

Research report

Loss of HCN1 subunits causes absence epilepsy in rats

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HIGHLIGHTS

- HCN1-deficient rat strain was developed by TALEN mutagenesis.
- The *Hcn1*-deficient rats exhibited reduced hyperpolarization-activated currents.
- The *Hcn1*-deficient rats exhibited spontaneous SWDs accompanying behavioral arrest.
- Both SWDs and behavioral arrest were suppressed by ethosuximide.
- These findings demonstrate that deficiency of HCN1 caused absence epilepsy in rats.

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ABSTRACT

Hyperpolarized-activated cyclic nucleotide-gated (HCN) channels underlie hyperpolarization-activated current (I_h) and are involved in controlling the excitability and electrical responsiveness of neurons. Absence epilepsy is clinically defined by a sudden, brief impairment of consciousness and behavioral arrest. Spike-and-wave discharges (SWDs) on electroencephalograms (EEG) are a diagnostic hallmark of absence epilepsy. In rat models of absence epilepsy, impaired function or expression of HCN1, a subtype of HCN channels, has been found. Here, to evaluate whether HCN1 deficiency causes absence epilepsy in rats, we developed *Hcn1*-knockout rats by transcription activator-like effector nuclease mutagenesis. The cortical and hippocampal pyramidal neurons of these rats displayed a significant reduction of I_h , a pronounced hyperpolarizing shift of the resting membrane potential, and increased input resistance, which indicated that the *Hcn1*-knockout rats were deficient in HCN1 function. The *Hcn1*-knockout rats were also more vulnerable to pentylentetrazol-induced acute convulsions. More importantly, they exhibited spontaneous SWDs, which were accompanied by behavioral arrest, both of which were suppressed by ethosuximide. These results confirm the involvement of the HCN1 subunit in the regulation of input resistance and provide direct evidence that a deficiency of HCN1 caused absence epilepsy in rats.

1. Introduction

Hyperpolarized-activated cyclic nucleotide-gated (HCN) channels underlie an inward current termed I_h , which is a mixed Na^+/K^+ current that is activated by hyperpolarization rather than depolarization (Biel

et al., 2009). I_h is conducted by four HCN channel subunits (HCN1–4) that assemble into homo- or heterotetrameric channels and are found in numerous excitable and non-excitable cell types, including many neurons of the central and peripheral nervous systems (Notomi and Shigemoto, 2004; Santoro et al., 2000) and in cardiomyocytes (Calejo

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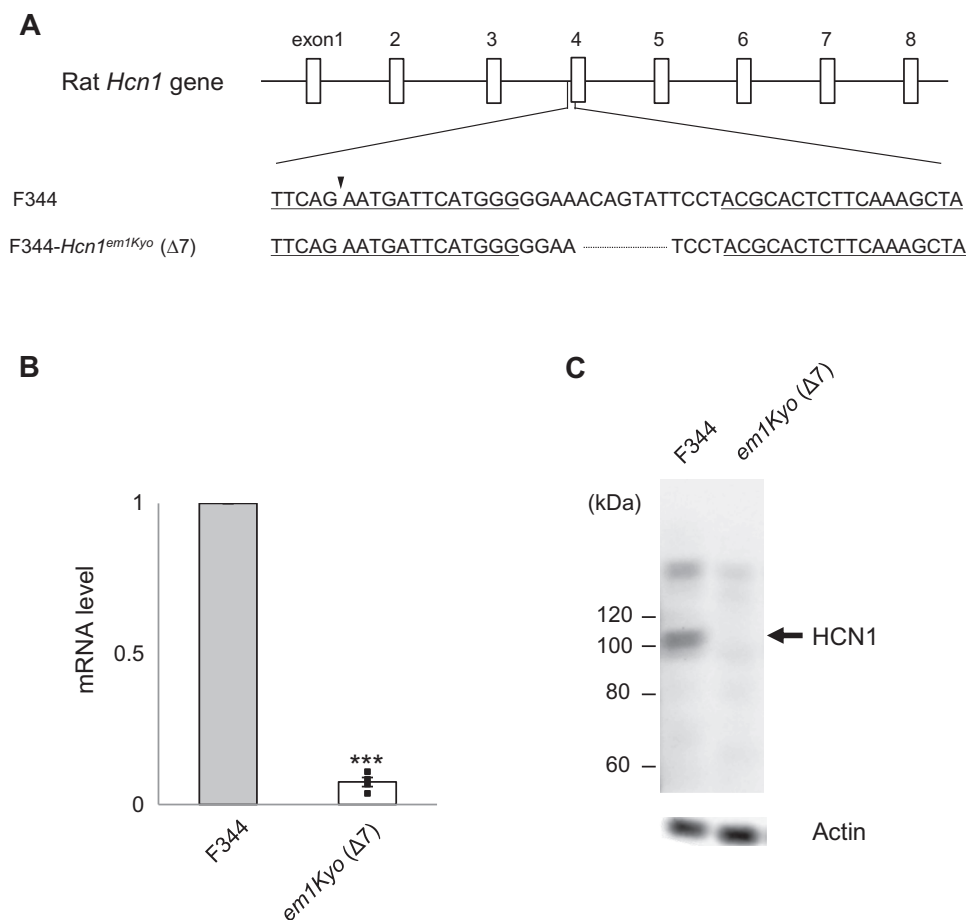


Fig. 1. Generation of *Hcn1*-knockout rats using TALEN technology. **A**, A schematic representation of the rat *Hcn1* gene and TALEN target site. Exons are represented by boxes. TALEN target sites are designated in exon 4 of the rat *Hcn1* gene and are underlined. The upper TALEN target site included the boundary of intron 3 and exon 4 (arrow head). The 7-bp deletion was found in F344-*Hcn1*^{em1Kyo} rats. **B**, Expression levels of *Hcn1* mRNA in brains of F344 (gray bar) and F344-*Hcn1*^{em1Kyo} (open bar) rats. F344-*Hcn1*^{em1Kyo} rats exhibited significantly reduced expression levels compared with F344. ***; $P < 0.001$. **C**, Western blot analysis of brain lysates from F344 and F344-*Hcn1*^{em1Kyo} rats. The HCN1 protein is detected in F344 but not in F344-*Hcn1*^{em1Kyo} rats. Actin is used as a loading control.

et al., 2014). In the nervous system, each HCN subunit displays a distinct pattern of distribution (Moosmang et al., 1999; Notomi and Shigemoto, 2004). HCN1 is expressed in the neocortex, hippocampus, cerebellar cortex, and brain stem, while HCN2 is distributed nearly ubiquitously throughout most brain regions. HCN3 is distributed at very low levels in the central nervous system. HCN4 is quite selectively expressed in various thalamic nuclei and in the mitral cell layer of the olfactory bulb.

There are two key characteristics of the physiological roles of HCN channels in neurons. First, HCN channels are partially open at rest and contribute to the setting of the membrane potential in many cells. Second, HCN channels inherently provide negative feedback as they can counteract both membrane hyperpolarization and depolarization. When the membrane is hyperpolarized, HCN channels are activated and produce a depolarizing inward current. When the membrane is depolarized, HCN channels are inactivated, which facilitates hyperpolarization. Thus, HCN channels can actively dampen both inhibitory and excitatory stimuli arriving at the cell membrane and thereby help to stabilize the membrane potential. Consequently, HCN channels are largely involved in controlling the excitability and electrical responsiveness of cells (Biel et al., 2009).

Absence seizures are clinically defined by a sudden, brief impairment of consciousness and behavioral arrest. Absence seizures mainly originate in the cortex but the thalamus is also involved in the pathology of the disease. Particularly, the generation of spike-and-wave discharges (SWDs), a diagnostic hallmark of absence seizures, is correlated with an increased prevalence of burst firing and synchronized oscillatory activity in the thalamocortical circuits (McCormick and Contreras, 2001).

Given the key role of HCN channels in controlling the firing behavior of thalamocortical neurons (Budde et al., 2005), abnormal HCN

channel expression may contribute to the generation of absence seizures. In agreement with this hypothesis, in two rat models of absence epilepsy, the Wistar Albino Glaxo Rij (WAG/Rij) and the Genetic Absence Epilepsy Rat from Strasbourg (GAERS), impaired expression and malfunction of the HCN1 channel is likely to be associated with the genesis of absence epilepsy. In WAG/Rij rats, HCN1 expression is reduced in the adult cortex (Strauss et al., 2004) and a rapid decline in expression of HCN1 channels precedes the onset of absence seizures (Kole et al., 2007). This suggests that the loss of HCN1 facilitates the initiation and propagation of spontaneous generalized seizures. By contrast, an increase in HCN1 expression, which is associated with a decrease in cAMP responsiveness of I_h in thalamocortical neurons, has been found in the epileptic thalamus of WAG/Rij and GAERS rats (Budde et al., 2005; Kuisle et al., 2006).

Inference about a causal relationship between the expression of the HCN1 channel and absence epilepsy is still controversial, as described above. In addition, *Hcn1*-knockout mice exhibit no spontaneous absence seizures (Huang et al., 2009). Thus, there is currently no direct evidence that dysfunction of HCN1 causes absence seizures.

In the present study, to evaluate whether *Hcn1* is a causative factor in absence seizures, we developed an *Hcn1*-knockout rat strain by transcription activator-like effector nuclease (TALEN) mutagenesis. The electrophysiological analyses of the brain slices demonstrated that *Hcn1*-knockout rats had deficits in HCN1 channel function. *Hcn1*-knockout rats were more vulnerable to pentylenetetrazol (PTZ)-induced acute convulsions, suggesting a higher baseline neuronal excitability. More importantly, they exhibited SWDs that could be suppressed by ethosuximide (ESM), an anti-epileptic drug (AED) effective in suppressing absence seizures in humans.

2. Results

2.1. Generation of *Hcn1*-knockout rats

Of 273 TALEN-injected embryos, 229 (83.9%) were transferred to the oviducts of pseudopregnant female rats. Of these implanted embryos, 42 (18.3%) successfully reached the end of normal gestation, and 40 rats were weaned. Sequence analysis of the TALEN target site of these 40 founder animals revealed that three males and eight females (27.5%) carried mutations. Among them, a founder animal (#84) was used to develop a *Hcn1*-knockout line. The F344-*Hcn1*^{em1kyo} strain that was derived from line #84 carried a 7-bp deletion in exon 4 (Fig. 1A). The 7-bp deletion was deduced to generate a premature stop codon because of frame shifting.

The level of expression of the *Hcn1* mRNA in the brain of F344-*Hcn1*^{em1kyo} rats decreased significantly compared with those in the control F344/NSIc rats (Fig. 1B). The western blotting results showed no expression of HCN1 proteins in the brains of F344-*Hcn1*^{em1kyo} rats (Fig. 1C). These findings indicated that F344-*Hcn1*^{em1kyo} rats had a null mutation of *Hcn1*. Rats homozygous for the *Hcn1*-null allele (*Hcn1*-knockout rats) developed normally and were fertile in both sexes.

2.2. Voltage and current responses of pyramidal neurons in *Hcn1*-knockout rats

To ascertain whether the knockout of *Hcn1* affected I_h , we investigated voltage and current responses of CA1 pyramidal neurons (CA1-PNs) in hippocampus layer 5 pyramidal neurons (L5-PNs) in the cortex. In F344 rats, CA1-PNs exhibited voltage responses characterized by a voltage sag—a rapid hyperpolarization followed by a slow depolarization—in response to current pulses. By contrast, the voltage sag was unclear in *Hcn1*-knockout rats (Fig. 2A). The sag ratios of *Hcn1*-knockout rats were significantly smaller than those of F344 rats in both L5-PNs ($5.92 \pm 0.488\%$ vs $16.1 \pm 1.32\%$, $P < 0.0001$) and CA1-PNs ($9.22 \pm 0.698\%$ vs $19.1 \pm 1.02\%$, $P < 0.0001$) (Fig. 2C). Expression of I_h in CA1-PNs was confirmed in F344 rats, but I_h expression was largely decreased in *Hcn1*-knockout rats (Fig. 2B). The current densities of *Hcn1*-knockout rats were significantly smaller than those of F344 rats

(200 ± 27.1 vs 90.7 ± 6.90 , $P < 0.0001$) (Fig. 2E). The resting membrane potentials of *Hcn1*-knockout rats were significantly hyperpolarized compared to those of F344 rats in both L5-PNs (-80.4 ± 0.743 mV vs -73.9 ± 0.612 mV, $P < 0.0001$) and CA1 PNs (-80.7 ± 0.876 mV vs -72.2 ± 0.663 mV, $P < 0.0001$) (Fig. 2F).

2.3. PTZ-induced seizures in *Hcn1*-knockout rats

PTZ has been used experimentally to induce acute seizures. When animals are more vulnerable to PTZ, their neurons are thought to be more easily activated (Klioueva et al., 2001; Löscher and Schmidt, 1988). To evaluate neuronal excitability, we administered PTZ to *Hcn1*-knockout rats in several different doses. We could not find any significant differences in latency or duration in each excitation behavior between *Hcn1*-knockout and F344 rats at any of the doses. However, when we injected PTZ at 50 mg/kg, we found a significant difference in the most severe excitation behavior. The most severe behavior of F344 rats was clonic seizure with falling back (score 5). By contrast, the behavior of *Hcn1*-knockout rats reached the level of tonic-clonic seizure (score 6) and 83% of these rats died without recovery from the tonic-clonic seizure (score 7). Maximum score at 50 mg/kg was 5.00 ± 0.00 in F344 rats and 6.83 ± 0.167 in *Hcn1*-knockout rats ($P < 0.001$) (Fig. 3A). These results indicated that *Hcn1*-knockout rats were susceptible to PTZ, which strongly suggested that *Hcn1*-knockout rats had a higher neural excitability than F344 rats.

2.4. SWDs in *Hcn1*-knockout rats

Behavioral absence seizures accompanying generalized SWDs have been observed in neurological mutant mice and rats (Serikawa et al., 2015). Although control F344 rats exhibited no SWDs, *Hcn1*-knockout rats exhibited 7–8 Hz SWDs in cortical electroencephalograms (EEGs) from 10 weeks of age. The SWDs were observed bilaterally (Fig. 4A) and synchronously in the cortex and the hippocampus (Fig. 4B). During the SWDs, *Hcn1*-knockout rats exhibited absence-like behavior, sudden and temporary behavioral arrest, and twitching of the vibrissae (Supplementary Video 1).



in both L5-PNs (0.498 ± 0.0363 vs 1.13 ± 0.0720 , $P < 0.0001$) and CA1-PNs (0.449 ± 0.0278 vs 1.32 ± 0.0861 , $P < 0.0001$) (Fig. 2D). These results indicated that the knockout of *Hcn1* decreased I_h in both the cerebral cortex and the hippocampal CA1 PNs.

We further investigated whether the decrease in I_h affected the input resistance and resting membrane potential. The input resistances of *Hcn1*-knockout rats were significantly larger than those of F344 rats in both L5-PNs (339 ± 44.8 vs 214 ± 18.6 , $P < 0.0001$) and CA1-PNs

The average number of SWDs during the 1 h observation period was 5.17 ± 0.550 at 10 weeks of age, 9.92 ± 1.72 at 15 weeks of age, 18.5 ± 4.16 at 20 weeks of age, and 25.1 ± 8.95 at 25 weeks of age. The average number at 20 weeks of age was significantly higher than that at 10 weeks of age. The average number of SWDs at 25 weeks was significantly higher than those at 10 and 15 weeks of age (Fig. 4C).

The duration of the SWDs was 2.06 ± 0.0981 sec at 10 weeks of age, 3.59 ± 0.237 sec at 15 weeks of age, 4.70 ± 0.283 sec at

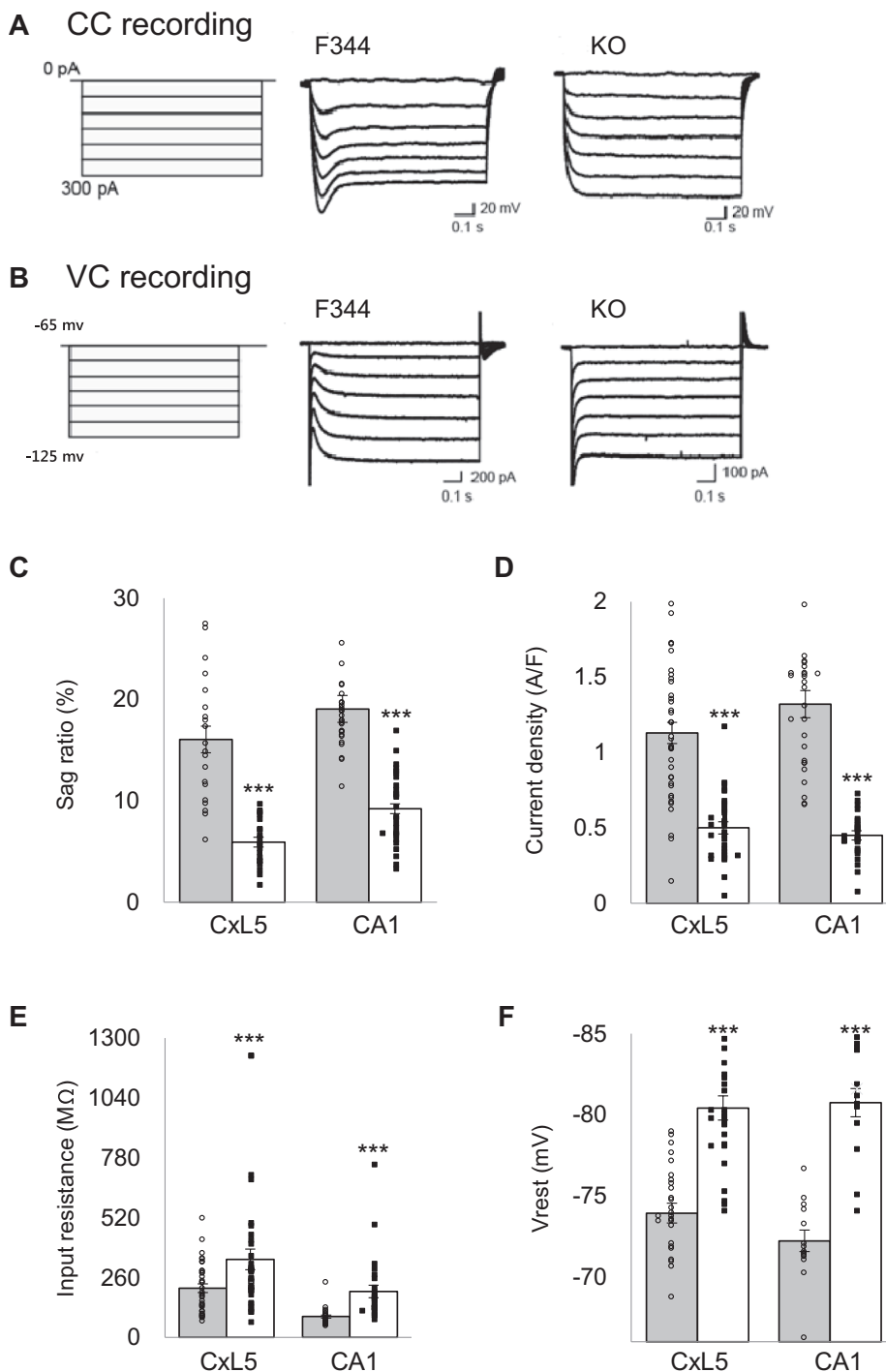


Fig. 2. Voltage and current responses of cortex and hippocampal pyramidal neurons in F344 and *Hcn1*-knockout rats. **A**, Voltage responses of hippocampal CA1 pyramidal neurons (CA1-PNs) in F344 (center) and *Hcn1*-knockout rats (right) in response to hyperpolarizing current pulses (left). **B**, Current responses of CA1-PNs in F344 (center) and *Hcn1*-knockout rats (right) in response to hyperpolarizing voltage pulses (left). **C**, Sag ratio of layer 5 pyramidal neurons (L5-PNs) and CA1-PNs in F344 (gray bars) and *Hcn1*-knockout (open bars) rats. **D**, I_h current density of L5-PNs and CA1-PNs in F344 (gray bars) and *Hcn1*-knockout (open bars) rats. **E**, Input resistance of L5-PNs and CA1-PNs in F344 (gray bars) and *Hcn1*-knockout (open bars) rats. **F**, Resting membrane potential of CA1-PNs and L5-PNs in F344 (gray bars) and *Hcn1*-knockout (open bars) rats. ***, $P < 0.0001$.

20 weeks of age, and 4.90 ± 0.861 sec at 25 weeks of age. The duration at 15 weeks of age was significantly longer than that at 10 weeks of age. The durations at 20 and 25 weeks of age were significantly longer than those at 10 and 15 weeks of age (Fig. 4D).

As a result, cumulative durations of the SWDs at 15 and 20 weeks of age were significantly longer than those at 10 weeks. The cumulative duration at 25 weeks of age was significantly longer than those at 10 and 15 weeks of age (Fig. 4E).

These results indicated that both the numbers and duration increased significantly with increasing age in *Hcn1* knockout rats (Fig. 4C–E).

2.5. Effects of AEDs on SWDs in *Hcn1*-knockout rats

To clarify the type of seizures in *Hcn1*-knockout rats, we evaluated the effects of two AEDs, phenytoin (PHT) and ESM. We found that neither the average numbers nor the cumulative duration of SWDs were different before and after administration of PHT (number: 26.0 ± 5.18 vs 16.3 ± 2.76 , $P = 0.130$, cumulative duration: 130.1 ± 31.5 sec vs 74.2 ± 16.3 sec, $P = 0.146$, mean $\pm$ SEM) (Fig. 5). By contrast, when we administered ESM to *Hcn1*-knockout rats, SWDs were completely suppressed (number: 25.0 ± 9.65 vs 0.00 ± 0.00 , $P < 0.05$, cumulative duration: 111.5 ± 41.4 vs 0.00 ± 0.00 , $P < 0.05$) (Fig. 5).

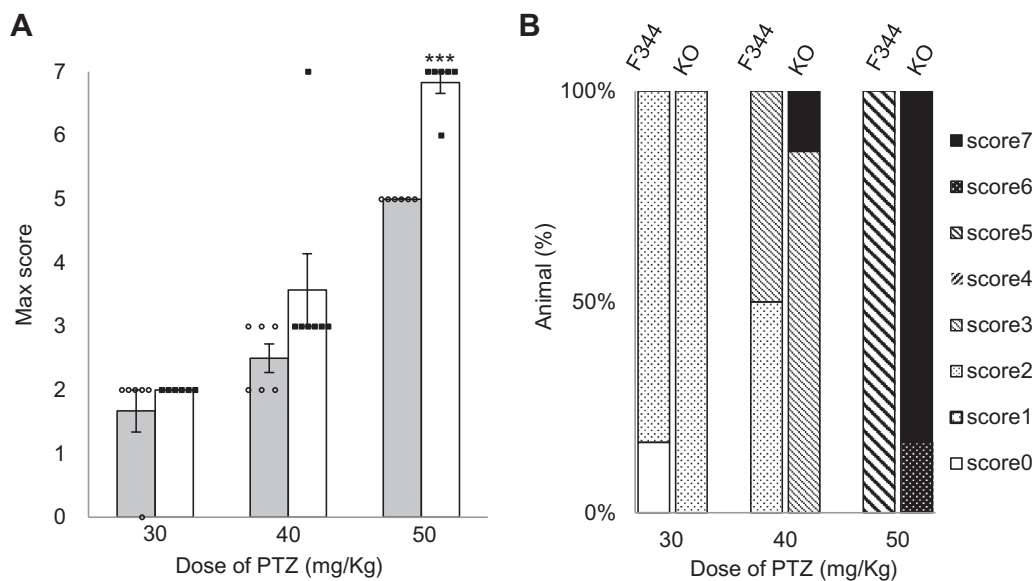


Fig. 3. PTZ-induced seizures in *Hcn1*-knockout rats. A, Average of scores recorded in PTZ-administered rats. Gray and open bars represent F344 ($n = 6$) and *Hcn1*-knockout ($n = 6$ –7) rats, respectively. Each injection was administered from the lowest to highest dose in the same animal on Day 1, 4, and 7. ***, $P < 0.001$. B, Proportions behavioral scores among PTZ-administered rats.

Thus, seizures occurring spontaneously in *Hcn1*-knockout rats had similar pharmacological and behavioral properties to those of absence seizures in humans.

3. Discussion

In the present study, we developed the *Hcn1*-knockout rat strain (F344-*Hcn1*^{em1kyo}). I_h currents were significantly reduced in *Hcn1*-knockout rats, and this effect is also seen in *Hcn1*-knockout mice (Nolan et al., 2003; Nolan et al., 2007). In addition, the *Hcn1*-knockout rats exhibited a significant reduction of the depolarizing voltage sag, a hallmark of I_h current activation (Pape, 1996). Thus, the *Hcn1*-knockout rats were deficient of HCN1 subunit function.

We observed that the resting membrane potential of cortex and hippocampus CA1 PNs from *Hcn1*-knockout rats was more hyperpolarized than those of wild-type cells, which was also seen in *Hcn1*-knockout mice (Nolan et al., 2007). HCN channels stabilize the resting membrane potential by lowering the input resistance (Nolan et al., 2007). In agreement with this, input resistance in the cortex and CA1 PNs from the *Hcn1*-knockout rats was higher than those from the wild-type rats. These findings indicated that HCN1 played a crucial role in the determination of resting membrane potential and suggested that membrane potentials of these cells would more easily fluctuate with any input current in *Hcn1*-knockout rats.

Hcn1-knockout rats were significantly vulnerable to PTZ-induced seizures, which builds upon the work of Huang et al. (2009) and indicates that a lack of HCN1 subunits influences neural excitability. Besides hyperpolarized resting membrane potential and increased input resistance, more action potentials were elicited in the *Hcn1*-knockout mice even when the resting membrane potential was adjusted to be hyperpolarized (-70 mV) (Huang et al., 2009). Such excitability has been thought to underlie the susceptibility to the convulsant in mice. Thus, the *Hcn1*-knockout rats may also exhibit greater action potentials, which explains why they were more susceptible to PTZ-induced seizures.

The *Hcn1*-knockout rats spontaneously exhibited SWDs on the cortical and hippocampal EEGs which were performed during behavioral arrest. Such seizures appeared as early as 10 weeks of age and continued throughout the experimental period. The SWDs as well as the behavioral arrest were suppressed by administration of ESM. These

findings indicated that the *Hcn1*-knockout rats spontaneously exhibited generalized absence epilepsy. Several studies suggest that synchronized oscillations in the thalamocortical network play a central role in the genesis of absence seizures (Kim et al., 2001; Maheshwari and Noebels, 2014; Sohal and Huguenard, 2003). It is probable that just as *Hcn2*-deficient mice exhibit absence seizures and elevated resting membrane potential in thalamocortical neurons (Ludwig et al., 2003), the *Hcn1*-knockout rats may have a pronounced alteration in the responsiveness of the thalamocortical neurons.

In humans, *de novo* mutations in *HCN1* have been found in families with early infantile epileptic encephalopathy (EIEE) (Nava et al., 2014), which are sporadic disorders characterized by recurrent seizures during the neonatal or infantile periods and are associated with impaired cognitive and motor development (O'Brien and Meisler, 2013). EIEE patients with *HCN1* mutations exhibited absence seizures, but these seizures appeared later in the progression of the disease. Thus, absence seizures in *HCN1* mutation-carrying patients are very likely a secondary effect of fever and the prolonged seizures, which occur during the first year in life. In addition, *Hcn1*-knockout mice exhibit no obvious absence epilepsy (Huang et al., 2009). Therefore, we concluded that our present study is the first report, to our knowledge, to provide direct evidence that the loss of function of the *Hcn1* gene caused absence epilepsy.

While the *Hcn1*-knockout rats exhibited absence epilepsy, *Hcn1*-knockout mice exhibit no obvious absence epilepsy (Huang et al., 2009). A likely explanation for this discrepancy may be that the expression of absence seizures is controlled by multiple genetic components in rodents, as it is in humans (Crunelli and Leresche, 2002; Gauguier et al., 2004). Alteration of the genetic background of *Hcn1*-knockout mice may lead to absence seizures in the resultant *Hcn1*-knockout mice. Indeed, alteration of genetic backgrounds have been shown to modify the absence seizure phenotype in *Gria4*-deficient mice, a genetic mouse model of absence epilepsy (Frankel et al., 2014). Alternatively, the significance of the HCN1 subunit may be different in the generation of I_h between mice and rats. Systematic gene-knockout experiments and expression analyses will be required to clarify this issue.

In summary, the *Hcn1*-deficient rats we developed in this study exhibited absence epilepsy, which indicated that the loss of function of the *Hcn1* gene caused absence epilepsy in rats.

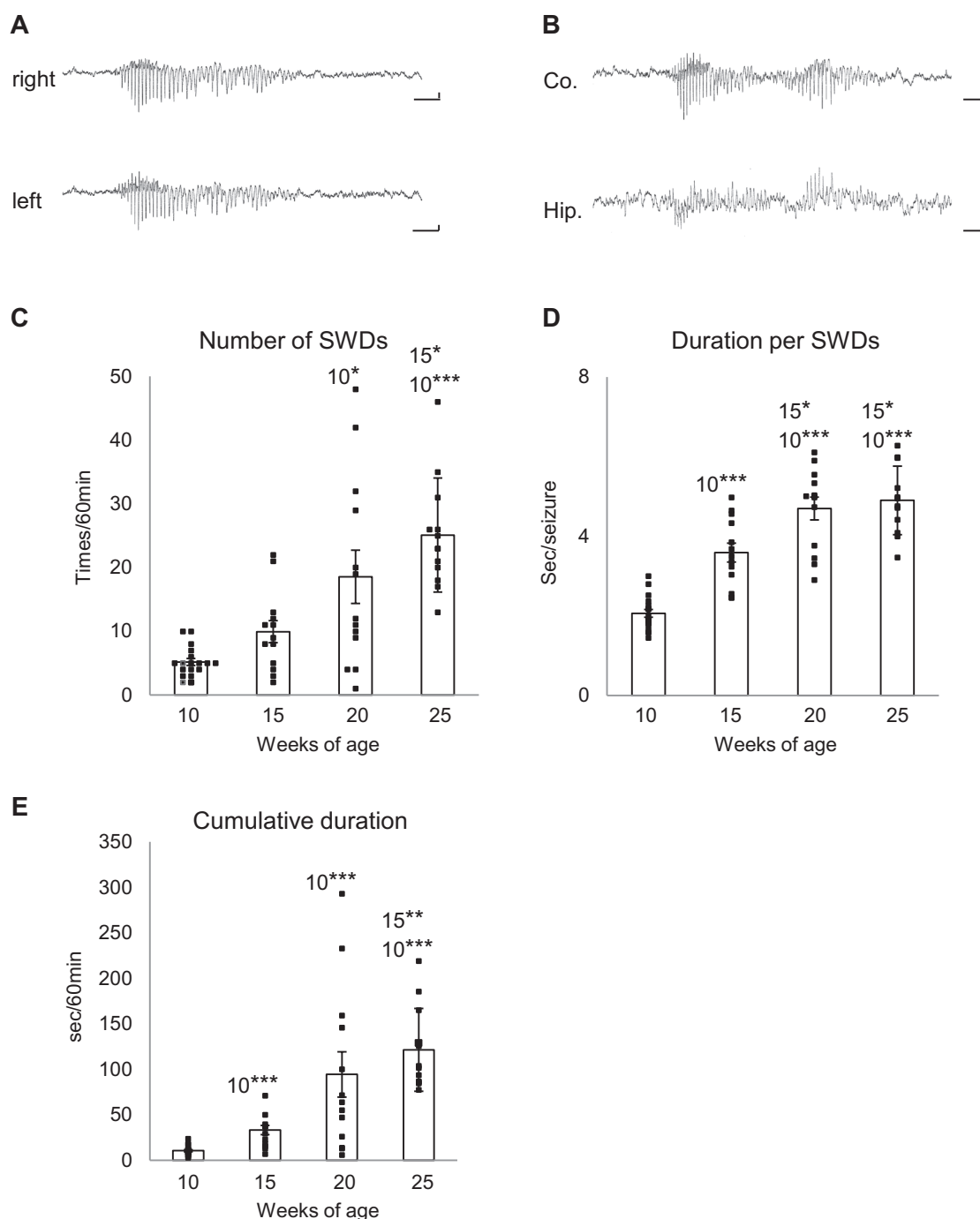


Fig. 4. EEG in *Hcn1*-knockout rats. A, EEGs of the *Hcn1*-knockout rats recorded at 25 weeks of age. Representative SWDs observed in the right (upper) and left (lower) cortical EEG. B, The cortical (upper; Co.) and hippocampal (lower; Hip.) EEGs. Calibration: Co. 200 μ V and 1 sec, Hip. 700 μ V and 1 sec. C–E, Ontogeny of SWDs in *Hcn1*-knockout rats. C, Number of SWDs; D, Duration per SWD; E, Cumulative duration of SWDs. Data were obtained from 10-, 15-, 20-, and 25-week-old rats ($n = 5$ –6) and represented as means $\pm$ SEM. Values on plots represented the weeks of age in which significant differences were detected. The EEG recordings were performed two or three times for each time point. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

4. Experimental procedure

4.1. Ethical use of animals

All animal experiments were approved by the Animal Research Committees of Kyoto University and Nara Medical University and were conducted according to their regulations on animal experimentation.

4.2. Animals

F344/NSlc rats were purchased from Japan SLC, Inc. (Hamamatsu, Shizuoka, Japan). F344-*Hcn1*^{em1Kyo} rats were obtained from the National Bio Resource Project for the Rat (Kyoto, Japan). In the present study, we used male rats, as it has been well-established that females have estrus cycles that affect neuron excitability and seizure

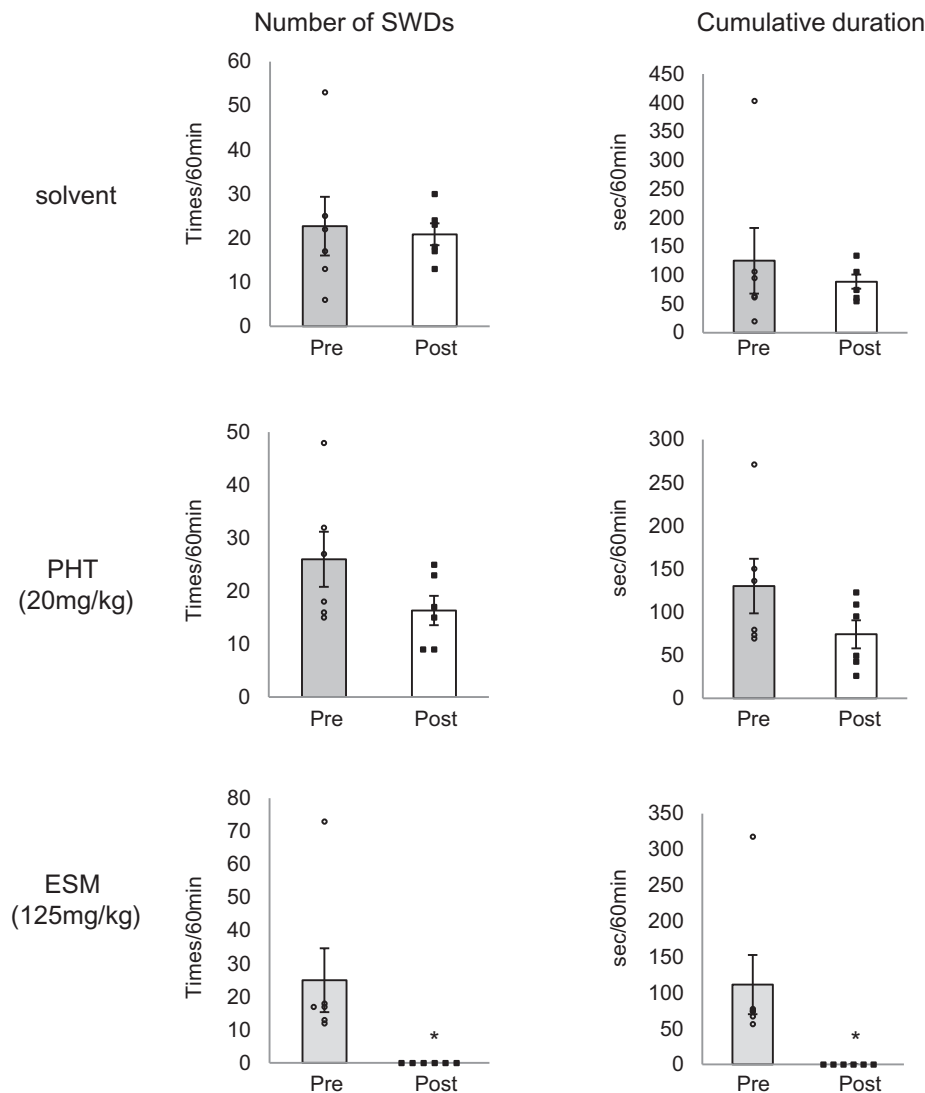


Fig. 5. Effect of AEDs on SWDs in *Hcn1*-knockout rats. Numbers (left) and cumulative duration (right) of SWDs for 60-min windows pre- and post-drug administration. The numbers and the mean cumulative duration of SWDs were significantly decreased by ESM, but not by PHT. Solvent did not affect the SWDs before or after administration. PHT: phenytoin, ESM: ethosuximide (n = 6, each; mean ± SEM). *P < 0.05.

susceptibility (Scharfman et al., 2003; Scharfman and MacLusky, 2006). In addition, males are commonly used to characterize seizure phenotypes in animal models of epilepsy and to develop anti-epileptic drugs (Kovács et al., 2015; Li et al., 2018).

4.3. TALEN plasmid construction and mRNA preparation

Platinum TALEN plasmids were constructed using the two-step Golden Gate cloning method as described previously (Sakuma et al., 2013b). The *Hcn1* TALEN target sequence, located in exon 4 of the *Hcn1* gene (NM_053375), is indicated in Fig. 1A. The TALEN mRNA was synthesized from plasmids linearized by *XhoI* digestion and purified as previously described (Sakuma et al., 2013a).

4.4. Microinjection of TALEN mRNA

TALEN mRNA was suspended in RNase-free water at 10 ng/μL. Approximately 2–3 pL of capped mRNA was microinjected into the male pronuclei of zygotes collected from F344/NSlc rats. Embryos were transferred to the oviducts of pseudopregnant Sprague Dawley female rats (Mashimo et al., 2013).

4.5. Quantitative PCR

Total RNA was isolated using ISOGEN II (Nippon Gene Co., Ltd, Tokyo, Japan) according to the manufacturer's instructions. Real-time PCR was performed using the Thermal Cycler Dice Real Time system (Takara Bio Inc., Otsu, Japan) with SYBR Premix Ex Taq II (Takara Bio Inc.). Primer sequences for quantification of *Hcn1* cDNA were 5'-GAG CTGACATGCGCCAGAA-3' and 5'-GGGATCCGCGTTAGCAAAGA-3'. The number of target molecules was normalized to that of peptidylprolyl isomerase A (*Ppia*) as an internal control (Kuramoto et al., 2011).

4.6. Western blotting

Western blotting was performed as described previously (Nishitani et al., 2016). The Anti-HCN1 antibody was purchased from Alomone Labs (Jerusalem, Israel) and used at a dilution of 1:1000 in Can Get Signal Immunoreaction Enhancer Solution (Toyobo, Osaka, Japan). The secondary antibody was an anti-rabbit IgG horseradish peroxidase (GE Healthcare, Buckinghamshire, UK) and was used at 1:100000 in Can Get Signal Immunoreaction Enhancer Solution. Signals were developed by ECL western blotting detection reagents (GE Healthcare) and detected using an image analyzer LAS-3000 (Fuji Film, Tokyo, Japan).

4.7. Whole-cell recordings in slice preparations

Male F344 ($n = 5$) and *Hcn1*-knockout rats ($n = 4$) at 18–19 weeks of age were deeply anesthetized with isoflurane and decapitated. Frontal slices (250 μ m thick) were made using a Microslicer (Pro 7, Dosaka EM, Kyoto, Japan) in an ice-cold sucrose solution. For the 14–35 recordings, the slice was submerged in the recording chamber and continuously perfused with the extracellular solution (125 mM NaCl, 2.5 mM KCl, 2 mM CaCl_2 , 1 mM MgCl_2 , 1.25 mM NaH_2PO_4 , 26 mM NaHCO_3 , and 25 mM glucose, aerated with 95% O_2 and 5% CO_2 (pH 7.4)).

L5-PNs in the cortical areas show a marked voltage sag induced by I_h (Kasper et al., 1994; Zhu, 2000; Zhang, 2004). In the hippocampal CA1, pyramidal neurons in the superficial region exhibit a larger somatic I_h than those in the deep region (Soltesz and Losonczy, 2018). Therefore, we performed whole-cell current-clamp and voltage-clamp recordings from L5-PNs in the sensorimotor cortex and CA1-PNs in the superficial region.

Patch pipettes were filled with an internal solution containing 120 mM K-methylsulfate, 10 mM KCl, 0.2 mM EGTA, 2 mM Mg-ATP, 0.3 mM Na-GTP, 10 mM HEPES, 10 mM Na_2 -phosphocreatine, and 0.1 mM spermine. The osmolality of the internal solution was 280–290 mOsm/l and the resistance of the patch electrodes was 4–8 M Ω in the bath solution. Voltage and current signals were low-pass filtered at 3–5 kHz and digitized at 10 kHz. Recordings were performed using an EPC-8 patch clamp amplifier (HEKA, Darmstadt, Germany) and the data were acquired using a pClamp9 system (Molecular Devices, Foster City, CA).

Neurons with membrane potentials below -50 mV immediately after patch membrane rupture and action potential peaks higher than 0 mV were used for further analyses. When voltage responses were investigated in current-clamp mode, hyperpolarizing current pulses (up to -300 pA in -50 pA increments, 1 sec in duration) were injected into the neurons (Fig. 2A). The membrane potentials of these neurons were maintained at -65 mV before the current pulses via the injection of constant currents. When current recordings were performed in voltage-clamp mode, a series of hyperpolarizing voltage pulses (from the holding potential of -65 mV to -125 mV in -10 mV increments, 1 sec in duration) were applied to the neurons (Fig. 2B).

4.8. Data analysis of whole-cell recordings

Off-line analysis was performed using AxoGraph X software (AxoGraph Scientific) and KaleidaGraph software (Hulinks, Tokyo, Japan). The input capacitance and input resistance were estimated based on the current induced by a 10-mV voltage step from a holding potential of -70 mV and the current induced by a voltage pulse of -10 mV applied at a holding potential of -65 mV, respectively. The resting membrane potential was determined as the voltage immediately after the membrane rupture. The voltage sag was analyzed from voltage traces induced by a -300 -pA current. The degree of the voltage sag was estimated by the sag ratio that was a percentage of the difference between the peak amplitude of the initial transient hyperpolarization and the mean amplitude of the steady-state hyperpolarization (940–990 ms after the onset of the current pulse) to the peak amplitude. The amplitude of an *h*-current was analyzed from a current trace induced by a voltage pulse to -125 mV (Fig. 2B). The current density was obtained by the amplitude normalized by the input capacitance.

4.9. PTZ-induced seizures

To evaluate the acute seizure susceptibility, *Hcn1*-knockout rats ($n = 6$ –7) or F344 rats ($n = 6$) were injected intraperitoneally with a dose of PTZ at 30, 40, or 50 mg/kg. Each injection was performed from the low to high dose in the same animal on Days 1, 4, and 7. Immediately after injection of PTZ, the animals were placed in an

observation cage (40 cm $\times$ 40 cm $\times$ 40 cm) and the incidence of excitation behaviors were continuously monitored by the time-sampling method (scored every 10 sec) for 15 min using an 8-point ranked score as follows: 0, no change; 1, recurrent head twitches or wet dog shakes; 2, myoclonic jerk of forepaws and/or upper trunk; 3, marked myoclonic jerk of forepaws and/or upper trunk with a rearing posture; 4, clonic seizures; 5, marked clonic seizures with falling backward; 6, tonic-clonic seizures; and 7, death, as described previously (Tokudome et al., 2016).

4.10. EEG recordings

For EEG recordings, rats of 9, 14, and 19 weeks of age ($n = 5$ –6, each) were anesthetized by intraperitoneal injection of an anesthetic mixture of medetomidine (0.15 mg/kg), midazolam (2.0 mg/kg), and butorphanol (2.5 mg/kg). With the animal's head fixed in a stereotaxic instrument (David Kopf Instrument, Tujunga, California, USA), silver ball-tipped and stainless-steel electrodes were chronically implanted bilaterally into the frontal cortex (approximately 2.0 mm lateral and 2.0 mm rostral to the bregma) and hippocampus (2.0 mm lateral and 3.8 mm caudal to the bregma, and 2.2 mm deep from the cortical surface). A reference electrode was placed on the frontal cranium. The electrodes were then connected to a miniature plug and fixed to the skull with dental cement. After a 1-week recovery period, cortical EEGs were recorded under free-moving conditions with an amplifier (MEG-6108; Nihon Kohden, Tokyo, Japan). The EEG recording was performed two or three times for each animal.

4.11. Effects of AEDs

The effects of AEDs on SWDs were examined as described previously (Tokuda et al., 2007). Briefly, male *Hcn1*-knockout rats ($n = 6$) aged 21–25 weeks were administered ESM (E7138 Sigma-Aldrich Japan K.K., Tokyo, Japan, 125 mg/kg) or PHT (D4505, Sigma-Aldrich Japan K.K., 20 mg/kg). It is known that ESM and PHT are effective in suppressing absence seizure and tonic convulsions, respectively. After a 15-min habituation, an EEG was recorded for 60 min pre- and post-AED administration, respectively.

4.12. Statistical analysis

Data are expressed as means $\pm$ SEM. The statistical significance of the difference between groups was determined by Student's *t*-test with Microsoft Office Excel 2007 (Microsoft Corporation, Redmond, WA, USA). For analysis of multiple groups, the Kruskal-Wallis test followed by the Steel-Dwass post hoc test was conducted with Excel Statistics 2015 software (Social Survey Research Information Co., Ltd., Tokyo, Japan). A *P* value of less than 0.05 was considered statistically significant.

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