Short Communication

Inhalation of 10% carbon dioxide rapidly terminates *Scn1a* mutation-related hyperthermia-induced seizures

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Number of words in summary: 229 words

Number of words in main text: 1731 words

Number of figures: 2

Key words: *SCN1A*, CO_2, Febrile seizure, Dravet syndrome, GEFS+

Introduction

Prolonged febrile seizures (FS) and status epilepticus are the most frequent neurological emergencies in children. Subjects with Dravet syndrome and genetic epilepsy with febrile seizures plus (GEFS+), which are caused by *SCN1A* mutations, often suffer from prolonged febrile seizures (Dravet et al., 2005; Scheffer & Berkovic, 1997). Early termination of prolonged seizures before arriving at the emergency department is associated with better patient outcomes. Therefore, it is important to establish a new therapy that is fast-acting, safe, and consistently achievable for paramedics.

Inhalation of 5% carbon dioxide (CO_2) strongly suppressed experimental FS in rats (Schuchmann et al., 2006). Thus, we investigated the efficacy of inhalation of a mixed gas containing 5% or 10% CO_2 to treat FSs using hyperthermia-induced seizure susceptible (HISS) rats that harbored an *Scn1a* missense mutation (Mashimo et al., 2010).

Materials and Methods

Animals

We used male HISS rats with a homozygous N1417H-*Scn1a* mutation (F344/NSlc-*Scn1a*^{Kyo811}) (National Bio Resource Project for the Rat in Japan, Kyoto University, Kyoto, Japan) (Mashimo et al., 2008) and male wild-type (WT) littermates. The N1417H-*Scn1a* mutation produced a hyperpolarizing shift in voltage-dependent inactivation that results in reduced excitability of the hippocampal GABAergic interneurons in HISS rats. The rats were maintained in standard laboratory conditions with a 12-h light/dark cycle, and food and water were provided *ad libitum*. All experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of Okayama University.

Experimental conditions

Induction of HIS and Electroencephalogram (EEG) recordings:

The methods used for induction of HIS and EEG analysis have been described previously (Hayashi et al., 2011). Briefly, at 4 weeks of age, stainless-steel screw electrodes were placed on the bilateral frontal cortex (AP, +0.5 mm; ML, ±3.0 mm from

bregma) and the bilateral occipital cortex (AP, −7.0 mm; ML, ±3.0 mm from bregma) for recordings and in the posterior end of the skull as a reference electrode. After a 1-week recovery period, HISs were evoked by hot water baths at 45°C. The rats were kept in water for a maximum of 5 min, or until a seizure occurred. The duration of the seizures was measured using EEG (Neurofax EEG-1200, Nihon Koden, Japan).

Inhalation of 5% CO_2 or 10% CO_2

Immediately after the induction of HISs, a rat was placed into a multi-gas incubator (MCO-5M-PJ, Panasonic Corporation, Osaka, Japan) at 28°C for 3 min. The chamber was filled with air or mixed gas, which was composed of 5% CO_2, 20% O_2, and 75% N_2, or 10% CO_2, 20% O_2, and 70% N_2.

Blood gas analysis

One hundred microliters of peripheral blood was collected from a tail vein. The blood gas was analyzed to measure the pH, partial pressure of CO_2 (pCO_2), partial pressure of oxygen (pO_2), and bicarbonate (HCO_3^-) using a blood gas analyzer (i-STAT Blood gas analyzer, Fuso Pharmaceutical Industries, Ltd, Osaka, Japan). To determine any changes in the biological parameters at the time of HISs, the blood gas analysis was performed

under normal conditions and immediately after HIS occurred. For the WT rats, the blood gas was measured at the end of a 5-min hot water bath because they did not experience HIS. To evaluate the efficacy of inhalation of 5% CO_2 or 10% CO_2 for treating HIS, the blood gas was measured after placing the *Scn1a* mutant rat in a chamber filled with air or mixed gas that contained 5% CO_2 or 10% CO_2 for 3 min.

Statistical analysis

The data are presented as mean ± SEM. Significant differences were tested using the unpaired *t*-test to compare two groups. The association between the CO_2 concentration and the anticonvulsant effect was evaluated using a one-way repeated measures ANOVA followed by a post hoc test (Fisher's PLSD). Statistical differences were accepted if p value was < 0.05.

Results

At 5 weeks of age, HISs did not occur in the WT rats (n = 19), whereas all of the *Scn1a* mutant rats (n = 19) experienced HISs within 5 min (Figure 1A, 1B). The rectal temperature at the onset of HISs in *Scn1a* mutant rats was significantly lower than that in the WT rats at the end of the 5-min hot water bath (Figure 1C). A representative EEG

in the WT demonstrated no epileptic events during hot water bathing, whereas HISs (tonic posturing and head nodding with brief twitching movements followed by repetitive myoclonic jerks) were accompanied by diffuse high-voltage spikes and sharp waves in the EEG of the *Scn1a* mutant rats (Figure 1D). The blood gas analysis demonstrated that respiratory alkalosis with a reduction of HCO_3^- levels was induced in both WT and *Scn1a* mutant rats because of hot water bathing (Figure 1E–H). The duration of hot water bathing was shorter for the *Scn1a* mutant rats than that in the WT rats; therefore, a significantly milder induction of respiratory alkalosis was observed in the *Scn1a* mutant rats than in the WT rats (Figure 1E and 1F).

We examined the anticonvulsant effects of inhalation of 5% CO_2 (n = 8) and 10% CO_2 (n = 12). Figures 2A, 2B, and 2C are representative EEGs during treatment with air, 5% CO_2, and 10% CO_2, respectively. Inhalation of 10% CO_2 shortened the duration of repetitive myoclonic jerks. Video data 1 and 2 are representative simultaneous video-EEG recordings during treatment with air and 10% CO_2, respectively. Inhalation of 10% CO_2 markedly shortens the duration of HIS from 62.6 ± 12.1 s to 15.5 ± 1.0 s, whereas no significant anticonvulsant effect on HISs with 5% CO_2 (48.4 ± 15.2 s) was observed (Figure 2D). Blood gas analysis demonstrated that inhalation of 5% or 10% CO_2 resulted in a significant lowering of the pH (Figure 2E), a significant elevation of

the pCO_2 level (Figure 2F), and an elevation of the pO_2 level (Figure 2G) compared to air treatment. However, the pH level of the 5% CO_2 treatment was almost the same as that at physiological level at room temperature.

After the suppression of HISs with the inhalation of 5% or 10% CO_2, the rats moved around normally, and their EEGs exhibited normal activity. Acidosis seemed to have no effect on the rats, except for the anticonvulsant effect.

Discussion

In general, experimental FS are difficult to evoke in rats after 3 weeks of age, therefore they were tested on postnatal days P8–P11. In a widely-used model using a hair dryer, HISs are caused by respiratory alkalosis following hyperventilation and loss of CO_2 in rat pups on postnatal days P8–P11 (Schuchmann et al., 2006). The expression of neuronal sodium channel $Na_v1.1$, which is encoded by *Scn1a*, becomes prominent in rats at approximately 5 weeks of age (Mashimo et al., 2010). FS associated with *SCN1A* mutations in humans persist beyond 6 years of age. On the basis of these factors, we used 5-week-old rats that were older than the usual model rats. Hot water bathing induced a significant reduction in the pCO_2 level, an elevation of the pO_2 level, and

alkalosis. Although a successful induction of respiratory alkalosis in the WT and *Scn1a* mutant rats at 5 weeks of age was observed, the WT rats did not experience HISs, whereas 100% of the *Scn1a* mutant rats experienced HISs. Differences of HIS susceptibility in the *Scn1a* mutant rats may have affected the age-dependent excitability of the brain, which reacts to alkalosis.

Inhalation of 10% CO_2 produced fast-acting and strong suppression of HISs in the *Scn1a* mutant rats at 5 weeks of age, whereas 5% CO_2 demonstrated no significant effects in them. In our experimental protocol, the delay during the transfer of the rat from the bath to the chamber was about 7–9 s; therefore, the estimated period when the HISs stopped after the inhalation of 10% CO_2 was less than 10 s. On the basis of the blood gas data after the end of HISs, respiratory acidosis because of the inhalation of 10% CO_2 might rapidly abolish HISs. However, the pCO_2 level after the 10% CO_2 treatment was still lower than that of the physiological pCO_2 level. This data indicates that partial recovery of pCO_2 may be sufficient to suppress HISs even though it does not reach the physiological level. The anticonvulsant action of CO_2 was demonstrated in epileptic humans over 80 years ago (Lennox, 1928). Recent studies have demonstrated that 5% CO_2 strongly suppressed experimental FS in rats on postnatal days P10–11, myoclonic epilepsy in a rat model, and intractable partial epilepsy in human subjects

(Schuchmann et al., 2006; Tolner et al., 2010). In this study, the differing effects of inhalation of 5% CO_2 may have been due to the insufficient induction of acidosis, the differences in animal models, age, genetic background, or the experimental procedures.

The molecular mechanisms that suppress seizures with the inhalation of CO_2 are largely unknown. The dilation of cerebral vessels with CO_2 or changes in the biophysical properties of *Scn1a* mutant channels against acidosis might contribute to the anticonvulsant action. An *SCN1A* mutation linked to the Dravet syndrome led to a significant reduction in the persistent sodium current with a decrease in intracellular pH (Rhodes et al., 2004).

The effects of antiepileptic drugs on HISs in *Scn1a* mutant rats reflect the results of a study conducted in human subjects with Dravet syndrome and GEFS+ (Hayashi et al., 2011). In our previous study, inhalation of 10% CO_2 exhibited approximately the same effect as diazepam on the termination of HISs. To improve the outcomes for patients with prolonged seizures, paramedics have begun to administer intramuscular or intravenous benzoziazepines to patients before arriving at the emergency departments (Warden et al., 2006; Silbergleit et al., 2012). Subjects with *SCN1A* mutation-related epileptic syndromes often have prolonged febrile seizures. Inhalation of a mixed gas containing CO_2 does not need specific medical training; therefore, not only paramedics

but also the patients' families can administer it. However, safety issues should be addressed before the clinical application of inhalation of CO_2 in epileptic patients. It is notable that 10% CO_2 rapidly terminates HISs in *Scn1a* mutant rats, which may lead to the development of a new therapy to treat *SCN1A* mutation-related epileptic syndromes.

Acknowledgments

We are grateful to the National BioResource Project for the Rat in Japan (http://www.anim.med.kyoto-u.ac.jp/NBR/) for providing rat strains (F344/NSlc-*Scn1a*^{Kyo811}).

Role of the funding source

This work was supported by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology (No. 21390312 and No. 23659522 to I.O. and No. 22591130 to T.O. and I.O.).

Figure legends

Figure 1. Induction of HIS and changes in the blood gas parameters.

A: WT rats (n = 19) did not experience HISs, whereas 100% of the homozygous *Scn1a* mutants (n = 19) experienced HISs B: Latency occurred before HIS. Hot water bathing was stopped after 300 s in the WT rats because HISs were not evoked. The *Scn1a* mutant rats experienced HIS at 224.1 ± 11.3 s C: The rectal temperature in the WT rats at the end of the hot water bath and at the induction of HISs in the *Scn1a* mutant rats D: Representative EEGs in the WT rats during hot water bathing and ictal EEG in the *Scn1a* mutant rats E–H: Blood gas analysis of the peripheral blood taken from a tail vein under normal conditions and immediately after 3 min of hot water bathing in WT (n = 12) and when HISs occurred in the *Scn1a* mutant rats (n = 12) E: pH F: pCO_2 G: pO_2 H: HCO_3^- $^{**}p < 0.001$, $^{*}p < 0.01$

Figure 2. The anticonvulsant effect of inhalation of 5% CO_2 or 10% CO_2 Immediately after the induction of HIS, a rat was placed in a multi-gas incubator filled with air (n = 12), 5% CO2 (n = 8), or 10% CO2 (n = 12) for 3 min. The blood gas parameters were then analyzed. Representative EEGs when the rat was placed in a chamber filled with air (A), mixed gas containing 5% CO_2, 20% O_2, and 75% N_2 (B), or mixed gas

containing 10% CO_2, 20% O_2, and 70% N_2 (C) The duration of the HISs in mixed gas containing 10% CO_2 was significantly shorter than that in air (10% CO_2 *vs.* air, $^{**}p = 3.7 \times 10^{-5}$) (D) Blood gas analysis of pH (5% CO_2 *vs.* air, $^{*}p = 0.0055$; 10% CO_2 *vs.* air, $^{**}p = 9.7 \times 10^{-7}$ (E), pCO_2 (5% CO_2 *vs.* air, $^{*}p = 0.014$; 10% CO_2 *vs.* air, $^{**}p = 2.2 \times 10^{-5}$) (F), pO_2 (5% CO_2 *vs.* air, $^{*}p = 0.045$; 10% CO_2 *vs.* air, $^{**}p = 1.2 \times 10^{-8}$) (G) , and HCO_3^- (H). $^{**}p < 0.001$, $^{*}p < 0.05$

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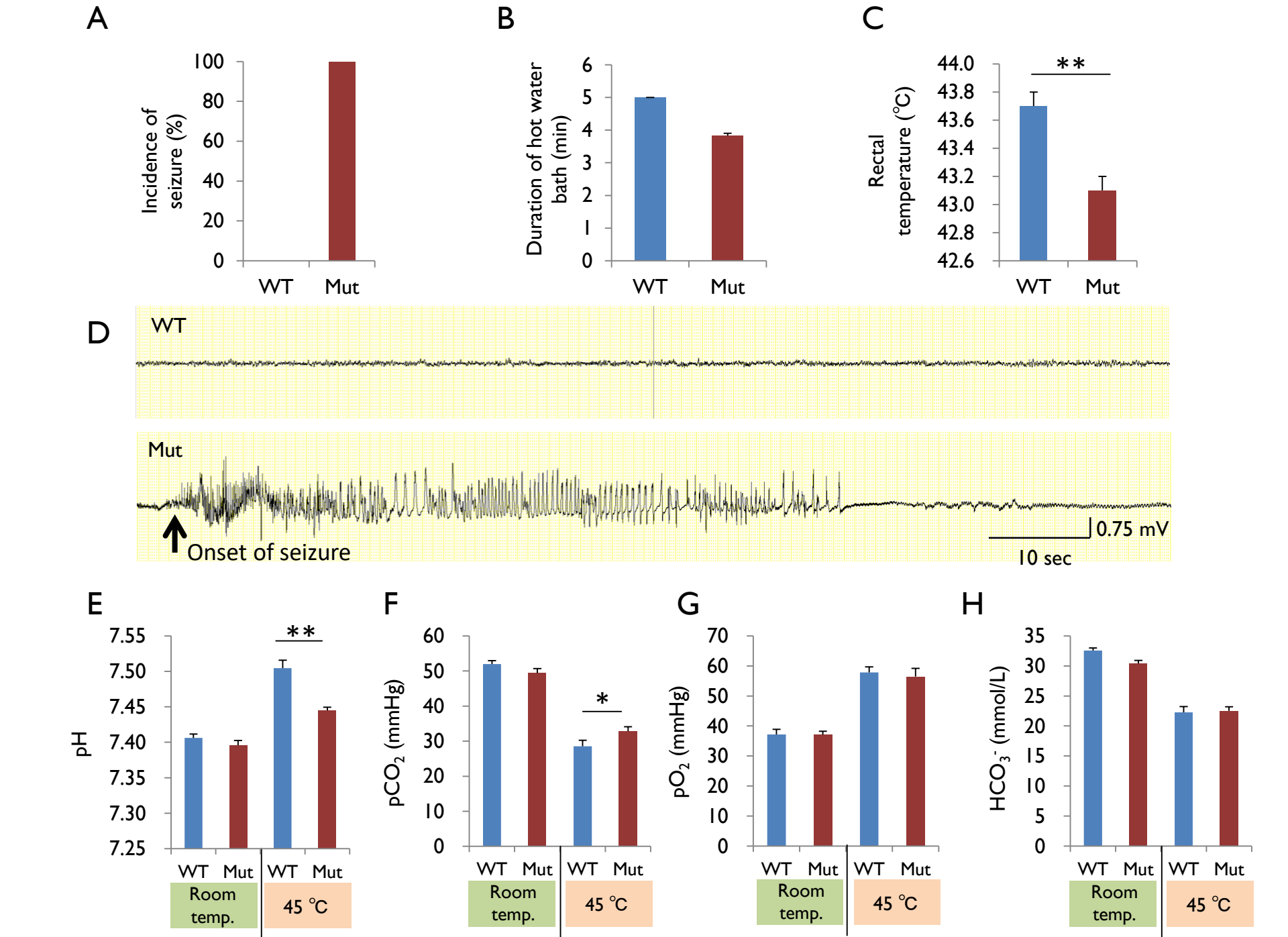
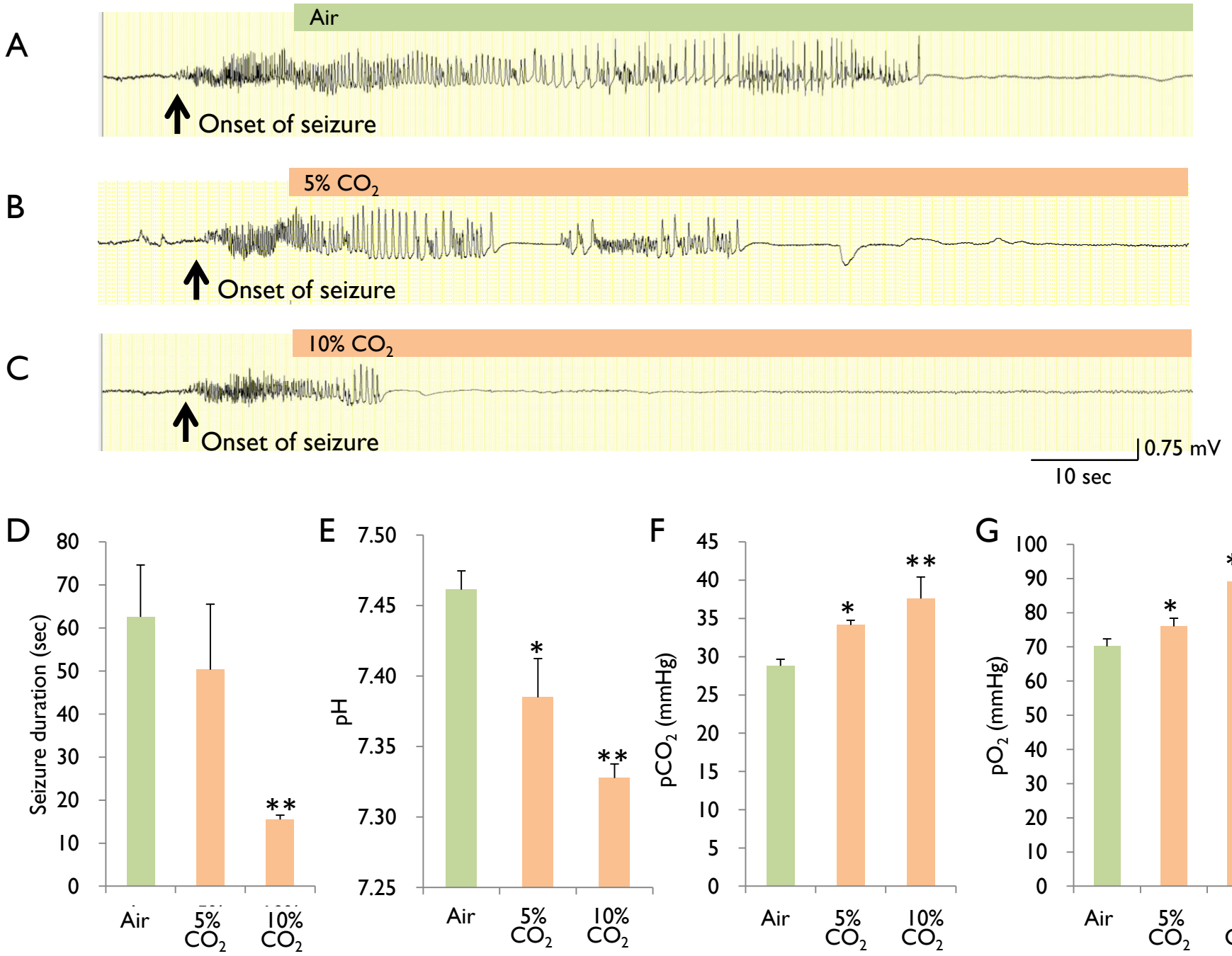

A
Incidence of seizure (%)
100
80
60
40
20
0
WT
Mut
B
Duration of hot water bath (min)
6
5
4
3
2
1
0
WT
Mut
C
Rectal temperature (°C)
44.0
43.8
43.6
43.4
43.2
43.0
42.8
42.6
**
WT
Mut
D
WT
Mut
Onset of seizure
0.75 mV
10 sec
E
pH
7.55
7.50
7.45
7.40
7.35
7.30
7.25
**
WT
Mut
WT
Mut
Room temp.
45 °C
F
pCO2 (mmHg)
60
50
40
30
20
10
0
*
WT
Mut
WT
Mut
Room temp.
45 °C
G
pO2 (mmHg)
70
60
50
40
30
20
10
0
WT
Mut
WT
Mut
Room temp.
45 °C
H
HCO3- (mmol/L)
35
30
25
20
15
10
5
0
WT
Mut
WT
Mut
Room temp.
45 °C

Figure 1.


A
Air
Onset of seizure
B
5% CO2
Onset of seizure
C
10% CO2
Onset of seizure
0.75 mV
10 sec
D
Seizure duration (sec)
80
70
60
50
40
30
20
10
0
**
Air
5% CO2
10% CO2
E
pH
7.50
7.45
7.40
7.35
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7.25
*
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Air
5% CO2
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F
pCO2 (mmHg)
45
40
35
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25
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15
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5
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*
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Air
5% CO2
10% CO2
G
pO2 (mmHg)
100
90
80
70
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40
30
20
10
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*
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Air
5% CO2
10% CO2

Figure 2.