



Original

Muscle weakness and impaired motor coordination in hyperpolarization-activated cyclic nucleotide-gated potassium channel 1-deficient rats

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Abstract: Hyperpolarization-activated cyclic nucleotide-gated potassium channel 1 (HCN1) contribute to spontaneous rhythmic activity in different tissues, including the heart and brain. Deficiency in HCN1 function is associated with sick sinus syndrome in mice and epilepsy in humans. We recently developed *Hcn1*-deficient rats and found that they exhibit absence epilepsy. While rearing *Hcn1*-deficient rats, we noticed loose muscle tension and abnormal gait. We therefore evaluated the muscle strength and motor functions of *Hcn1*-deficient rats. When subjected to the wire hang test, *Hcn1*-deficient rats fell down more easily than control F344 rats. Grip strength of *Hcn1*-deficient rats was significantly smaller than F344 rats. In the inclined plane test, they exhibited a smaller maximum angle. In the rotarod test, the latency to fall was shorter for *Hcn1*-deficient rats than F344 rats. In the footprint analysis, *Hcn1*-deficient rats exhibited smaller step length and wider step width than F344 rats. Instead of poor motor coordination ability and muscle weakness, *Hcn1*-deficient rats exhibited normal electromyograms, muscle histology, and deep tendon reflex. These findings suggest that HCN1 channels contribute to motor coordination and muscle strength, and that the muscle weakness of *Hcn1*-deficient rats results from the involvement not of the peripheral but of the central nervous system.

Key words: hyperpolarization-activated cyclic nucleotide-gated potassium channel 1 (HCN1), motor coordination, muscle strength, rat

Introduction

Hyperpolarization-activated cyclic nucleotide-gated potassium channels (HCN) underlie an inward cationic current *I_h*, which is activated by hyperpolarization rather than depolarization [1]. These channels also contribute to spontaneous rhythmic activity in both the heart and brain [22]. HCN1 is one of four subunits (HCN1–4)

that make up HCN channels and mainly expresses in the central nervous system (CNS) [15, 21] and in cardiomyocytes [2]. In *Hcn1*-deficient mice, functional defects have been shown in both the heart and brain. For example, *Hcn1*-deficient mice exhibited profound motor learning and memory deficits in swimming and elevated-speed rotarod tasks [14] and are more susceptible to kainic acid-induced seizures [7]. They also exhibited

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sick sinus syndrome, which is characterized by bradycardia and sinus dysrhythmia [5].

Recently, we developed *Hcn1*-deficient rats by transcription activator-like effector nuclease mutagenesis [12]. Neither *Hcn1* transcript nor HCN1 protein could be detected in the CNS. The cortical and hippocampal pyramidal neurons of these rats displayed a significant reduction in *I_h*, a pronounced hyperpolarizing shift of the resting membrane potential, and increased input resistance. Thus, the functions of HCN1 channels are lost in *Hcn1*-deficient rats. *Hcn1*-deficient rats are more susceptible to pentylenetetrazol-induced seizures, which suggests that CNS neurons are activated more easily. *Hcn1*-deficient rats also exhibited spontaneous absence seizures that were accompanied by spike-and-wave discharges (SWDs) and behavioral arrest. These findings indicated that a deficiency in HCN1 function caused absence epilepsy in rats [12].

In this study, we evaluate the muscle strength and motor functions of *Hcn1*-deficient rats, because they exhibited abnormal gait.

Materials and Methods

Ethical use of animals

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

Animals

Male *Hcn1*-knockout rats (F344-*Hcn1*^{em1Ky}) with body weights between 250–350 g at 12–15 weeks of age were obtained from the National Bio Resource Project for the Rat (Kyoto, Japan). Male F344/NSlc rats (250–350 g) at 12–15 weeks of age were purchased from Japan SLC, Inc. (Shizuoka, Japan) and were used as control animals.

Wire hang test

A rope was stretched horizontally 50 cm above floor level and each rat was allowed to grasp the rope with its forelimbs. Body positions were examined with a six-point scale system: 0, falling off; 1, hanging on the rope by two forelimbs; 2, same as 1 but attempting to climb on the rope; 3, same as 1 plus one or both hind limbs; 4, same as 3 plus tail wrapped around the rope; and 5, escaping. Latency to fall was also measured. The test was performed three times for each animal. The maximum score and duration achieved among the three trials was recorded.

Grip strength test

The grip strength was measured with a digital force gauge (A&D Co., Ltd., Tokyo, Japan) attached to a piece of grid. Each rat was held by the base of its tail and allowed to grasp the grid with its forelimbs. The rat was slowly moved backwards until it released the grid. The test was performed three times for each animal and the greatest force (N) among the three trials was recorded.

Inclined plane test

The inclined plane test was performed using a sliding apparatus described by Rivlin and Tator [19]. Each rat was placed on a sliding plate covered with cardboard. The angle of the sliding plate was gradually increased by one degree per two seconds. The maximum angle was determined at the moment when the rat could no longer support its body position. The test was performed five times for each animal. The greatest angle measured among the five trials was recorded.

Balance beam test

The balance beam test was performed as described previously with slight modifications [26]. Four 1.2-m-long wooden planks of different widths (4.5 cm, 3.0 cm, 2.0 cm, 1.5 cm) were elevated about 30 cm above floor level. We placed rats on the end of the plank and put them through the test three times for each plank width. The narrowest plank on which each rat could walk from end to end without falling was recorded and scores were assigned as follows: 1 for 4.5 cm, 2 for 3.0 cm, 3 for 2.0 cm, and 4 for 1.5 cm.

Rotarod test

The rotarod test was performed with a rotarod apparatus (O'hara & Co., Ltd., Tokyo, Japan). Before the test, rats were trained to walk on the rotating rod at 5 rpm. Thirty min after the training, rats were tested on the rotating rod at 10 rpm fixed speed for 300 s. If rats fell down before 300 s, the time was recorded. The test was performed three times and the maximum time among the three trials was recorded.

Footprint analysis

Rats were placed in a 9-cm-wide and 100-cm-long corridor of which the floor was covered with white absorbing paper. Rats were first trained to explore the corridor before their hind paws were dipped in ink. Two trials were performed for each animal. Distances between two subsequent feet on *y*-axis (length) and on *x*-axis (width) were measured [25].

Electromyogram (EMG)

EMG was recorded from the *musculus gastrocnemius* and *musculus triceps brachii*. Rats were anesthetized with isoflurane to permit EMG electrode implantation. Thirty-gauge concentric needle electrodes (Ambu, Copenhagen, Denmark) were implanted into the muscle. Twenty-seven-gauge needles were inserted subcutaneously on the back and used as ground electrodes. After recovery from anesthesia, EMG activity was recorded. The knee was bent to record the gastrocnemius muscular EMG. The forelimb was stretched to record the triceps brachii muscular EMG. EMG activity was amplified (MEG-6108; Nihonkohden, Tokyo, Japan) and observed with a thermal alley recorder (RTA-1100; Nihonkohden). The recorded signals were analyzed using PowerLab ML845 software (AD Instruments, Bella Vista, Austria).

Histology

Histological analysis was performed as described previously [13]. The *musculus gastrocnemius* and *musculus triceps brachii* were collected from 12-week-old *Hcn1*-deficient and F344 rats. Tissue samples were fixed in 10% neutral-buffered formalin and embedded in paraffin. Sections were cut at 4 μm and stained with hematoxylin and eosin.

Deep tendon reflex test

Rats were postured in the supine position. The knee was bent to 90 degrees and the ankle was kept in the neutral position. Deep tendon reflex was evoked by tapping the tendon of the tibias anterior muscle with a wooden pencil hammer. Deep tendon reflexes were scored as previously described [18]: 0 for no reflex, 1 for somewhat diminished, 2 for normal reflex, 3 for brisker than average, and 4 for hyperactive, associated with clonus.

Statistical analysis

Data were expressed as mean \pm SEM. Statistical significance of differences between groups was determined by Student's *t*-test with Microsoft Office Excel 2007 (Microsoft Corp., Redmond, WA, USA). In the wire hang score test and balance beam test, Wilcoxon rank sum test was conducted with exactRanktests package of R3.3.3 (R project Contributors) [24]. In the deep tendon reflex test, Welch's two sample *t*-test was conducted with R3.3.3. A *P* value less than 0.05 was considered statistically significant.

Results

Wire hang, grip strength, and inclined plane tests

To assess muscle strength, we performed wire hang and grip strength tests. *Hcn1*-deficient rats were unable to wrap their tails around the rope and fell down more easily from the rope than control F344 rats. The average maximum score and latency to fall of *Hcn1*-deficient rats ($n=6$) were significantly smaller than those of F344 rats ($n=6$): score, 2.67 ± 0.422 vs. 4.00 ± 0.00 , $P=0.0152$; latency, 30.3 ± 8.83 vs. 54.7 ± 4.13 , $P=0.0317$ (Fig. 1A). The average maximum grip strength of the forepaws of *Hcn1*-deficient rats ($n=10$) was significantly smaller than that of F344 rats ($n=10$): 3.92 ± 0.0909 N vs. 4.56 ± 0.194 N, $P=0.0125$ (Fig. 1B). In the inclined plane test, the average maximum angle of *Hcn1*-deficient rats ($n=6$) was significantly smaller than that of F344 rats ($n=6$): 27.2 ± 0.925 degrees vs. 36.5 ± 0.697 degrees, $P<0.001$ (Fig. 1C).

Balance beam and rotarod tests

To assess motor ability, we performed balance beam and rotarod tests. In the balance beam test, F344 rats could walk across a 1.5-cm plank, while *Hcn1*-deficient rats often could not walk the same plank. The average maximum score of *Hcn1*-deficient rats ($n=6$), however, did not differ significantly from that of F344 rats ($n=6$): 3.17 ± 0.307 vs. 4.00 ± 0.00 , $P=0.0606$ (Fig. 2A). In rotarod test, rats were tested on a rotarod at a constant speed of 10 rpm. The average maximum latency to fall of *Hcn1*-deficient rats ($n=6$) was significantly shorter than F344 rats ($n=6$): 36.8 ± 7.96 s vs. 182 ± 27.8 s, $P<0.001$ (Fig. 2B).

Footprint analysis

Footprint analysis showed smaller step length and wider step width in *Hcn1*-deficient rats ($n=9$) compared with F344 rats ($n=9$): length, 7.00 ± 0.107 cm vs. 7.64 ± 0.154 cm, $P<0.001$; width, 4.73 ± 0.136 cm vs. 3.94 ± 0.0911 cm, $P<0.001$ (Fig. 2C).

EMG, histology, and deep tendon reflex

We performed EMG and histological analyses on *Hcn1*-deficient rats as they exhibited weaker muscle strength. *Hcn1*-deficient rats exhibited similar EMGs as F344 rats (Fig. 3A). Maximum action potentials were not significantly different in the *musculus gastrocnemius* and *triceps brachii* between *Hcn1*-deficient rats ($n=5$) and F344 rats ($n=5$). For the *musculus gastrocnemius*, 0.116 ± 0.00850 vs. 0.104 ± 0.00565 , $P=0.277$; for the *musculus triceps brachii*, 0.108 ± 0.00217 vs. 0.105 ± 0.00352 , $P=0.511$ (Fig. 3B).

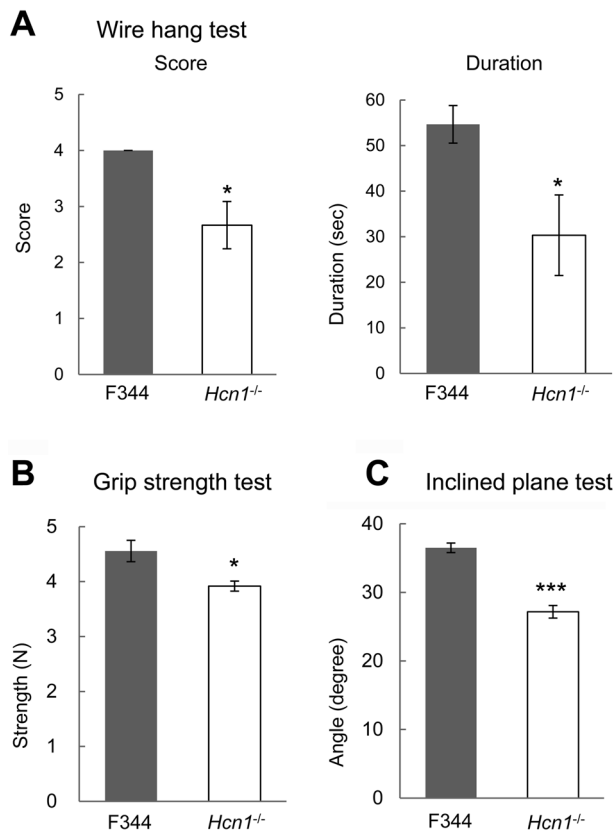


Fig. 1. *Hcn1*-deficient rats exhibited weaker muscle strength. A: Wire hang test (n=6, each). B: Grip strength test (n=6, each). C: Inclined plane test (n=6, each). Results for *Hcn1*-deficient rats are shown as white bars, while those for F344 rats are shown as black bars. *: $P < 0.05$, ***: $P < 0.001$.

In terms of histopathology, *Hcn1*-deficient rats exhibited no apparent abnormal alternation in the muscles (Fig. 3C). *Hcn1*-deficient rats (n=5) exhibited normal or symmetrical deep tendon reflexes similar with F344 rats (n=5): 2.0 ± 0.0 vs. 2.2 ± 0.4 , $P = 0.37$ (Fig. 4).

Discussion

In this study, we evaluated the muscle strength and motor functions of *Hcn1*-deficient rats. The behavioral tests that we performed revealed apparent muscle weakness and defects in motor coordination and balance in *Hcn1*-deficient rats. Instead of muscle weakness, however, we observed that *Hcn1*-deficient rats exhibited normal EMG, muscle histology, and deep tendon reflex.

Hcn1-deficient rats exhibited a smaller score in the wire hang and grip strength tests, in addition to lower posture maintenance compared with control rats in the inclined plane test. The wire hang and grip strength tests measure forelimb strength and coordination in small rodents [20, 23], while the inclined plane test determines limb motor function of laboratory animals [4]. Our findings therefore indicate that *Hcn1*-deficient rats suffer from

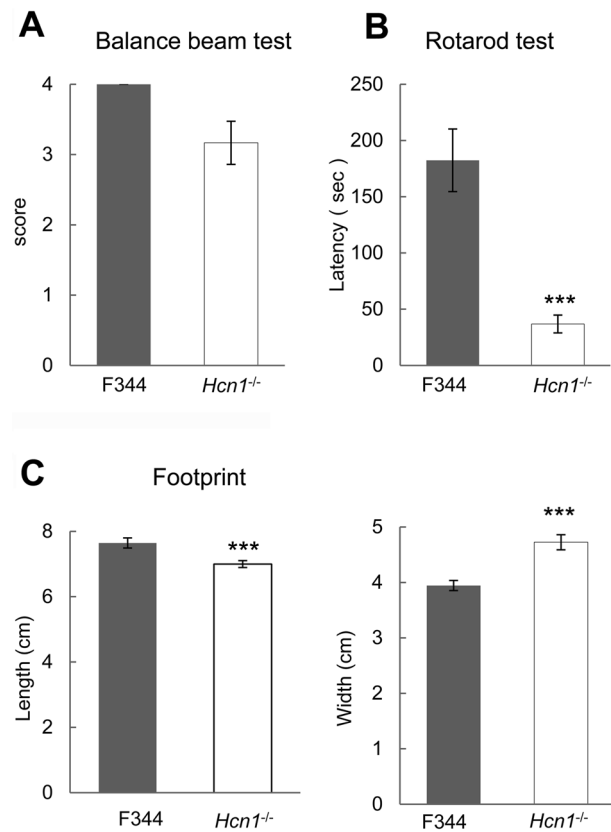


Fig. 2. *Hcn1*-deficient rats exhibited impaired motor ability. A: Balance beam test (n=6, each). B: *Hcn1*-deficient rats exhibited shorter latency to fall from the rod than F344 rats (n=6, each). C: Footprint analysis. Results for *Hcn1*-deficient rats are shown as white bars, while those for F344 rats are shown as black bars (n=9, each). ***: $P < 0.001$.

reduced muscle strength and defects in motor function.

The balance beam and rotarod tests, as well as footprint analysis, are well established and widely used protocols for measuring motor coordination and balance in mice and rats [3]. Although *Hcn1*-deficient rats exhibited similar scores to control rats in the balance beam test, they demonstrated significantly lower performance in the rotarod test. Our footprint analysis revealed abnormal gait i.e. shorter step length and wider step width in *Hcn1*-deficient rats. These findings indicated that *Hcn1*-deficient rats had defects in motor coordination and balance and suggested that HCN1 channels contribute to muscle strength and motor coordination and balance.

Loose muscle tension, decreased muscle strength, and abnormal gait i.e. short step length and wide step width suggest that *Hcn1*-deficient rats suffer from hypotonia, a symptom of diminished skeletal muscle tone associated with decreased resistance of the muscles to passive stretching [10]. Hypotonia can be caused by abnormalities of the CNS, known as central hypotonia, or any element of the lower motoneuron, known as peripheral

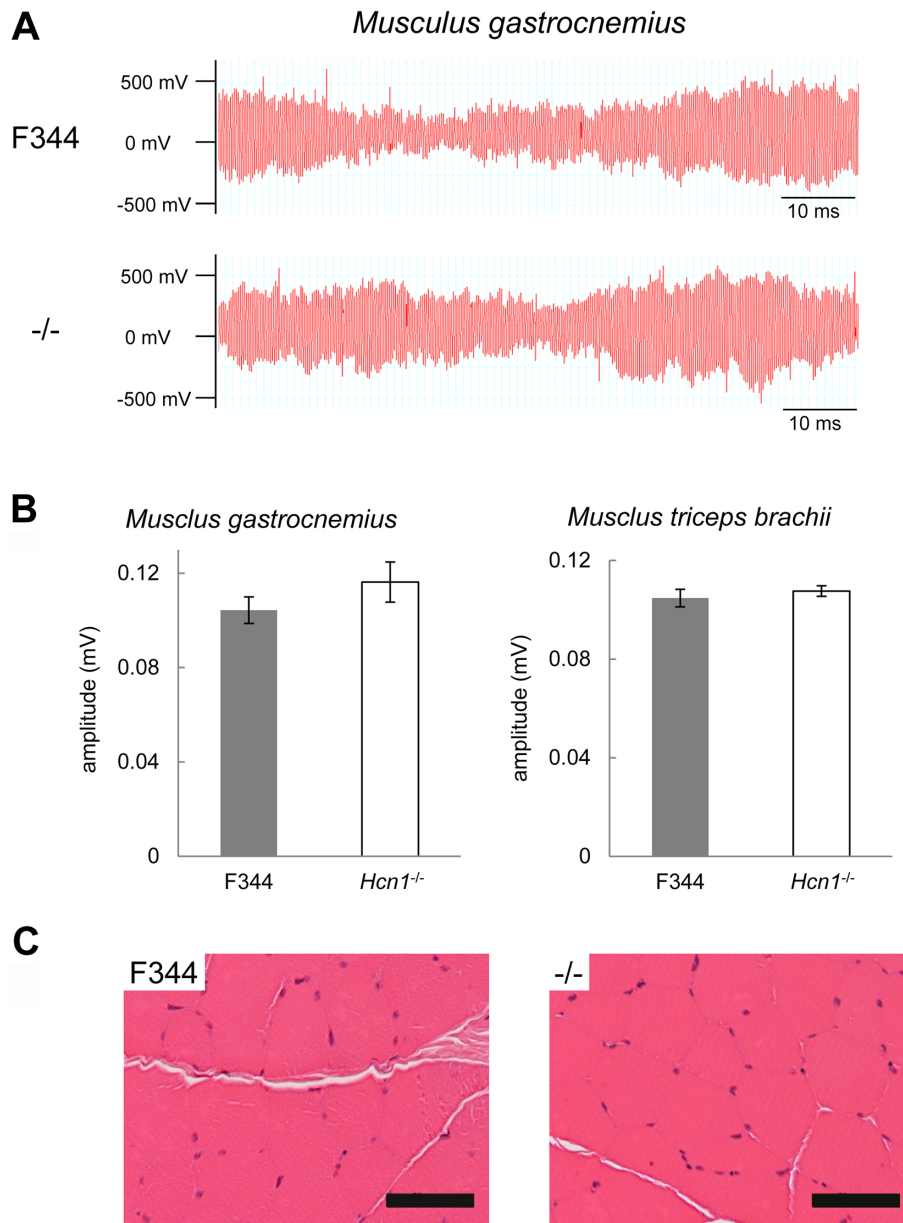


Fig. 3. Electromyogram and histology of muscles of *Hcn1*-deficient rats. **A:** Electromyograms of the musculus gastrocnemius of F344 (upper) and *Hcn1*-deficient rats (lower). **B:** Maximum action potential in the musculus gastrocnemius (left) and the musculus triceps brachii (right). Filled and open bars represent F344 (n=5) and *Hcn1*-deficient rats (n=5), respectively. **C:** Histology of the musculus triceps brachii in F344 (left) and *Hcn1*-deficient rats (right). Bar: 50 μ m.

hypotonia [6, 9, 17]. There are several clinical tests to differentiate central from peripheral hypotonia. Among these tests, the deep tendon reflex test is simple but critical. The deep tendon reflex is absent in peripheral hypotonia, but is not noticeably affected in central hypotonia [6]. *Hcn1*-deficient rats exhibited normal deep tendon reflexes similar to those of F344 rats. In addition, they exhibited normal EMG and muscle histology. We therefore considered the hypotonia observed in *Hcn1*-deficient rats to be central hypotonia.

Central hypotonia is associated with a lack of the cer-

ebellum's facilitatory efferent influence on the fusimotor system that innervates intrafusal muscle fibers [16]. HCN1 channels are expressed in cerebellar Purkinje cells and are thus the major determinant of *I_h* in these cells [14]. In addition, HCN1 channels are also expressed in the spinal cord and the medulla oblongata [11]. Given the important roles of the cerebellum and spinal cord in the neuromuscular system [8], it is likely that HCN1 channel dysfunction in these tissues may be involved largely in the observed hypotonia of *Hcn1*-deficient rats.

Hcn1-deficient rats exhibit absence epilepsy from 10

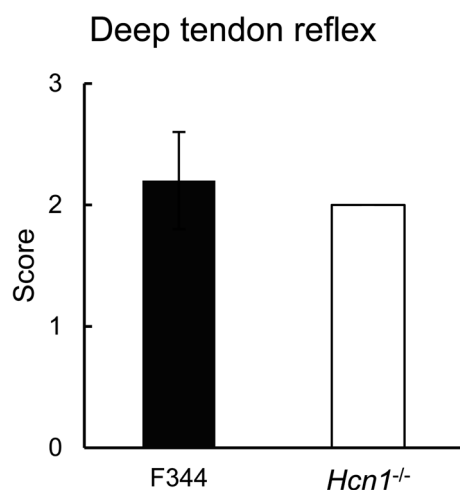


Fig. 4. *Hcn1*-deficient rats exhibited normal deep tendon reflexes similar with F344 rats. Average scores of deep tendon reflexes. Filled and open bars represent F344 (n=5) and *Hcn1*-deficient rats (n=5), respectively.

weeks of age. The average number of SWDs, a hallmark of absence epilepsy, was 5.2 and the average duration of SWDs was 2.1 s during the 1 h observation period at this age [12]. As the *Hcn1*-deficient rats used in this study were around 9–14 weeks of age, they were likely to exhibit absence seizures. Although there is the possibility that the absence seizures are associated with muscle weakness, the relatively short cumulative duration of the SWD i.e. 10.9 s/h in *Hcn1*-deficient rats suggests HCN1 channel dysfunction contributes to the muscle weakness without depending on absence seizures.

In summary, *Hcn1*-deficient rats exhibit muscle weakness and defects in motor coordination and balance. As they exhibit normal deep tendon reflex, the cause of their muscle weakness appears to originate from the CNS. The muscle weakness itself may cause the motor coordination defects observed in *Hcn1*-deficient rats.

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