Scn1a and Cacna1a mutations mutually alter their original phenotypes in rats

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A B S T R A C T

This study aimed to examine the effects of Cacna1a mutation on the phenotype of Scn1a-associated epilepsy in rats. We used rats with an N1417H missense mutation in the Scn1a gene and others with an M251K mutation in the Cacna1a gene. Scn1a/Cacna1a double mutant rats were generated by mating both Scn1a and Cacna1a mutants. We investigated general health and the epileptic phenotype in all these genotypes. The onset threshold of hyperthermia-induced seizures was examined at 5 weeks and spontaneous seizures were monitored using video-EEG recordings from 6 to 12 weeks of age. Scn1a/Cacna1a double mutants showed significantly reduced threshold for hyperthermia-sensitive seizures onset compared with the Scn1a mutants and had absence seizures having 6–7 c/s spike-wave bursts with changes in the spike-wave pattern, whereas Cacna1a mutants had regular 6–7 c/s spike-wave bursts. In Scn1a/Cacna1a double mutants, 6–7 c/s spike-wave bursts were accompanied with eyelid myoclonia and continuously shifting generalized clonic seizures, which were not observed in either Scn1a or Cacna1a mutants. Although a curvature of the spine was observed in rats of all these genotypes, the degree of curvature was more pronounced in Scn1a/Cacna1a double mutants, followed by Cacna1a and Scn1a mutants. Our results indicate that Cacna1a and Scn1a mutations mutually alter their original phenotypes in rats. The phenotype of absence seizures with eyelid myoclonia, generalized clonic seizures, and of spine curvature in the Scn1a/Cacna1a double mutants were similar to that observed in patients with Dravet syndrome.

1. Introduction

Mutations in the SCN1A gene, which encodes the voltage-gated sodium channel Na1,1, cause a spectrum of epileptic disorders ranging from a benign form of genetic epilepsy with febrile seizure plus (GEFS+) to an intractable form of Dravet syndrome (Catterall et al., 2010; Oliva et al., 2012). The type of SCN1A and mosaic variants impact epileptic severity (Zuberi et al., 2011; de Lange et al., 2018). Frameshift, nonsense, and nonfunctional missense mutations have mostly been reported in patients with Dravet syndrome, while missense mutations with residual function of the protein have often been reported in GEFS+ patients (Harkin et al., 2007). However, it remains unclear as to why similar pathogenic SCN1A mutations cause a spectrum of phenotypes. One explanation is the presence of genetic modifiers, which are a common feature of monogenic diseases with variable clinical phenotypes among individuals with the same mutation. Indeed, previous studies have investigated genetic modifiers to better understand the underlying mechanism and severity prediction of GEFS+ and Dravet syndrome (Singh et al., 2009; Gaily et al., 2013; Ohmori et al., 2008, 2013; Hammer et al., 2017).

Abbreviations: SCN1A, sodium voltage-gated channel alpha subunit 1; CACNA1A, calcium voltage-gated channel subunit alpha1 A; GABA, gamma-aminobutyric acid; GEFS+, genetic epilepsy with febrile seizure plus; SCN8A, sodium voltage-gated channel alpha subunit 2; POLG, DNA polymerase gamma, catalytic subunit; CACNB4, calcium voltage-gated channel auxiliary subunit beta 4; KCNQ2, potassium voltage-gated channel subfamily Q member 2; EEG, electroencephalogram; CACNA1G, calcium voltage-gated channel subunit alpha1 G; CT, computed tomography; KO, knockout.

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2. Materials and methods

2.1. Animals

We used F344/NSc-Lcn1a Kyo811 rats with a homozygous N1417H missense mutation in the Scn1a gene, henceforth referred to as Scn1a mutant rats, and GRY/Idr rats with a homozygous M251K mutation in the Cav2.1 gene, henceforth referred to as Cav2.1 mutant rats. N1417H-Scn1a and M251K-Cacna1a mutations are found in the pore regions of voltage-gated channels, which are highly selective for permeant cations such as sodium and calcium. Pore-missense mutations have been linked to human channelopathies due to alternation in electrophysiological properties (Catterall et al., 2010, Simms and Zamponi, 2014). The original strains of Scn1a and Cacna1a mutant rats were F344-NSc and Wistar, respectively. Cacna1a mutant rats were repeatedly crossed with wild type (WT) F344/NSc rats (Nippon SLC, Hamamatsu, Japan) for more than 15 generations to minimize the impact of strain-specific genetic differences on the phenotype. After backcrossing Cacna1a mutant Wistar rats to WT F344/NSc rats, the resultant heterozygous M251K-Cacna1a mutant offspring were mated with homozygous Scn1a mutant rats to generate Scn1a/Cacna1a double mutant rats. Intercrossing these double mutant rats generated offspring carrying the homozygous N1417H-Scn1a and homozygous M251K-Cacna1a mutations.

Scn1a mutant rats exhibited susceptibility to hyperthermia-induced seizures (Mashimo et al., 2010). Hippocampal neurons of Scn1a mutant rats demonstrated impaired biophysical properties of inhibitory GABAergic neurons (Mashimo et al., 2010). Cacna1a mutant rats exhibited ataxia and absence seizures from 6 weeks of age (Tokuda et al., 2007). In acutely dissociated Purkinje cells of Cacna1a mutant rats, the high-voltage-activated Ca2+ channel, Cav2.1, showed an increased current density and a depolarizing shift in the activation and inactivation curves compared with those of WT rats (Tanaka et al., 2007).

In this study, we examined four groups: WT rats, Scn1a mutant rats with a homozygous N1417H mutation (Scn1a mutants), Cacna1a mutant rats with a homozygous M251K mutation (Cacna1a mutants), and double mutant rats with a double homozygous N1417H-Scn1a and M251K-Cacna1a mutation (Scn1a/Cacna1a double mutants).

Rats were maintained under standard laboratory conditions with a 12-h light/dark cycle and food and water ad libitum. Animal experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of Okayama University. The 3R (replacement, reduction, and refinement) concept was considered when planning the experiments.

2.2. Genotyping

At 3 weeks of age, DNA was extracted from the tail snips with DNeasy Blood & Tissue kit (Qiagen). The fragment of interest with the mutation was amplified with a pair of primers as follows: Scn1a-sense 5′-TGACCTTCTCTTCTTCGGTGG-3′ and Scn1a-antisense 5′-TGGGTGAAAAATCATCCTTGTGTT-3′ for the N1417H-Scn1a mutation and Cacna1a-sense 5′-TCTCTGTTTCGAGGTTAC-3′ and Cacna1a-antisense 5′-GTTGCTAAAGACACAGGTTGC-3′ for the M251K-Cacna1a mutation. Resultant PCR products (380 bp for Scn1a and 350 bp for Cacna1a) were digested with BclI and PsI restriction enzymes, respectively. The genotype was determined using the size of the digested fragments on a 3% agarose gel. Scn1a fragment of 380 bp was divided into two fragments of 276 bp and 104 bp if it had a mutation. Cacna1a fragment of 350 bp was divided into two fragments of 220 bp and 130 bp if it had a mutation (Supplementary Fig. 1).

2.3. General health

Body weight was measured at 6, 8, 10, and 12 weeks of age in WT controls, Scn1a mutants, Cacna1a mutants, and double mutant rats. An X-ray examination (MBR-1520R-3, HITACHI) and a computed tomography (CT) scan (LaTheta LCT-200, HITACHI ALOKA) of the skeletal structure was performed in 9-month old rats (two rats per genotype) under pentobarbital sodium anesthesia or vaporized isoflurane.

2.4. Video-electroencephalogram recordings

The rats were implanted with electrodes for electroencephalogram (EEG) recordings as previously described (Hayashi et al., 2011). Following a recovery period of at least 1 week, hyperthermia-induced seizures, absence seizures, and spontaneous seizures were recorded through a video-EEG (Neurofax EEG-1200, Nihon Kohden).

2.5. Hyperthermia-induced seizures

We evaluated hyperthermia-induced seizures in Scn1a mutants and Scn1a/Cacna1a double mutants to compare and examine the effects of the additional Cacna1a mutation in Scn1a mutant rats. Hyperthermia-induced seizures were induced by placing rats in a water bath filled with hot water at 45 °C as described previously (Hayashi et al., 2011).

2.6. Absence seizures

The total duration of spike-wave discharges were assessed for 60 min in awake EEG recordings and in a single seizure duration at 6, 8, 10, and 12 weeks of age. Based on the absence seizures’ frequency in these rats, a 60-min duration was deemed appropriate to evaluate the associated severity. Number of rats used per group is shown in supplementary table 1.

2.7. Spontaneous seizures other than absence seizures

For assessment of spontaneous seizures in the three mutant genotypes (Scn1a mutants, Cacna1a mutants, and double mutant rats), long-term video-EEG recordings were collected in 13–15 h overnight sessions (regularly from 6:00 p.m. to 9:00 a.m.), once every 2 weeks for up to 12 weeks of age (Supplementary Table 1).

2.8. Statistical analysis

All data are presented as the mean ± SD (standard deviation) and
reported as significant at \( P < 0.05 \). Data were compared using a paired \( t \)-test.

3. Results

3.1. General health and skeletal abnormality

The growth curve from 6 to 12 weeks of age for each genotype is shown in Fig. 1A. \( Cacna1a \) mutants and \( Scn1a/Cacna1a \) double mutants exhibited diminished growth with a body weight significantly lower than that of WT rats \( ( P < 0.001) \). There was no significant difference in body weight between WT rats and \( Scn1a \) mutants \( ( P = 0.16) \). \( Cacna1a \) mutants and \( Scn1a/Cacna1a \) double mutants showed different neurological phenotypes including hypotonia, wide based hindlimb position, and ataxic gait. Since these two groups were inactive during an experimental apparatus, no behavioral evaluation was conducted.

During the breeding of experimental rats, we observed an excessive spinal curvature in older \( Cacna1a \) mutants; therefore, we examined skeletal abnormalities in 9-month old rats using X-ray and CT scan. \( Cacna1a \) mutants and \( Scn1a/Cacna1a \) double mutants exhibited excessive spinal curvature between the cervical and thoracic vertebrae (Fig. 1B and C). \( Scn1a \) mutants showed a mild spinal curvature. \( Scn1a/Cacna1a \) double mutants tended to have a more severe spinal curvature compared to \( Cacna1a \) mutants.

3.2. Impact of Cacna1a mutation on hyperthermia-induced seizures

\( Scn1a \) mutants exhibit susceptibility to hyperthermia-induced seizure. WT and \( Cacna1a \) mutants did not develop hyperthermia-induced seizures in this study. To evaluate the possible impact of \( Cacna1a \) mutation on hyperthermia-induced seizures, we compared the latency to seizure onset, rectal temperature at seizure onset, and seizure duration between \( Scn1a \) mutants and \( Scn1a/Cacna1a \) double mutants. The time interval until a seizure occurred after placing a double mutant rat in hot water \( (45^\circ C) \) was significantly shorter than that observed for \( Scn1a \) mutants (Fig. 2A; \( Scn1a \) mutants, \( 222.6 \pm 5.3 \) s vs. \( Scn1a/Cacna1a \) double mutants, \( 146.0 \pm 17.5 \) s, \( P < 0.01 \)). The rectal temperature at the time of seizure in double mutants rats was significantly lower than that in \( Scn1a \) mutants (Fig. 2B; \( Scn1a \) mutant rats, \( 43.4 \pm 0.07^\circ C \) vs. \( Scn1a/Cacna1a \) double mutant rats, \( 42.4 \pm 0.19^\circ C \), \( P < 0.01 \)). These results indicate that the threshold of hyperthermia-induced seizure onset in \( Scn1a/Cacna1a \) double mutant rats is lower than that in \( Scn1a \) mutants. Seizure duration was longer in \( Scn1a/Cacna1a \) double mutants compared to \( Scn1a \) mutants, though statistically insignificant (Fig. 2C; \( Scn1a \) mutants, \( 64.7 \pm 8.0 \) s vs. \( Scn1a/Cacna1a \) double mutants, \( 82.6 \pm 17.4 \) s, \( P = 0.42 \)). The generalized tonic-clonic seizure and EEG patterns were similar in the two groups (Fig. 2D and E).

3.3. Impact of Scn1a mutation on absence seizures caused by Cacna1a mutation

We recorded EEGs for 60 min in \( Cacna1a \) mutants and \( Scn1a/Cacna1a \) double mutants at 6, 8, 10, and 12 weeks of age. We previously demonstrated that \( Scn1a \) mutants had no absence seizures by using a long-term video-EEG monitoring, (Ohmori et al., 2014).

The total duration of absence seizure between the two groups was not significantly different (Fig. 3A), whereas differences in ictal EEG patterns of absence seizure were observed. At 6 weeks of age, 6–7 c/s (cycles per second) spike-and-wave bursts were regular in both groups (Fig. 3B and C). At 10 weeks of age, the ictal EEG pattern in \( Cacna1a \) mutants was the same as that at 6 weeks (Fig. 3D), while patterns in \( Scn1a/Cacna1a \) double mutants often exhibited a change from the regular 6–7 c/s spike-wave bursts to a gradually slowing pattern in the latter half, as shown in Fig. 3E.

3.4. New type of seizures in Scn1a/Cacna1a double mutant rats

\( Scn1a/Cacna1a \) double mutants exhibited new types of spontaneous seizures that have never been observed in either \( Scn1a \) or \( Cacna1a \) single mutants. In double mutants, absence seizures were accompanied with myoclonic components involving the eyelids and limbs (Fig. 4A, Video 1). Some spike-and-wave bursts sequentially developed to generalized clonic seizures (Fig. 4B, Video 2). Myoclonic jerks accompanied with polyspikes on EEGs were also detected (Fig. 4C, Video 3). Generalized,

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**Fig. 1.** Comparison of growth curves and skeletal malformations. The body weight of rats (WT, \( n = 10 \); \( Scn1a \) mutants, \( n = 10 \); \( Cacna1a \) mutants, \( n = 12 \); \( Scn1a/Cacna1a \) double mutants, \( n = 10 \) ) was measured from 6 weeks to 12 weeks of age. The skeletal structure was evaluated by X-ray examination and a computed tomography scan in 9-month old rats (\( n = 2 \) per genotype); ** \( P < 0.01 \).
Fig. 2. The threshold of hyperthermia-induced seizures is lowered in Scn1a/Cacna1a double mutants. (A) The time to onset of a seizure after placing rats in hot water (45° C). (B) Rectal temperature at the onset of hyperthermia-induced seizure and (C) the seizure duration. Scn1a mutants, n = 10; double mutant, n = 13; **P < 0.01. (D) and (E) demonstrate representative EEG patterns of hyperthermia-induced seizures in Scn1a mutants and in double mutant rats, respectively.

Fig. 3. The pattern of spike-and-wave discharges in Scn1a/Cacna1a double mutants is altered. (A) The total seizure duration was measured for 60 min in Cacna1a mutants and Scn1a/Cacna1a double mutants at 6, 8, 10, and 12 weeks of age. A representative EEG pattern of absence seizure showed regular spike-and-wave discharges in Cacna1a mutants (B) and in Scn1a/Cacna1a double mutants at 6 weeks of age (C); and in Cacna1a mutants at 10 weeks of age (D). (E) The ictal EEG pattern of Scn1a/Cacna1a double mutants at 10 weeks of age showed slowing cycles of spike-and-wave discharges. Black dots indicate regular cycles of spike-and-wave discharges. Red dots indicate slowing cycles of spike-and-wave discharges.
sudden, clonic seizures were observed twice at 12 weeks of age (Fig. 4D, Video 4). The seizure types observed for each genotype are summarized in Table 1.

![Fig. 4. Ictal EEG of spontaneous seizures in double mutant rats. (A) The rat was motionless with half-closed eyelids accompanied by a 6 c/s (cycles per second) spike-wave discharges on an EEG. Blinking and myoclonic jerks appeared in the latter half of the seizures (black border). (B) The 6 c/s spike-and-wave discharges began to appear during sleep. These discharges continuously developed big spikes with a slower cycle. A jerk of the head appeared at the time-point indicated by the arrow, followed by generalized clonic seizures. The rat stood up and thrashed its forelimbs, while opening and shutting the mouth, like oral automatism. (C) Myoclonic seizures and (D) generalized clonic seizures appeared at 12 weeks of age. Sleeping rats had a jerk accompanied by polyspikes on an EEG (C). The sleeping rat suddenly had a jerk of the head followed by clonic movements of the forelimbs. An ictal EEG showed a burst of spikes and big sharp waves with gradually increasing amplitude.](https://doi.org/10.1016/j.neuint.2020.104859)

Table 1

| Type of seizures observed in each genotype | Hyperthermia-induced seizure | Absence seizure | Absence seizure with myoclonic components | Myoclonic-like seizure | Spontaneous clonic seizure |
|-------------------------------------------|-----------------------------|----------------|------------------------------------------|------------------------|---------------------------|
| Scn1a mutants                            | +                           | none           | none                                     | none                   | none                      |
| Cacna1a mutants                           | none                        | none           | none                                     | none                   | none                      |
| Scn1a/Cacna1a double mutants              | ++                          | +              | +                                        | +                      | +                         |

Supplementary video related to this article can be found at [https://doi.org/10.1016/j.neuint.2020.104859](https://doi.org/10.1016/j.neuint.2020.104859)
4. Discussion

4.1. Hyperthermia-sensitive seizures

In Dravet syndrome, epileptic seizures are easily triggered by fever. It remains unclear as to why Cacna1a mutation lowers the threshold of hyperthermia-sensitivity seizures. The molecular mechanisms linking SCN1A mutations with hyperthermia-sensitive seizures are also largely unknown. The temperature-dependent electrophysical properties of recombiant epilepsy-associated SCN1A missense mutation gene product are different from those for the WT SCN1A gene product (Peters et al., 2016), which partly explains the hyperthermia-sensitivity of mutant SCN1A. However, approximately half SCN1A mutations found in patients with Dravet syndrome are truncating mutations that result in a nonfunctional gene product, while the remaining are missense mutations that often lead to complete loss of function (Catterall et al., 2010; Ohmori et al., 2006). Thus, it is unlikely that only biophysical alterations of the voltage-gated sodium channel Na1.1, encoded by SCN1A, in response to hyperthermia are responsible for the hyperthermia-sensitive seizures in Dravet syndrome. An electrophysiological study using neurons from mutant animals or cells derived from patients with Dravet syndrome may be helpful to understand the mechanism and exacerbation factors of hyperthermia-sensitive seizures.

4.2. Absence seizures with myoclonia and clonic seizures

Some patients with Dravet syndrome exhibit absence seizures with myoclonia (Dravet, 2011). However, to date, absence seizures and myoclonic seizures have not been reported in Dravet syndrome mice models, Scn1a KO mice, or Scn1a mutants (Yu et al., 2006; Ogivara et al., 2007; Ohmori et al., 2014). The absence seizures in double mutant rats are characterized by 6–7 s bursts associated with eyelid myoclonia and a transition to clonic convulsions. To our knowledge, no animal models of absence epilepsy exhibiting these phenomena have been reported to date. Absence seizures in patients with Dravet syndrome are characterized by frequent association of eyelid myoclonia and generalized myoclonia (Dravet, 2011; Tsuda et al., 2013). When the myoclonic component is pronounced, it is difficult to differentiate absence seizures from myoclonic seizures, since both are considered the same epileptic phenomena with different intensity and duration (Dravet, 2011). The characteristics of absence seizures observed in double mutant rats are similar to those observed in patients with Dravet syndrome. A recent study of whole exome sequencing in 87 patients with SCN1A-related epilepsy suggested potential modifying associations in genes such as SCN8A, POLG, SCN2A, CACNA1A, and CACNA1G, in several patients who carried variants in these genes (de Lange et al., 2020). Further studies are warranted to determine whether absence seizures and myoclonic seizures are associated with other modifier genes in patients with Dravet syndrome.

4.3. Orthopedic abnormality

We first observed that Cacna1a mutants began displaying curvature of the spine with increasing age. Although differences in spinal curvature were not significant as measured by X-ray and CT scan, it was visually more pronounced in the Cacna1a mutant group than in the Scn1a mutant group, while the double mutants had maximum spinal curvature. The progressive crouching gait in patients with Dravet syndrome has attracted significant attention (Rilstone et al., 2012; Rodda et al., 2012). Currently, two hypotheses have been proposed to explain the cause of gait disturbance (Kalume et al., 2007; Pasano et al., 2014). The hypothesis that gait disturbance is caused by cerebellar dysfunction is supported by experimental results in Scn1a KO mice (Kalume et al., 2007). Na1.1 channels are expressed in cerebellar Purkinje neurons, which firing rates in Scn1a KO mice are substantially reduced (Kalume et al., 2007). Cav2.1 channels are also expressed on Purkinje cells and play a critical role for neurotransmitter release (Mori et al., 1991). Gait disturbance can be exacerbated by Scn1a and Cacna1a double damage in the sequential excitation process of Purkinje neurons. The alternative hypothesis is that gait disturbance in adults is a symptom of parkinsonism rather than cerebellar ataxia. Treatment with levodopa has been reported to improve parkinsonism and antiepileptic conditions in patients with Dravet syndrome (Pasano et al., 2014). We have previously reported a significantly lower level of dopamine in the striatum in Scn1a mutant rats compared to that in WT (Ohmori et al., 2014), partially supporting the parkinsonism-caused gait disturbance in Dravet syndrome hypothesis.

4.4. Scn1a and Cacna1a share the same physiological pathway

Exacerbation of symptoms linked to Scn1a and Cacna1a mutations may be attributed to the same physiological pathway, since both Na1.1 and Cav2.1 are expressed in parvalbumin-positive GABAergic interneurons (Yu et al., 2006; Ogivara et al., 2007; Jiang et al., 2016) and cerebellar Purkinje cells (Kalume et al., 2007; Mori et al., 1991). A positron emission tomography study using flumazenil, a selective GABA-A receptor ligand, reported GABA-A receptor impairment in patients with CACNA1A mutations (Kono et al., 2014). GABA-A receptor dysfunction in the cerebral cortex was also reported in an animal model with a Cacna1a mutation (Tehrani et al., 1997). A Cacna1a mutation causes a decrease in GABAergic neuronal activity attributed to GABA-A receptor dysfunction. Several candidate modifier genes were reported in a mouse model of Dravet syndrome included genes encoding for GABA-A receptor, voltage-gated calcium channels, and the Na+/K+-ATPase, ATP1A3 (Miller et al., 2014). SCN1A, CACNA1A, and ATP1A3 are causative genes of hemiplegic migraine and cerebellar ataxia (Dichgans et al., 2005; Ducros et al., 1999; Potic et al., 2015; Holm and Lykke-Hartmann, 2016). ATP1A3 is primarily expressed in GABAergic neurons (Böttger et al., 2011). Defective ATPases may cause sodium and potassium ions leakage across the cell membrane, reducing neuronal excitability. These findings suggest that SCN1A, CACNA1A, and ATP1A3A share same physiological function, specifically when expressed in the same type of neurons or neuronal circuit.

5. Conclusions

Herein, we showed that Cacna1a and Scn1a mutations mutually alter the phenotype of single mutant Scn1a and Cacna1a epileptic rats, respectively. The Cacna1a mutation lowers the threshold of hyperthermia-sensitive seizures in Scn1a mutants, while Scn1a mutation adds myoclonic components to absence seizures in Cacna1a mutants. Orthopedic abnormality was observed in Scn1a, Cacna1a, and Scn1a/Cacna1a double mutants, while the degree of spinal curvature was the most pronounced in Scn1a/Cacna1a double mutants. Exacerbation of symptoms linked to Scn1a and Cacna1a mutations may share the same pathway. Using next-generation sequencing, large amounts of genetic data can be easily, and cost effectively, obtained, which will help to better manage epilepsy. Identifying modifier genes, associated with absence seizures or gait disturbance in Dravet syndrome, will help with more accurate prognosis and treatment strategy.

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Declaration of competing interest

The authors report no competing interests in relation to the research covered in the submitted manuscript.
Data availability statement
All data related to this paper are available from the corresponding author.
Scn1a mutant rats and Cacna1a mutant rats are available from the National BioResource Project for Rats in Japan, Kyoto University.

CRediT authorship contribution statement
Iori Ohmori: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.
Kiyoka Kobayashi: Investigation, Data curation, Writing - review & editing.
Mamoru Ouchida: Methodology, Validation, Formal analysis, Investigation, Data curation, Visualization, Writing - review & editing.

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Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuint.2020.104859.

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