), all extend excitatory projections to the cortex that help suppress sleep onset (Siegel, 2004). IHC showed that 43% and 24% of the serotonergic cells in the DRN and MnR, respectively, also express Na$_v$1.1 channels (Table 1, Fig. 2); however, none of the tyrosine hydroxylase-positive noradrenergic cells of the LC showed co-localization with Na$_v$1.1 (Table 1, Fig. 2). Finally, the pedunculopontine tegmental nucleus (PPT) and the laterodorsal tegmental nucleus (LDT) are components of the ascending reticular activating system that send cholinergic signals to the thalamus to enhance cortical arousal (Rye, 1997). IHC analysis revealed that 38% and 31% of the choline acetyltransferase (ChAT)-immunoreactive cholinergic cells were Na$_v$1.1-positive in the PPT and LDP, respectively (Table 1, Fig. 2). Together, these results show that the Na$_v$1.1 channels are broadly expressed in cell types involved in epilepsy and sleep-wake physiology.

**RH mutants show increased wakefulness and decreased NREM and REM sleep during the dark phase of baseline recordings**

The sleep and wake amounts of RH mutants were compared to WT littermates. The 12-hour light and 12-hour dark phase of EEG recordings from two consecutive days were averaged. The total times spent in wakefulness, NREM, and REM sleep were quantified as percentages of the total recording time.

Diurnal sleep-wake rhythms were present in both genotypes, with expected overall increases in sleep during the light phase and wakefulness during the dark phase (Fig. 3).

During wakefulness, rANOVA indicated a main effect of phase of day ($F_{(1,10)}=227.3; p<0.001$) and an interaction between phase of day and genotype ($F_{(1,10)}=5.7; p<0.03$). Similar wake amounts were observed between genotypes during the light phase (WT, 35%; RH, 36%; Tukey’s $p>0.05$; Fig. 3A); however, during the dark phase, the RH mutants exhibited 15% more wakefulness when compared to WT littermates (WT, 64%; RH, 74%; Tukey’s $p<0.05$, Fig. 3A).

For the time spent in NREM sleep, rANOVA indicated a main effect of phase of day ($F_{(1,10)}=193.2; p<0.001$) and an interaction between phase of day and genotype ($F_{(1,10)}=5.3; p<0.04$). During the light phase, comparable amounts of NREM were observed between genotypes (WT, 54%; RH, 54%; Tukey’s $p>0.05$; Fig. 3C); however, the RH mutants showed 24% less NREM sleep during the dark phase when compared to WT littermates (WT, 33%; RH, 25%; Tukey’s $p<0.05$, Fig. 3C). Finally, for REM sleep, rANOVA indicated a main effect of genotype ($F_{(1,10)}=6.8; p<0.02$), phase of day ($F_{(1,10)}=313; p<0.001$), and an interaction between genotype and phase of day ($F_{(1,10)}=5.5; p<0.04$). Comparable amounts of REM sleep were observed between genotype during the light phase (WT, 10%; RH, 10%; Tukey’s $p>0.05$; Fig. 3E); however, during the dark phase, the RH mutants experienced 48% less REM sleep when compared to the WT littermates (WT, 3.6%; RH, 1.9%; Tukey’s $p<0.05$, Fig. 3E).

To perform a more detailed sleep-wake analysis, the different states of vigilance were also analyzed in two-hour intervals (Fig. 3B, 3D, 3F). Analysis of the light phase revealed that the percentages of time spent awake, and in NREM and REM sleep, were comparable between WT littermates and RH mutants ($p>0.05$ for all comparisons). During the dark phase, the RH mutants spent more time awake compared to WT littermates; however, these differences were not statistically significant (Fig. 3B). In contrast, the RH mutants spent significantly less time in NREM sleep from 3 am to 5 am (Tukey’s, $p<0.05$; Fig. 3D) and in REM sleep from 11 pm to 5 am (Tukey’s $p<0.05$; Fig. 3F).

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To determine whether the mutants showed alterations in sleep fragmentation, the number of arousals and transitions (stage shifts) between the states of vigilance (wakefulness to NREM, REM to wakefulness, REM to NREM, NREM to wakefulness, and NREM to REM) were quantified during the light and dark phases (Supplementary Table 1). The RH mutants showed a similar number of awakenings and stage shifts compared to the WT littermates (p>0.05 for all comparisons). Wake, NREM, and REM sleep duration per episode were also quantified, and there were no statistically significant differences between WT littermates and RH mice (Supplementary Table 1).

**RH mutants show unaltered delta power rhythm during basal NREM sleep**

NREM sleep, characterized by high-amplitude slow oscillations (0.5–4 Hz), is the restorative phase of sleep. The duration of NREM sleep and the amplitude of delta waves are positively correlated with the amount of prior wakefulness (Franken et al., 2001; Tobler & Borbely, 1986). Since the RH mutants showed increased wakefulness and a decrease in NREM sleep during the dark phase, we sought to determine whether the delta power intensity was also altered. The EEG power intensity during NREM sleep was determined in RH mutants and WT littermates by fast Fourier transformation (FFT) of the slow-wave delta (0.5–4 Hz) frequency in 10-second intervals, as previously described (Papale et al., 2010). The EEG recordings from two consecutive days were averaged and the normalized delta power bands were quantified as percentages of the total recording time in two-hour intervals of the 12-hour light and 12-hour dark periods. The RH mutants showed similar delta power intensity during light and dark phases compared to the WT littermates (p>0.05 for all comparisons, data not shown).

**Response to sleep deprivation was similar between RH mutants and WT littermates during the first 12 hours of sleep rebound**

The effect of six hours (7 am to 1 pm) of sleep deprivation (SD) on the amount of wake and sleep during the subsequent 18 hours (in three 6-hour bins) of the recovery period, compared to the equivalent period during baseline recordings, was first determined within each genotype and then compared between genotypes (Fig. 4). For the first six hours of the rebound period (Fig. 4A), a main effect of SD was observed on wake and NREM sleep amounts (rANOVA for wake: F(1,10)=47.9, p<0.001 and NREM sleep: F(1,10)=15.7, p<0.003). Specifically, the WT littermates and the RH mutants showed similar statistically significant decreases in wake amounts (WT, 24% decrease; RH, 21% decrease), and increases in NREM sleep amounts (WT, 11% increase; RH, 13% increase) (Tukey’s, p<0.05; Fig. 4A). During the next six hours of sleep rebound (7 pm to 1 am, Fig. 4B), which started at the beginning of dark phase, rANOVA indicated a main effect of SD on NREM (F(1,10)=12.4, p<0.005) and REM sleep (F(1,10)=33, p<0.001) amounts. A statistically significant increase in NREM sleep amount was only observed for the RH mutants (WT, 14% increase; RH, 73% increase; Tukey’s, p<0.05 for RH mutants; Fig. 4B); however, both genotypes exhibited statistically significant increases in REM sleep amount (WT, 81% increase; RH, 329% increase; Tukey’s, p<0.05 for both genotypes; Fig. 4B). Surprisingly, when wake and sleep amounts during rebound were compared between RH mutants and WT littermates, post hoc analysis indicated that the amounts of wake and sleep during both of these six-hour rebound periods were not statistically different between RH mutants and WT littermates (Tukey’s, p>0.05 for both rebound periods). These results show that although the mutants have altered sleep-wake amounts during baseline recordings, following SD they are able to compensate for the loss of sleep and generate sleep-wake amounts that are comparable to WT levels.
**RH mutants begin to return to baseline levels during the last six hours of sleep rebound**

During the last six hours of the recovery period (Fig. 4C), rANOVA indicated a main effect of SD on wake amounts ($F_{(1,10)}=14.8, p<0.005$). Both WT littermates and RH mutants showed statistically significant decreases in wake amounts when compared to baseline values (WT, 9% decrease; RH, 8% decrease; Tukey’s, $p<0.05$, Fig. 4C). For the time spent in sleep, rANOVA detected a main effect of SD on NREM ($F_{(1,10)}=9.7, p<0.01$) and REM sleep ($F_{(1,10)}=24.2, p<0.001$) amounts. A statistically significant difference in NREM sleep amount was only observed for the WT littermates (WT, 9% increase; RH, 8% increase; Tukey’s, $p<0.05$ for WT littermates; Fig. 4C); however, both genotypes exhibited statistically significant increases in REM sleep amounts (WT, 31% increase; RH, 77% increase; Tukey’s, $p<0.05$ for both genotypes; Fig. 4C).

When wake and sleep amounts were compared between RH mutants and WT littermates during this period, rANOVA indicated a main effect of genotype on wake ($F_{(1,10)}=9.1, p<0.01$) and NREM sleep ($F_{(1,10)}=8.1, p<0.01$), and post hoc analysis showed that the percentage of time spent in wake (WT, 49% versus RH, 57%) and NREM sleep (WT, 44% versus RH, 37%) were statistically different between WT littermates and RH mutants. This suggests that during this period the RH mutants began to return to baseline levels, where greater wakefulness and reduced sleep amounts are observed in the mutants.

**Delta power intensity in response to extended wakefulness is similar for RH mutants and WT littermates**

The need for sleep is directly related to the amount of prior wakefulness (Borbely & Achermann, 1999). As the duration of continued wakefulness lengthens, the homeostatic drive for sleep intensifies, leading to an increase in delta power density during NREM sleep. To examine the homeostatic sleep response to the six hours of SD, the densities of the delta power band during NREM sleep in the subsequent 18-hour period were determined in six-hour intervals and compared to the corresponding time periods at baseline for RH mutants and WT littermates (Fig. 5). Overall, the RH mutants exhibited similar homeostatic response to SD as the WT littermates. As expected, both genotypes exhibited a statistically significant increase in delta power intensity during the first 6-hour period following SD (rANOVA: main effect of SD: $F_{(1,10)}=106.8, p<0.001$; Tukey’s, $p<0.05$ for both genotypes; Fig. 5A). During the subsequent 6-hour period, although both genotypes showed decreased delta power intensity, the values were comparable to baseline (Tukey’s, $p>0.05$, Fig. 5B). For the last 6-hour period, the decrease in delta power intensity was statistically significant for both genotypes when compared to baseline (rANOVA: main effect of SD: $F_{(1,10)}=116.1, p<0.001$; Tukey’s, $p<0.05$ for both genotypes, Fig. 5C).

**Discussion**

We recently reported that heterozygous RH mutants exhibit infrequent spontaneous seizures and reduced thresholds to hyperthermia- and flurothyl-induced seizures. In addition, electrophysiological analysis revealed that Na$_{v}$1.1 function in GABAergic interneurons of the hippocampus and cortex is critical to the maintenance of normal neuronal excitability, and reduced neuronal inhibition is implicated in seizure generation in SCN1A-derived epilepsies (Martin et al., 2010; Ogiwara et al., 2007; Ohno et al., 2010; Yu et al., 2006).

Results from this study show that in addition to GAD-positive cells of the hippocampus and cortex, Na$_{v}$1.1 is also expressed in serotonergic (5-HT) neurons of the DRN and MnR, as well as in cholinergic neurons of the PPT and LDT. We speculate that alterations in these neurotransmitter systems may also contribute to seizure generation in patients with SCN1A mutations. 5-HT and cholinergic nuclei have extensive projections to regions such as the
hippocampus, thalamus, and cortex that are known to play a role in epilepsy (Frankel et al., 2005; Juruska et al., 2010; Saper et al., 2005; Sorman et al., 2011). Pharmacological modifications to the 5-HT system with selective serotonin reuptake inhibitors SSRIs (Torta & Monaco, 2002) or with recreational use of 3,4-methylenedioxymethamphetamine (MDMA; Ecstasy) which acts as an agonist and reuptake inhibitor of 5-HT (Ben-Abraham et al., 2003) have been found to impact seizure susceptibility. Alterations to the cholinergic system have similarly been associated with seizures. Increased hippocampal kindling (Ferencz et al., 2001) and susceptibility to proconvulsants (Silveira et al., 2000) have been reported following cholinergic lesioning. Recently, reduced ACh immunoreactivity was observed in patients with West syndrome and Lennox-Gastaut syndrome (Hayashi et al., 2012). Thus, the expression of Na\textsubscript{v}1.1 in 5-HT and cholinergic neurons raises the possibility that perturbation of these systems may contribute to the seizures observed in patients with SCN1A mutations.

Additionally, Na\textsubscript{v}1.1 channels were also found to co-localize with orexinergic cells in the lateral HT. Activation of the orexinergic network reinforces the monoaminergic control of wakefulness (Saper et al., 2005). The expression of Na\textsubscript{v}1.1 in orexinergic neurons involved in these pathways is consistent with a functional role for Scn1a in sleep physiology.

The sleep-wake architecture of RH mice during baseline recordings showed more wakefulness and less NREM and REM sleep. It is important to note that although the RH mutants experience infrequent spontaneous seizures (Martin et al., 2010) the animals reported in this manuscript were seizure-free during the EEG recordings. A recent study demonstrated that heterozygous Scn1a knockout mice, which model Dravet syndrome, have a longer circadian period, with delayed onset of activity during the dark phase (Han et al., 2012). We observed that the latency to the onset of sustained wakefulness in response to the dark phase (beginning at 7 pm) was comparable between RH mutants and WT littermates, suggesting that the abnormal sleep-wake amounts in the RH mutants are the result of altered sleep and wake regulatory mechanisms, and not an impaired circadian rhythm. It may be that loss-of-function mutations have a greater impact on neuronal function, thereby resulting in more extensive alterations in sleep architecture and circadian rhythms.

Since comparable amounts of sleep were observed in WT littermates and RH mutants during the light phase, we challenged the animals to six hours of extended wakefulness to determine whether sleep homeostatic mechanisms are conserved in the mutants. Interestingly, while the mutants experienced less NREM and REM sleep during baseline recordings, sleep amounts during the first 12 hours of sleep rebound were largely comparable with levels observed in WT littermates. However, during the last six hours of sleep rebound, the RH mutants exhibited more wakefulness and less NREM sleep than WT littermates. We speculate that, if the EEG recordings were continued for a longer period of time, the RH mutants would also begin to show a decreased amount of REM sleep, resulting in a return to baseline sleep architecture.

Patients with epilepsy often report insufficient sleep amounts and excessive daytime sleepiness (Bazil, 2000; Hoeppner et al., 1984; Klobucnikova et al., 2009). Interestingly, normalization of sleep-wake patterns in epilepsy patients has been reported to also reduce seizure frequency (Beran et al., 1999; Malow et al., 1997; Vaughn et al., 1996). The reduced amounts of sleep observed in RH mutants during baseline conditions raises the possibility that patients with SCN1A mutations may have altered sleep architecture, thereby contributing to the overall seizure phenotype and potentially creating negative outcomes over time.
We recently reported that mice with mutations in the VGSC Scn8a which serve as a model of absence epilepsy (Papale et al., 2009), experience reduced amounts of wakefulness, increased NREM sleep, and an impairment of REM sleep generation and maintenance during the light phase compared to WT littermates (Papale et al., 2010). In a recent review, Cirelli reported that very few mouse mutants show alterations in both NREM and REM sleep (Cirelli, 2009). Interestingly, both NREM and REM sleep are affected in Scn1a and Scn8a mutants, highlighting an important role for VGSCs in the neuronal networks involved in sleep regulation. Differences between the specific sleep alterations observed in the Scn1a and Scn8a mutants may reflect the unique biophysical properties of each VGSC, differences in cell type and regional expression patterns, or subcellular localization.

In addition to establishing a role for SCN1A in the regulation of sleep physiology, the demonstration of Na\textsubscript{v}1.1 channels in specific cell types/brain regions that are known to play important roles in seizure generation and sleep regulation, now provides a possible explanation for the constellation of clinical features (seizures and sleep abnormalities) associated with human SCN1A mutations. In addition to SCN1A, mutations in SCN2A and SCN3A are also found in patients with epilepsy. SCN2A mutations have been reported in families with benign familial neonatal infantile seizures (Striano et al., 2006) and in several patients with severe forms of epilepsy (Ogiwara et al., 2009; Shi et al., 2009), and a SCN3A mutation was reported recently in a child with partial epilepsy (Holland et al., 2008). Further studies are therefore warranted to determine whether other VGSCs are also associated with alterations in sleep.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. \(\text{Na}_v1.1\) co-localization with GAD-positive interneurons in the hippocampus (HIPP), cortex, thalamic reticular nucleus (TRN), and hypothalamus (HT). Arrows indicate examples of \(\text{Na}_v1.1\)-immunoreactive neurons that co-localize with GAD. Scale bars, 100 microns.
Na\textsubscript{v}1.1 expression in brain regions involved in sleep regulation and epilepsy

Na\textsubscript{v}1.1 immunoreactivity with choline acetyltransferase (ChAT) in pedunculopontine tegmental nucleus (PPT), serotonin (5-HT) in dorsal raphe nucleus (DRN), and orexin-A in lateral hypothalamus (LH), are shown. None of the tyrosine hydroxylase (TH)-positive adrenergic cells of the locus coeruleus (LC) showed co-localization with Na\textsubscript{v}1.1 channels. Arrows indicate cells that are immunoreactive for both Na\textsubscript{v}1.1 and the specific cell type marker. Closed arrowheads indicate cells that are immunoreactive for Na\textsubscript{v}1.1 but not the specific cell-type marker. Open arrowheads indicate cells that are positive for the specific cell-type marker but not Na\textsubscript{v}1.1. Scale bars, 100 microns.

Figure 2. Na\textsubscript{v}1.1 expression in brain regions involved in sleep regulation and epilepsy

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Figure 3. The RH mutation enhances time spent in wakefulness and decreases time spent in non-REM (NREM) and rapid eye movement (REM) sleep during the dark phase of baseline EEG recordings.

Percentage of time spent in wakefulness (A and B), NREM sleep (C and D), and REM sleep (E and F) during the baseline-recording period. Data were averaged over light and dark phases (A, C, and E). The circadian variation of the sleep-wake cycle was obtained by dividing the light and dark periods into two-hour intervals (B, D, and F). WT, white bars and squares; RH, black bars and squares. Values are presented as mean±SEM. rANOVA followed by Tukey’s post hoc test, * p<0.05, ** p<0.01 between genotype.
Figure 4. Sleep architecture following six hours of total sleep deprivation in RH mutants and WT littermates

Comparison of the amount of time spent in wakefulness, NREM sleep, and REM sleep during 18 hours of the recovery period with the equivalent time period during baseline recording. The recovery period consisted of six hours of the light period (1 pm to 7 pm, A), first 6 hours of the dark period (7 pm to 1 am, B), and second 6 hours of the dark period (1 am to 7 am, C). Baseline recordings, white bars and squares; sleep rebound recordings, black bars and squares. Values are presented as mean±SEM., rANOVA followed by Tukey’s post hoc test, * p<0.05, ** p<0.01, *** p<0.001 within genotype.

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Figure 5. NREM sleep delta power intensity in response to extended wakefulness is similar for RH mutants and WT littermates
The recovery period consisted of six hours of the light period (1 pm to 7 pm, A), first 6 hours of the dark period (7 pm to 1 am, B) and second six hours of the dark period (1 am to 7 am, C). Baseline recordings, white bars and squares; sleep rebound recordings, black bars and squares. Values are presented as mean±SEM. rANOVA followed by Tukey’s post hoc test, *** p<0.001 within genotype.
Table 1

Expression of Na\textsubscript{v}1.1 channels in different brain regions/cell types associated with sleep and epilepsy.

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>Cell Marker</th>
<th>No. positive for cell type marker</th>
<th>No. Na\textsubscript{v}1.1 positive</th>
<th>Co-localization (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hipocampus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CA3</td>
<td>GAD</td>
<td>60</td>
<td>59</td>
<td>98</td>
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<tr>
<td>CA1</td>
<td>GAD</td>
<td>80</td>
<td>78</td>
<td>98</td>
</tr>
<tr>
<td>DG</td>
<td>GAD</td>
<td>50</td>
<td>47</td>
<td>94</td>
</tr>
<tr>
<td>Str. rad.</td>
<td>GAD</td>
<td>86</td>
<td>84</td>
<td>98</td>
</tr>
<tr>
<td>DH</td>
<td>GAD</td>
<td>41</td>
<td>40</td>
<td>98</td>
</tr>
<tr>
<td>Cortex</td>
<td>GAD</td>
<td>212</td>
<td>207</td>
<td>98</td>
</tr>
<tr>
<td>TRN</td>
<td>GAD</td>
<td>212</td>
<td>202</td>
<td>95</td>
</tr>
<tr>
<td>HT</td>
<td>GAD</td>
<td>188</td>
<td>182</td>
<td>97</td>
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<tr>
<td>LH</td>
<td>Orexin-A</td>
<td>69</td>
<td>13</td>
<td>19</td>
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<tr>
<td>DRN</td>
<td>5-HT</td>
<td>143</td>
<td>62</td>
<td>43</td>
</tr>
<tr>
<td>MnR</td>
<td>5-HT</td>
<td>54</td>
<td>13</td>
<td>24</td>
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<tr>
<td>LC</td>
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<td>ChAT</td>
<td>113</td>
<td>43</td>
<td>38</td>
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<tr>
<td>LDT</td>
<td>ChAT</td>
<td>45</td>
<td>14</td>
<td>31</td>
</tr>
</tbody>
</table>

Data show number of cells positive for Na\textsubscript{v}1.1 and cell type markers located in the hippocampus (CA3, CA1, dentate gyrus (DG), stratum radiatum (STR), and dentate hilus (DH)), cortex, thalamic reticular nucleus (TRN), hypothalamus (HT), lateral hypothalamus (LH), dorsal raphe nucleus (DRN), median raphe nucleus (MnR), locus coeruleus (LC), pedunculopontine tegmental nucleus (PPT) and laterodorsal tegmental nucleus (LDT). Markers examined: glutamic acid decarboxylase (GAD), orexin-A, serotonin (5-HT), choline acetyltransferase (ChAT), tyrosine hydroxylase (TH).