Table 1
Effects of antiepileptic drugs on body (rectal) temperature in Hiss rats.

Drugs	Dose (mg/kg, i.p.)	Rectal temperature (°C)	
		Before	After
Sodium valproate	300	37.8 ± 0.09	37.9 ± 0.17
Diazepam	0.5	37.7 ± 0.06	37.8 ± 0.06
	1.0	37.9 ± 0.09	38.0 ± 0.04
Phenytoin	20	37.8 ± 0.15	37.4 ± 0.05*
	40	37.8 ± 0.07	34.9 ± 0.41*
Ethosuximide	100	37.9 ± 0.05	37.7 ± 0.15

Values are the mean ± SEM.
* $P<0.05$, Statistically different from the control value before the treatment (paired Student's *t*-test).

amygdala, hippocampus, and perirhinal–entorhinal cortex, suggesting that the hyperexcitability of limbic neurons associated with Scn1a missense mutation plays a crucial role in the pathogenesis of febrile seizures. In addition, Hiss rats showed a significantly lower threshold in generating epileptiform discharges upon local stimulation of the hippocampus. These results indicate that functional abnormalities within the hippocampal circuit are at least partly involved in generation of hyperthermic seizure in Hiss rats. Our findings are consistent with the notion that the initial precipitating FS is generated in the limbic formation, which consequently leads to secondarily generalized seizures (e.g., temporal lobe elipeptogenesis) (Baram et al., 1997; Scantlebury and Heida, 2010; Toth et al., 1998; VanLandingham et al., 1998).

In our previous studies (Mashimo et al., 2010), the N1417H mutation of Scn1a caused a hyperpolarized shift in the voltage dependency of $Na_v1.1$ channels inactivation concomitantly with a slight increase in persistent leak currents. The N1417H mutation also caused a significant decrease in the spike amplitude in the hippocampal GABAergic interneurons, but not in the pyramidal neurons, indicating a specific impairment of GABAergic activities (Mashimo et al., 2010). These changes in $Na_v1.1$ channel properties were relatively mild as compared to the GEFS+ mutation (R1648H) (Martin et al., 2010) or the SMEI nonsense mutation in mice (Yu et al., 2006) since the N1417H mutation did not significantly affect the sodium current density (Mashimo et al., 2010). Consistent with these findings, Hiss rats did not show severe behavioral deficits (e.g., ataxia) or premature death. Conversely, our studies suggest that even a mild loss-of-function mutation in $Na_v1.1$ channels can cause a significant vulnerability to FS. For this reason, the following possibilities seem plausible. 1) Inhibition of the axonal excitability (i.e., propagation of action potentials) with the N1417H mutation also inhibited the GABAergic activity, since recent analysis revealed that Scn1a are expressed not only in the somata of GABAergic neurons but also in their proximal and distal axons (Ogiwara et al., 2007). In addition, 2) *in vivo* influences due to the mutation may turn out to be more intensive by multiple and convergent projections of the GABAergic neurons onto the pyramidal neurons, leading to synchronized or magnified influences of the GABAergic impairment. Finally, since hyperthermic stimuli per se are known to reduce the activity of hippocampal GABAergic interneurons (Kwak et al., 2008; Tsai and Leung; 2006; Wang et al., 2000), 3) even relatively small changes in GABAergic functions may cause more pronounced influences on the animals' vulnerability to FS.

Information on the actions of antiepileptic drugs in the Scn1a mutant animals is very limited probably due to their short survival time. The present study showed that hyperthermic seizures in Hiss rats were effectively alleviated by diazepam and sodium valproate. Since both agents are known to enhance GABAergic neurotransmission by allosteric potentiation of $GABA_A$ receptor-mediated inhibition or by inhibiting GABA transaminase, respectively (Sasa, 2006), these actions may counteract the dysfunction of GABAergic interneurons in Hiss rats. In fact, these agents are reportedly effective in treating human FS (Fetveit, 2008; Incorpora, 2009; Lux, 2010). In contrast, phenytoin and ethosuximide which are clinically used for generalized tonic–clonic seizures and absence seizures in humans, respectively, did not significantly affect the incidence and magnitude (i.e., duration) of hyperthermic seizures in Hiss rats. Although a slight prolongation in the seizure latency was found with phenytoin and ethosuximide, this might be due to the nonspecific CNS depressive actions of these agents (e.g., sedation and hypothermia) (Davis et al., 1978). It should be noted that phenytoin did not reduce seizures even though it significantly lowered the body temperature of Hiss rats, implying that the agent has a proconvulsive action against FS. These responses of Hiss rats to antiepileptic drugs are consistent with those of human FS patients (e.g., GEFS+ and SMEI), supporting the notion that the Na_v channel inhibiting antiepileptics (e.g., phenytoin and carbamazepine) should be avoided in the treatment of FS (Fetveit, 2008; Incorpora, 2009; Lux, 2010). Since no appropriate pharmacotherapy for human FS, especially GEFS+ and SMEI, has been established, Hiss rats seems to be useful for searching new treatments.

Although Hiss rats exhibited hyperthermic seizures soon after birth (1–5 weeks old) (Mashimo et al., 2010), we employed 4-week-old Hiss rats in the present study. This is based on our previous findings that the expression of Scn1a was very low until P14 and developmentally matured at 3–5 weeks old and that the phenotypic (i.e., seizure susceptibility) contrast to the control animals are prominent at this age (Mashimo et al., 2010). Since perinatal development of the central nervous system in rats is known to be much slower than in humans, such that many of the neural elements (e.g., ion channels, receptors and enzyme) are expressed around or after the birth in rats, but mostly during prenatal periods in humans (Dobbing and Sands, 1979; Romijn et al., 1991; Clancy et al., 2001; Vinay et al., 2005), the susceptibility to hyperthermic seizures associated with the Scn1a mutation may occur earlier (e.g., early after birth) in humans. In addition, the Scn1a mutation may also increase seizure susceptibility to stimuli other than hyperthermia since Hiss rats showed a reduced threshold for the electrically induced hippocampal afterdischarges. Further studies using the animals of younger ages or the different seizure models with non-hyperthermic (e.g., chemoconvulsants, acoustic and kindling) stimuli will be required to further validate the utility and specificity of Hiss rats as an epilepsy model.

In conclusion, the present study demonstrated that Hiss rats carrying a missense mutation in Scn1a showed markedly high susceptibility to hyperthermic seizures, which was associated with a region-specific increase in the excitability of limbic neurons (e.g., hippocampus and amygdala). Furthermore, hyperthermic seizures in Hiss rats were significantly alleviated by diazepam and sodium valproate, but not by ethosuximide or phenytoin, which mimic the responses of human FS patients. The present findings support the notion that Hiss rats are useful as a novel animal model of FS and suggest that hyperexcitation of limbic neurons associated with the Scn1a mutation plays a crucial role in the pathogenesis of febrile seizures.

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Research Report

Inhibitory effects of levetiracetam on absence seizures in a novel absence-like epilepsy animal model, Groggy rat

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ABSTRACT

Levetiracetam (LEV) is known to inhibit convulsive seizures and is clinically used for treating both partial and generalized seizures. The study was performed to determine whether LEV possesses an inhibitory effect on absence seizures in a novel genetic animal model of absence epilepsy, Groggy (GRY) rats. Single injections of LEV at doses ranging from 20 to 160 mg/kg i.p. markedly inhibited absence seizures in GRY rats. The anti-absence action of LEV was potent and the cumulative duration of spike and wave discharges (SWD) in GRY rats was almost completely suppressed even at 20 mg/kg (i.p.). When the time-course of the inhibitory action of LEV (80 mg/kg i.p.) was examined up to 24 h after the treatment, the appearance of SWD was suppressed for over 6 h after injection of LEV in contrast to the action of sodium valproate (200 mg/kg i.p.) which had a very short effect (<2 h). The maximum level of blood concentration of LEV was attained within 2 h after administration, and the drug disappeared from the blood in 24 h with $T_{1/2}$ of 2.7 h. These results revealed that LEV displays potent and relatively long-lasting inhibitory effects on absence seizures in GRY rats.

1. Introduction

Levetiracetam (LEV, Keppra®) is a novel antiepileptic drug (AED) approved as adjunctive therapy for partial-onset seizures and for primary generalized tonic–clonic seizures and myocloninc seizures of juvenile myoclonic epilepsy. It is also approved for use as an initial monotherapy in the European Union. LEV was found to possess a unique preclinical profile in animal models of epilepsy. It has antiseizure effects in kindling and genetic models of epilepsy but lacks anticonvulsant activity against electroshock- or chemically induced convulsions (Klitgaard et al., 1998). This appears to correlate with a novel antiepileptic mechanism pertaining to binding with the synaptic vesicle protein 2A (SV2A), which is involved in the regulation of neurotransmitter release (Lynch et al., 2004; Sasa, 2006; Kaminski et al., 2008). LEV has also been reported to inhibit N-type calcium ion channel currents while not affecting other ion channels (e.g., sodium or potassium channels) nor involving GABA receptor functions (Klitgaard, 2001; Lynch et al., 2004; Kaminski et al., 2009). With its broad-spectrum profile, LEV has anticonvulsant effects against both partial and generalized seizures in experimental and spontaneous animal models of epilepsy (Gower et al., 1992; Loscher and Honack, 1993; Gower et al., 1995). Besides the anticonvulsive actions, LEV has also been reported to significantly reduce spike and wave discharges (SWD) in two absence epileptic animal models, the genetic absence epilepsy rats from Strasbourg (GAERS) and WAG/Rij rats (Gower et al., 1995;

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Bouwman and van Rijn, 2004). Our previous studies demonstrated that LEV inhibited both convulsive and absence-like seizures in the spontaneously epileptic rat (SER), a genetic rat model expressing both seizure types (Ji-qun et al., 2005). Similarly, LEV also suppressed spontaneous SWD in DBA/2J mice, which show complex seizures as well as SER (Marrosu et al., 2007). The outcome from these rodent models suggests the potential application of LEV as a treatment for absence epilepsy. Indeed several clinical trials have shown considerable reductions or elimination of absence seizure incidents in patient groups treated with LEV without demonstrating any major side-effects (Cavitt and Privitera, 2004; Di Bonaventura et al., 2005; Striano et al., 2008; Verrotti et al., 2008).

The groggy (GRY) rat is a spontaneous neurological mutant that was originally found in the closed colony of Slc:Wistar by the characterization of an ataxic phenotype (Takeuchi et al., 1994). We have previously mapped the causative gene, *gry*, on the rat chromosome 19 and identified a missense mutation (M251K) of the gene encoding the α_{1A} subunit of the P/Q type voltage-dependent Ca^{2+} channel (*Cacna1a*) (Tokuda et al., 2007). Cortical electroencephalograph (EEG) recording revealed that GRY rats exhibited 7- to 8-Hz SWD concomitant with sudden immobility and staring, consistent with the phenotype in the known *Cacna1a* mutant, *tottering* mice (Fletcher et al., 1996; Tokuda et al., 2007). Conventional AEDs, such as ethosuximide (ESM) and sodium valproate (VPA), inhibited both the incidence and duration of SWD in GRY rats, but phenytoin (PHT) did not, suggesting that the pharmacological profile against seizures in this strain is very similar with that of absence seizures in humans (Tokuda et al., 2007). The objectives of the present study were to determine whether LEV possesses an inhibitory activity against SWD in the novel absence model GRY rats and to compare its effects to those of VPA. The blood concentrations of LEV in GRY rats were also measured.

2. Results

2.1. *Effects of levetiracetam on spike and wave discharges*

We previously demonstrated that absence seizures in GRY rats are not affected by the vehicle injection at post-treatment 15 min and 45 min (Tokuda et al., 2007). In the present study, when the acute effects of LEV on absence seizures in GRY rats were examined at doses from 20 to 160 mg/kg i.p., an inhibition of SWD occurrence was observed with LEV from the minimum dose of 20 mg/kg. LEV significantly reduced the cumulative duration of SWD at post-drug 15 and 45 min, compared with those of the control period ($p<0.01$, Fig. 1A and B). The number of appearances of SWD also significantly decreased after LEV administration. While the average duration of each SWD appearance remained unaltered (Table 1): the number was significantly decreased from 40.4±8.6 to 14.4±6.4, 15–30 min after the injection of LEV 20 mg/kg ($n=5$). Dose-dependency in the inhibitory effects of LEV was not evident in the range of examined 20 to 160 mg/kg i.p. Obvious behavioral changes such as sedation were not observed during the recordings.

Next, the time-course of the inhibitory effects of LEV (80 mg/kg i.p.) on SWD in GRY rats were examined before injection and at 2, 6, 8, and 24 h after the injection of the drug. VPA (200 mg/kg i.p.) and physiological saline were also tested as a comparative drug and control, respectively. The SWD appearance was almost completely suppressed 2 h after LEV treatment and the inhibition lasted for at least 6 h (Fig. 2). Two of five animals exhibited one seizure episode during the 2- or 6-h postdrug recording with the same duration of SWD as the respective average value in the control period (data not shown). Statistical analysis showed that LEV had significant effect on SWD compared to saline and VPA [$F(2,70)=12.91$, $p<0.01$]. The interaction of treatment and time was also significant [$F(8,70)=1.36$, $p<0.01$]. Post hoc comparisons revealed that in the LEV treatment group the mean cumulative durations of SWD of post-drug 2 h and 6 h were significantly lower than those of pre-drug session and of post-drug 24 h (Fig. 2). In addition, they were also significantly lower than the corresponding values in the saline and VPA treatments within each session (Fig. 2). The cumulative duration of SWD at 8 h after LEV treatment was still decreased compared to the reference value, although it was statistically non-significant. The SWD occurrence was restored 24 h after LEV treatment. On the other hand, VPA completely suppressed SWD 30 min after the treatment (Fig. 2, inset). This inhibitory effect, however, disappeared in 2 h. No significant change in the seizure appearance was observed in the saline group throughout the 24 h observation period.

2.2. *Blood concentration of LEV*

LEV blood concentrations were measured in a different animal group of GRY rats under the same schedule and dosage as in the time-course experiment. The maximum level of LEV was obtained at 2 h after the drug (80 mg/kg i.p.) administration, followed by an exponential decline to 8 h with an apparent elimination half-life ($T_{1/2}$) of 2.7 h (Table 2). LEV was not detected in the blood at 24 h after the injection.

3. Discussion

We have previously demonstrated that absence seizures in GRY rats were suppressed by ESM and VPA while neither PHT nor vehicle affected seizure incidence and duration (Tokuda et al., 2007). Thus, the AED actions against seizures in GRY rats are considered to mimic those against human absence seizures. In the present study, LEV significantly suppressed SWD appearance in GRY rats at the dose range of 20–160 mg/kg i.p. in a dose-independent manner. A significant suppression of SWD was obtained from 15 min to 6–8 h after the LEV injection (80 mg/kg), indicating that LEV has potent and long-lasting inhibitory effects on absence seizures. The efficacy of LEV on absence seizures has been indicated in other animal models mimicking absence epilepsy such as GAERS, WAG/Rij rats and SER. In addition, no behavioral side effects were observed in GRY rats after LEV treatment, corresponding to previous findings (Gower et al., 1995; Bouwman and van Rijn, 2004; Ji-qun et al., 2005). Taken together, the present finding further supports the effectiveness of LEV against absence seizures in human epilepsy.

LEV has no or negligible actions against acute electroshock- or pentylenetetrazole-induced seizures (MED or ED50

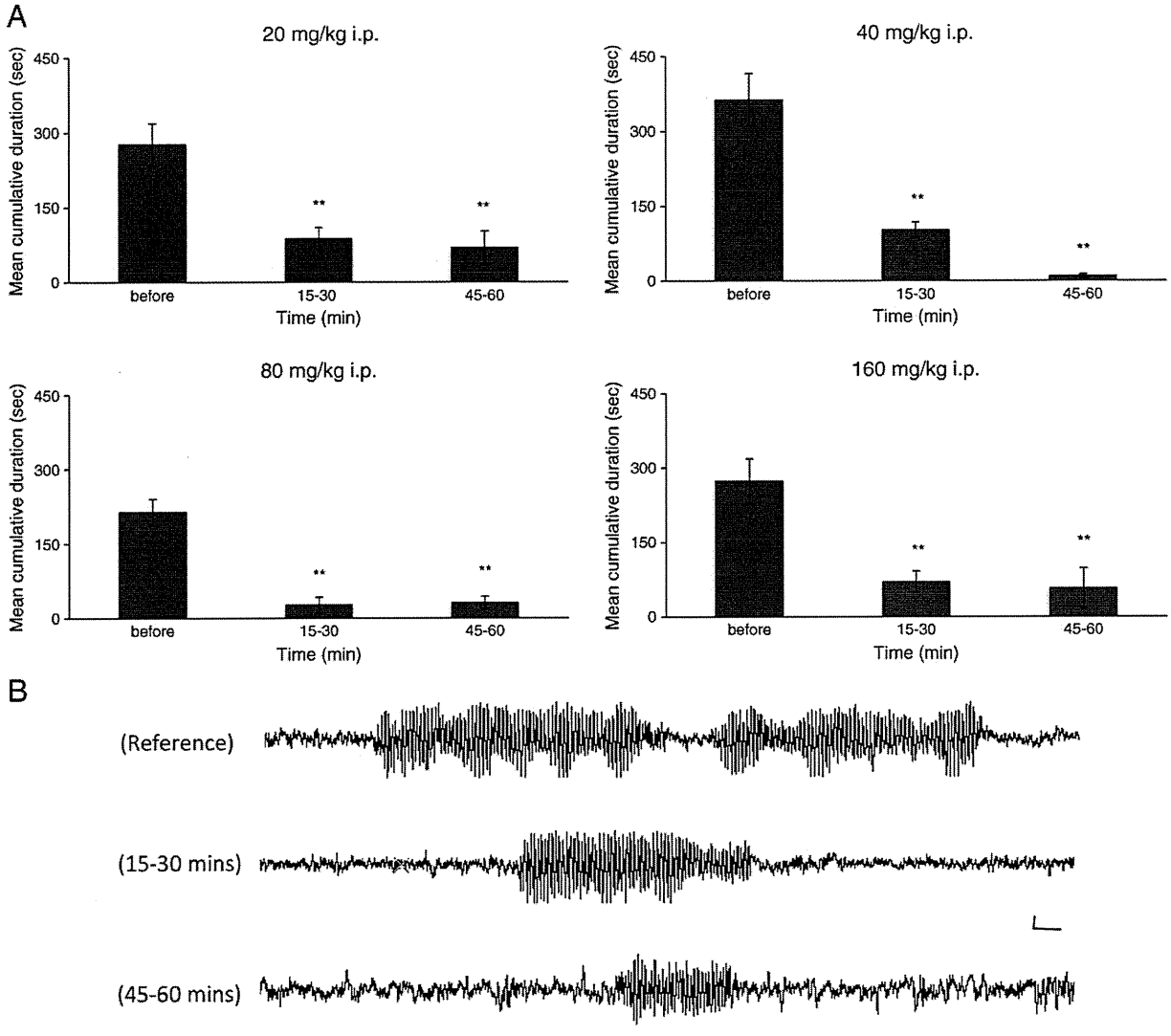

Fig. 1 – Acute effects of LEV, 20 to 160 mg/kg i.p., on spike and wave discharges in GRY rats. (A) The mean cumulative duration for each 15-min post-drug session was compared to that for the reference period. The vertical lines indicate the S.E.M values ($n=5$ per dose-group). $^{**}p<0.01$. (B) Representatives of cortical EEG trace recorded from a GRY rat before and after the injection of LEV 80 mg/kg. Calibration: 50 μV and 1 s.

values > 100 mg/kg, i.p.). However, LEV effectively inhibits seizures in pentylenetetrazole-kindled, amygdala-kindled models or pilocarpine-induced focal seizure model with MED or ED50 values of about 30–50 mg/kg (i.p.) (Klitgaard et al., 1998; Ohno et al., 2010). The inhibitory actions of LEV in GRY rats were equally potent among the doses examined, and the incidence of SWD was almost suppressed even at 20 mg/kg (i.p). These actions of LEV were similar to those reported in GAERS model, where LEV suppressed absence seizures at doses of 5.4 to 170 mg/kg with no significant dose-effect relationship. In

Table 1 – The mean number of SWD incidence and the mean length of a SWD incidence.

	Mean SWD incidence (number/15 min)			Mean SWD duration (s)		
	Before	15–30 min	45–60 min	Before	15–30 min	45–60 min
20 mg/kg	40.4±8.6	14.4±6.4*	10.2±4.6*	7.3±0.7	7.7±2.2	6.9±1.8
40 mg/kg	43.6±3.6	17.2±2.5**	2.4±0.7**	8.1±0.6	6.2±0.9	3.3±1.2
80 mg/kg	23.2±4.2	3.0±1.6**	3.2±1.0**	9.8±0.9	4.8±2.1	8.4±1.3
100 mg/kg	28.2±3.7	9.0±2.1**	6.0±3.5**	9.9±1.3	7.5±1.3	8.0±2.0

Mean±S.E.M, $n=5$ in each group. * $p<0.05$, ** $p<0.01$, compared with the corresponding value in the control period before the LEV treatment.

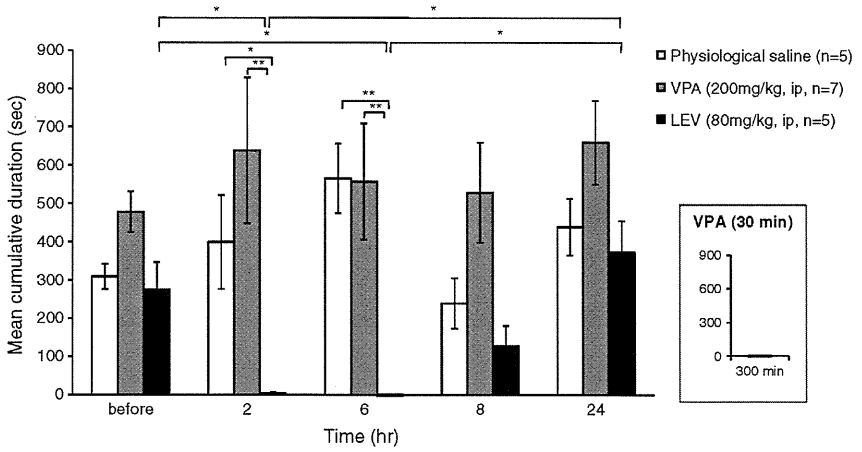

Fig. 2 – Long-lasting effects of LEV (80 mg/kg i.p.) and VPA (200 mg/kg i.p.) on spike and wave discharges in GRY rats. The mean cumulative durations were compared among treatments and each session. Inset: the mean cumulative duration of SWD after 30 min in the VPA group. The vertical lines indicate the S.E.M values. White bars: saline group ($n=5$), gray bars: VPA group ($n=7$), black bars: LEV group ($n=5$). $^{*}p<0.05$, $^{**}p<0.01$.

addition, in accordance with the previous findings on absence-like seizures of SER (Ji-qun et al., 2005), the inhibitory effects of LEV were more pronounced 2 and 4 h after the injections than 1 h post-treatment. Interestingly, LEV reduced only SWD incidence in GRY rats but not duration unlike other most animal models of absence seizure. This may suggest that mechanisms regulating seizure events are unique in GRY rats. Further investigations at molecular levels would be required to address this difference.

It is noteworthy that the suppression of SWD by the single LEV lasted to 6–8 h after the injection in contrast to the duration of action of VPA. Doheny's and other groups have studied the blood pharmacokinetics of LEV in the Sprague–Dawley rats, showing the maximum serum level (T_{max}) and the half life ($T_{1/2}$) of LEV was 0.25–0.5 h and 1–3 h, respectively (Loscher and Honack, 1993; Doheny et al., 1999; De Smedt et al., 2007). Although the blood sample prior to 2 h was not collected in the present study, the blood pharmacokinetics of LEV in GRY rats (apparent $T_{1/2}=2.7$ h) seemed to be similar to the previous data. Since the effective blood concentration of LEV in human epilepsy is reported to be 6–20 mg/L, the blood concentration obtained from GRY rat at 6 h after the treatment (21.6 μg/ml) is considered to be effective (Johannessen et al., 2003; Bialer et al., 2004). On the other hand, the T_{max} and $T_{1/2}$ of VPA (200 mg/kg i.p.) are reported to be about 0.25 h and 1 h (Mesdjian et al., 1982), respectively, which are similar to those of LEV. Despite the similarity of the pharmacokinetics of LEV and VPA, the duration of anti-absence seizure effects of LEV in GRY rats was much longer than that of VPA. The longer duration of the LEV effects might be due to the difference of the brain pharmacokinetics: the cerebrospinal fluid (CSF) $T_{1/2}$ of LEV was reported to be 4.4–4.9 h, much slower than that of VPA whose mean $T_{1/2}$ in various brain areas was in the range of 0.6–0.8 h (Mesdjian et al., 1982; Doheny et al., 1999). In addition, there are other possibilities such as LEV affecting gene expression related to the epileptic and/or epileptogenic factors, since the inhibition of seizures by the drug actually lasted for several days after cessation of the 5-days drug treatment in SER (Yan et al., 2005). Interestingly, the study by Russo and his colleagues demonstrated that early treatment with LEV, before seizure onset, can significantly modify the development of absence epilepsy in the WAG/Rij model (Russo et al., 2009). On the other hand, chronic treatments in early life did not prevent the expression of SWD in GAERS (Dedeurwaerdere et al., 2005). Further studies are required to delineate the mechanisms underlying anti-absence seizure and antiepileptogenic actions of LEV in GRY rats. In conclusion, our results demonstrated that LEV has potent and long-lasting effects against absence seizures in the novel genetic rat model of absence epilepsy, GRY, supporting a treatment potential of LEV against absence seizures in humans.

Table 2 – Blood concentration of LEV in GRY rats after a single intraperitoneal injection at dose of 80 mg/kg.

	2 h	6 h	8 h	24 h
(μg/ml)	61.0±2.4	21.6±1.8	12.9±1.1	0.0±0.0

Mean±S.E.M, $n=6$.

4. Experimental procedures

All GRY rats were obtained from the National BioResource Project-Rat, Kyoto University (Kyoto, Japan). Animals were kept and bred at the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University in the condition of constant temperature (24±2 °C) and humidity (50±10%) under a 14-h light/10-h dark cycle (light on at 7:00 a.m.). All experimental procedures were conducted according to the Guideline for Animal Experiments of Kyoto University. Surgical procedures for brain electrodes implantation and EEG recordings were performed as described before with slight modifications (Tokuda et al., 2007). Briefly, 35 male rats aged 16–30 weeks were implanted with four screw electrodes in subdural space under

ketamine/xylazine (75 and 10 mg/kg, i.p., respectively) anesthesia. The reference electrode was fixed on the frontal cranium, the other two were located bilaterally over the frontal cortex (3.0 mm lateral and 3.0 mm caudal to the bregma, according to the brain atlas of Paxinos and Watson), and the final one was fixed over the cerebellum to act as a ground. One week after the surgery, cortical EEGs were recorded according to experimental schedules, following 30-min habituation periods. During the experiments animals were placed in a shielded box and permitted to move freely. The EEG and behavioral changes before and after LEV administration were continuously observed and no obvious changes were confirmed during the observation periods. LEV and VPA were dissolved in the physiological saline. For the dose-response experiments of LEV, EEG was recorded for 15 min before drug administration as a reference period, and then recorded two times: 15–30 and 45–60 min after the administration of a single injection of LEV at doses of 20, 40, 80 or 160 mg/kg i.p. ($n=5$ per a group). For the time-course experiments of LEV, following a reference recording for 30 min, post-drug EEGs were recorded for 30 min at four separate time-points: 2, 6, 8, and 24 h after an injection of LEV (80 mg/kg i.p., $n=5$), VPA (200 mg/kg i.p., $n=7$), or physiological saline ($n=5$). When the same animal was used repeatedly, more than 1 week elapsed before the subsequent test. Absence seizures were determined by the appearance of SWD on EEG accompanied by a resting pose in the animals. Both the number and duration of seizure episodes were measured, and then the cumulative duration of seizures was calculated for each recording session. The data were analyzed by one-way ANOVA, followed by Tukey's test if required. In the time-course experiment, the cumulative durations of seizures were compared among three treatments and five time sessions by two-way ANOVA, followed by post hoc Fisher's test or paired t-tests ($\alpha=0.05$). To measure the blood concentration of LEV, blood samples were repeatedly collected from the cervical veins of six GRY rats under anesthesia before the injections of LEV (80 mg/kg, i.p.) and at 2, 6, 8 and 24 h post-injection. Samples were centrifuged for 15 min at 1800 × g to separate the cells from sera. Sera were then transferred into new tubes and stored −80 °C until analyzed. The LEV concentration in sera was analyzed by Mitsubishi Chemical Medience Corporation (Tokyo, Japan). LEV was a generous gift from UCB Pharma S.A. (Belgium) and VPA was purchased from Sigma.

Acknowledgments

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Full Paper

Serotonergic Modulation of Absence-Like Seizures in Groggy Rats: a Novel Rat Model of Absence Epilepsy

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Abstract. To explore the role of the serotonergic system in modulating absence seizures, we examined the effects of 5-HT_{1A} and 5-HT_2 agonists on the incidence of spike-and-wave discharges (SWD) in Groogy (GRY) rats, a novel rat model of absence-like epilepsy. GRY rats exhibited spontaneous absence-like seizures characterized by the incidence of sudden immobile posture and synchronously-associated SWD. The total duration of SWD in GRY rats was about 300 – 400 s/15-min observation period under the control conditions. However, the incidence of SWD was markedly reduced either by the 5-HT_{1A} agonist (±)-8-hydroxy-2-(di-*n*-propylamino)-tetralin [(±)8-OH-DPAT] or the 5-HT_2 agonist (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane [(±)DOI]. The 5-HT reuptake inhibitors, fluoxetine and clomipramine, also inhibited the SWD generation. In addition, the inhibitory effects of (±)8-OH-DPAT and (±)DOI were reversed by WAY-100135 (5-HT_{1A} antagonist) and ritanserin (5-HT_2 antagonist), respectively. The present results suggest that the serotonergic system negatively regulates the incidence of absence seizures by stimulation of 5-HT_{1A} and 5-HT_2 receptors.

Keywords: absence seizure, 5-HT_{1A} receptor, 5-HT_2 receptor, Groggy rat, spike-and-wave discharge

Introduction

It is now known that serotonergic neurotransmission plays an important role in regulating not only psycho-emotional functions, but also motor functions including epileptic seizures (1). 5-HT neurons modulate a wide variety of experimentally-induced seizures in various animal models of epilepsy. Previous studies have shown that the agents which enhance serotonergic activity, such as the 5-HT reuptake inhibitors (e.g., fluoxetine), inhibit generalized and partial convulsive seizures (2 – 5). Conversely, agents (e.g., *p*-chlorophenylalanine) that deplete the 5-HT level in the brain enhance the seizure induction (6, 7). It is also known that many antiepileptic drugs (e.g., valproic acid, carbamazepine, and phenytoin) enhance 5-HT release in the brain, which has been suggested to be partly involved in their antiepileptic actions (8 – 10). All these findings imply that overall stimulation of the serotonergic system reduces seizure generation.

5-HT neurons derived from the raphe nuclei send their axons to various brain regions including the cerebral cortex, limbic regions, and diencephalon, where serotonergic neurotransmission is mediated by multiple 5-HT receptors that can be divided into 7 families (5-HT_1 – 5-HT_7) encompassing 14 subtypes (11). Among these 5-HT receptor subtypes, the roles of 5-HT_{1A} and 5-HT_2 (5-$HT_{2A, 2B, 2C}$) receptors in the control of seizure generation have been widely studied in various models. It has been demonstrated that 5-HT_{1A} agonists [e.g., 8-hydroxy-2-(di-*n*-propylamino)-tetralin (8-OH-DPAT)] inhibited chemoconvulsions induced by bicuculline, picrotoxin, or pilocarpine (12 – 14). In addition, 5-HT_2 agonists [e.g., *m*-chlorophenylpiperazine (mCPP)] reportedly inhibited both chemically (e.g., pentylenetetrazole)- and

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electrically-evoked convulsions (5, 15). Thus, it is conceivable that the serotonergic system negatively regulates the incidence of convulsive seizures by activating 5-HT_{1A} and 5-HT_2 receptors. However, information on their roles in modulating the absence seizures is very limited and still elusive. A line of studies have shown that 5-HT_{1A} receptors augment, while 5-HT_2 receptors inhibit, the absence seizures (16 – 18). However, all the results to date were obtained from Wistar Albino Glaxo/Rijswijk (WAG/Rij) rats, a genetic rat model of absence-like epilepsy. Therefore, further studies using different animal models are necessary to validate the roles of 5-HT_{1A} and 5-HT_2 receptors in controlling absence seizures.

The Groggy (GRY) rat is a spontaneous mutant with an ataxic phenotype originally found in the closed colony of Slc:Wistar (19). More recently, Tokuda et al. (20) showed that GRY rats carry a missense (M251K) mutation in the gene encoding the α_{1A} subunit of the P/Q type voltage–dependent Ca^{2+} channel (*Cacna1a*) and, like the *Cacna1a* mutant *tottering* mice (21), exhibit absence-like seizures associated with 7 – 8 Hz spike and wave discharges (SWD) and concomitant immobile postures. In addition, absence-like seizures in GRY rats were significantly alleviated by two antiepileptic drugs effective for human absence seizures, ethosuximide and sodium valproic acid, but not by phenytoin that lacks efficacy for absence seizures (20). These analyses of seizure phenotypes reveal that GRY rats are useful as a novel model of human absence epilepsy. In the present study, therefore, we studied the effects of 5-HT_{1A} and 5-HT_2 agonists, (±)8-OH-DPAT and (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane [(±)DOI], respectively, on the incidence of SWD in GRY rats to clarify the roles of 5-HT_{1A} and 5-HT_2 receptors in modulating absence seizures.

Materials and Methods

Animals

GRY rats (GRY/Idr) were obtained from the National BioResource Project for the Rat (NBRPR#0368) in Japan. The animals were kept in air-conditioned rooms under a 12-h light/dark cycle and allowed ad libitum access to food and water. The experimental protocols of this study were approved by the Experimental Animal Research Committee at Osaka University of Pharmaceutical Sciences.

Drug treatments and EEG recording

Surgical procedures for the electrode implantation and EEG recordings were performed as described previously (20, 22). Since it was confirmed in the preliminary experiments that both male and female rats exhibited SWD with a similar frequency, GRY rats of either sex (250 – 350 g) were employed. The animals were anesthetized with pentobarbital (40 mg/kg, i.p.) and fixed in a stereotaxic instrument (SR-6; Narishige, Tokyo). Small holes were made in the skull and silver ball electrodes were placed on the surface of the right or left frontal and occipital cortex. A reference electrode was placed on the surface of 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, animals underwent the subsequent experiments.

On the day of experiments, the animals were placed in an electrically-shielded observation cage (28 × 45 × 20 cm). After a 15-min habituation period, EEG and behavior of the animals were simultaneously monitored under freely-moving conditions using an amplifier (MEG-6108; Nihon Kohden, Tokyo) and a thermal alley recorder (RTA-1100, Nihon Kohden). To evaluate 5-HT agonists, EEG was recorded for 15 min before drug administration (pre-drug control) and 15 – 30 and 45 – 60 min after the administration of (±)8-OH-DPAT (0.3 and 1 mg/kg) or (±)DOI (0.3 and 1 mg/kg). In the experiments using 5-HT antagonists, following the 15-min EEG recording (pre-drug control), animals were first treated with either WAY-100135 or ritanserin (each at 3 mg/kg) and 30 min later given 1 mg/kg of (±)8-OH-DPAT or (±)DOI. EEG was recorded for 15 min before and 15 – 30 min after the administration of (±)8-OH-DPAT or (±)DOI. Each animal was normally used twice or 3 times with a drug-recovery period of about 1 week and each subjected to the different treatment (different dose or drug).

Drugs

The drugs used in this study were as follows: (±)8-OH-DPAT hydrochloride, (±)DOI hydrochloride, fluoxetine hydrochloride, clomipramine hydrochloride, and ritanserin (Sigma-Aldrich, St. Louis, MO, USA); (±)WAY-100135 hydrochloride (TOCRIS, Bristol, UK). (±)DOI was dissolved in saline. (±)8-OH-DPAT, fluoxetine, clomipramine, (±)WAY-100135, and ritanserin were first dissolved in 1% lactate solution and then diluted with saline. Since ritanserin solution was acidic, the agents were orally administered. All other agents were administered intraperitoneally except for WAY-100135 that was given subcutaneously.

Statistical analyses

For the EEG analysis, total duration of SWD during each 15-min observation period was calculated. Data are expressed as the mean ± S.E.M. Statistical significance of differences in the total SWD duration was determined by unpaired one-way ANOVA and Tukey's *post-hoc* comparison test (multiple group comparison) or unpaired Student's *t*-test (two group comparison). A *P* value of

less than 0.05 was considered statistically significant.

Results

Effects of 5-HT agonists on SWD in GRY rats

GRY rats showed ataxia (e.g., unstable gait and occasional extension of the hind limbs) when moving, but other behaviors (e.g., alertness, exploring, and spontaneous activity) were generally normal. GRY rats often exhibited spontaneous absence-like seizures, which are characterized by a sudden immobile posture with vacuous staring and a synchronously-associated 7 – 8 Hz SWD in EEG (Fig. 1). The total duration of SWD in GRY rats was about 300 – 400 s/15-min observation during the pre-drug control period (Fig. 2). The incidence of SWD was not significantly affected by the vehicle alone (control group), while it tended to decrease with time.

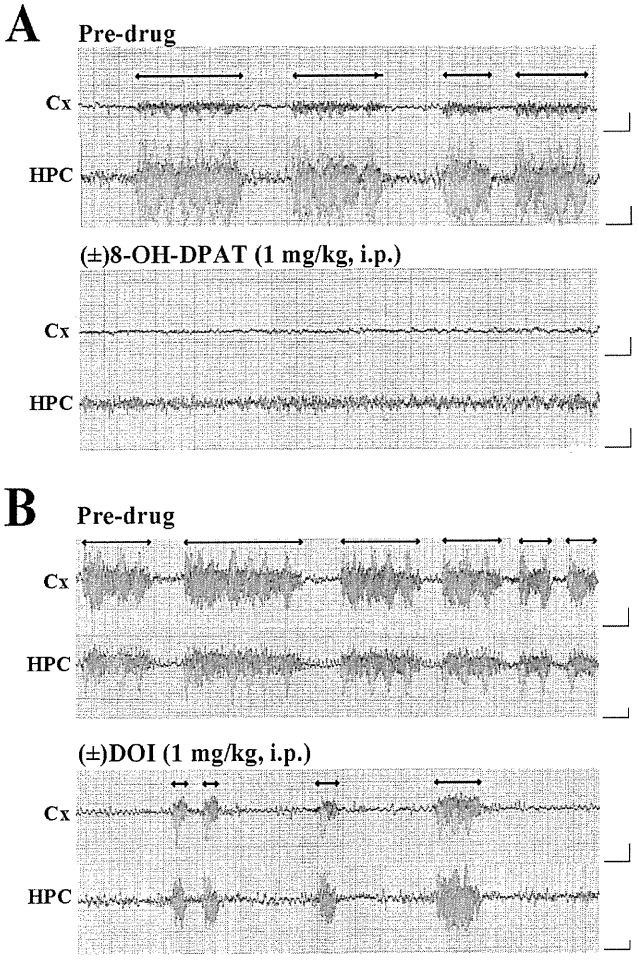

Fig. 1. Typical SWD in GRY rats before and after (±)8-OH-DPAT or (±)DOI treatment. Cortical and hippocampal EEG were recorded from GRY rats chronically implanted with recording electrodes. A: EEG recorded before (Pre-drug) and 15 min after administration of (±)8-OH-DPAT (1 mg/kg, i.p.). B: EEG recorded before and 15 min after administration of (±)DOI (1 mg/kg, i.p.). Cx: cerebral cortex. HPC: hippocampus. Lines with arrows indicate the period of each SWD. Calibration: 50 μV and 5 s.

Treatment of animals with the 5-HT_{1A} agonist (±)8-OH-DPAT (0.3 and 1 mg/kg, i.p.) transiently induced a flat body posture in GRY rats, which usually subsided within 15 – 30 min after the treatment. (±)8-OH-DPAT caused no apparent alterations in EEG by itself, but markedly inhibited the incidence of SWD in a dose-related manner (Fig. 2A). The inhibitory effects of (±)8-OH-DPAT were most prominent at 15 – 30 min after the injection and the total duration of SWD fell to about 15% of the control level with 1 mg/kg of (±)8-OH-DPAT. Similarly, the 5-HT_2 agonist (±)DOI (0.3 and 1 mg/kg, i.p.) also produced a dose-related inhibition of SWD in GRY rats (Fig. 2B). The total duration of SWD was markedly reduced to about 10% – 20% of the control level by 1 mg/kg (±)DOI. No abnormal EEG or behaviors (e.g., head twitches and wet dog shakes) were found with (±)DOI at the tested doses.

We also examined the 5-HT stimulants fluoxetine and clomipramine, which increase the extracellular 5-HT level by inhibiting 5-HT reuptake. As shown in Fig. 3, both fluoxetine and clomipramine at 20 mg/kg (i.p.) significantly inhibited the incidence of SWD in GRY rats, which resembled the actions of (±)8-OH-DPAT and (±)DOI.

Effects of 5-HT antagonists on (±)8-OH-DPAT– and (±)DOI-induced inhibition of SWD

We next examined the effects of 5-HT antagonists, WAY-100135 (5-HT_{1A} antagonist) and ritanserin (5-HT_2 antagonist), on (±)8-OH-DPAT– and (±)DOI-induced inhibition of SWD in GRY rats, respectively. Treatment with WAY-100135 (3 mg/kg, s.c.) did not affect the SWD incidence by itself (Fig. 4). However, the subsequent treatment with (±)8-OH-DPAT (1 mg/kg, i.p.) no longer significantly inhibited SWD [cf. Fig. 2A: 15 – 30 min after 1 mg/kg (±)8-OH-DPAT], indicating that WAY-100135 antagonized (±)8-OH-DPAT–induced SWD inhibition. Similarly, ritanserin (3 mg/kg, p.o.) did not alter the SWD per se, but antagonized (±)DOI (1 mg/kg, i.p.)–induced inhibition of SWD (Fig. 4) [cf. Fig. 2B: 15 – 30 min after 1 mg/kg (±)DOI].

Discussion

Although previous studies have shown that both 5-HT_{1A} and 5-HT_2 receptors inhibit convulsive seizures in various models of epilepsy (e.g., bicuculline-, picrotoxin-, and pentylenetetrazole-induced convulsions) (5, 12 – 15), the roles of these receptors in modulating ab-

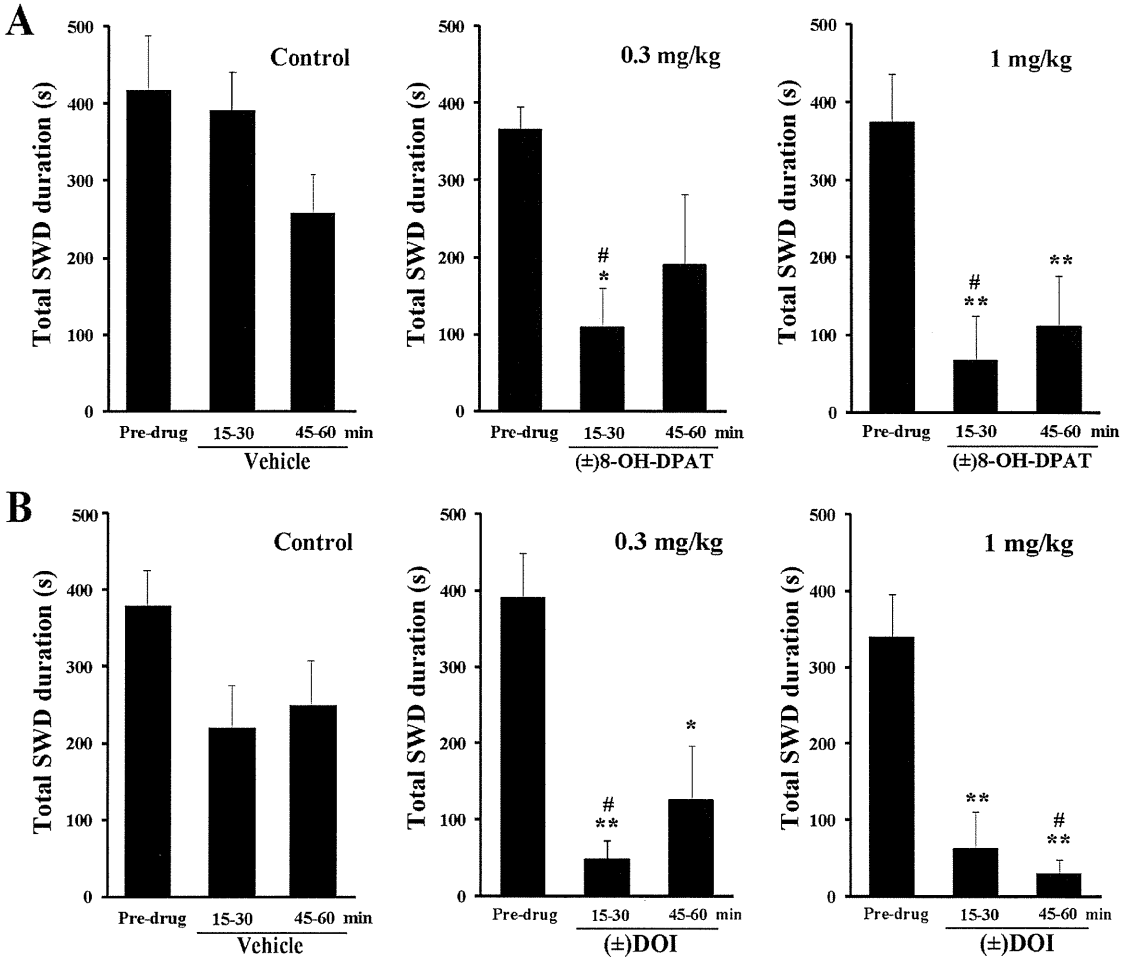

Fig. 2. Effects of (±)8-OH-DPAT and (±)DOI on the incidence of SWD in GRY rats. After a 15-min habituation period, EEG was recorded for 15 min before drug administration (Pre-drug) and 15 – 30 and 45 – 60 min after the administration of the drugs. The incidence of SWD was evaluated as the total SWD duration (s) during each 15-min observation period. A: Effects of (±)8-OH-DPAT (0.3 and 1 mg/kg, i.p.). B: Effects of (±)DOI (0.3 and 1 mg/kg, i.p.). Each column represents the mean ± S.E.M. of 4 – 6 GRY rats. $^{*}P < 0.05$, $^{**}P < 0.01$: Significantly different from the pre-drug values (one-way ANOVA and Tukey's test). $^{\#}P < 0.05$: Significantly different from the respective value in the control group (Student's *t*-test).

sence epilepsy remain elusive. The present study using GRY rats, a novel rat model of absence epilepsy (20), demonstrated that both (±)8-OH-DPAT and (±)DOI markedly inhibited the incidence of SWD associated with the absence-like behavior. In addition, stimulation of the serotonergic system by the 5-HT reuptake inhibitors fluoxetine and clomipramine also inhibited the SWD generation. Furthermore, the inhibitory actions of 8-OH-DPAT and DOI were reversed by WAY-100135 (5-HT_{1A} antagonist) and ritanserin (5-HT_2 antagonist), respectively. These results strongly suggest that the serotonergic system inhibits the absence seizure activity via activating 5-HT_{1A} and 5-HT_2 receptors in GRY rats.

A previous study using WAG/Rij rats, a widely used model of absence epilepsy, showed that the 5-$HT_{2B/2C}$ agonist mCPP reduced the incidence of SWD and these actions were antagonized by the selective 5-HT_{2C} antagonist SB-242084 (17). Although we did not specifically differentiate the actions (±)DOI into 5-HT_{2A}, 5-HT_{2B}, or 5-HT_{2C} subtypes in this study, our results in GRY rats are consistent with the above studies, supporting the notion that 5-HT_2 (probably 5-HT_{2C}) receptors inhibit the absence seizures. 5-HT_{2C} receptors are densely distributed in the choroid plexus and in various brain regions including the cerebral cortex, limbic regions, and basal ganglia (11). The involvement of 5-HT_{2C} receptors in the regulation of seizures is strongly supported by the facts that animals lacking 5-HT_{2C} receptors exhibit a marked vulnerability to seizure induction (23 – 25). In addition, earlier studies showed that 5-HT_2 receptors are involved in 5-HT–induced inhibition of rhythmic burst firing of the thalamic neurons, which are closely related to the

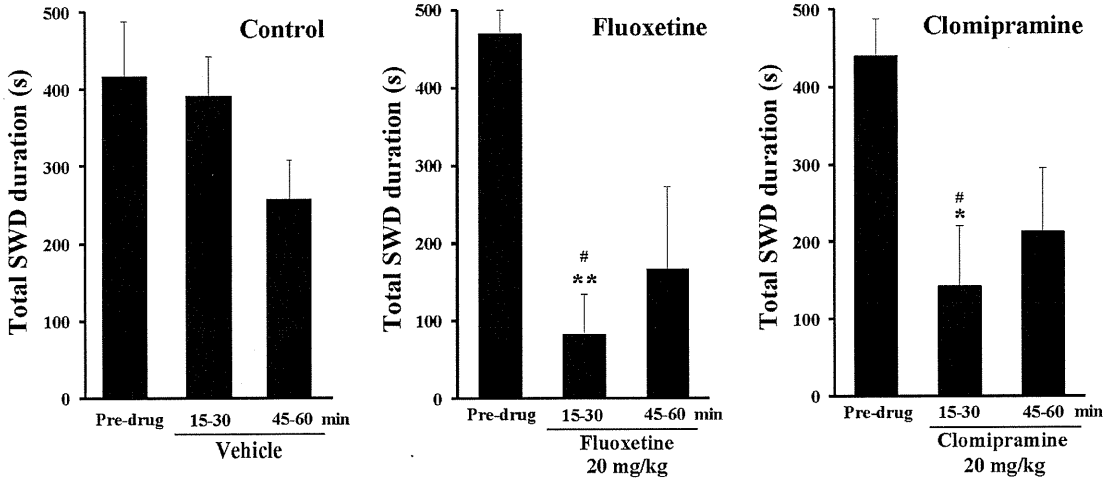

Fig. 3. Effects of fluoxetine and clomipramine on the incidence of SWD in GRY rats. After a 15-min habituation period, EEG was recorded for 15 min before drug administration (Pre-drug) and 15 – 30 and 45 – 60 min after the administration of fluoxetine (20 mg/kg, i.p.) or clomipramine (20 mg/kg, i.p.). The incidence of SWD was evaluated as the total SWD duration (s) during each 15-min observation period. The control graph is the same as in Fig. 2A and is quoted for comparisons. Each column represents the mean ± S.E.M. of 4 – 7 GRY rats. $^{*}P < 0.05$, $^{**}P < 0.01$: Significantly different from the pre-drug values (one-way ANOVA and Tukey's test). $^{\#}P < 0.05$: Significantly different from the respective value in the control group (Student's *t*-test).

generation of SWD and/or cortical spindle activity (26 – 28). Thus, these inhibitory functions of 5-HT_2 receptors on the thalamic neurons seem to be responsible for the anti-absence activity of 5-HT_2 agonists, although the precise mechanisms remain uncertain.

5-HT_{1A} receptors are expressed in the limbic areas, septum, and the raphe nuclei at high density and also expressed in the cerebral cortex, diencephalon, and basal ganglia at low to moderate density (11, 29, 30). 5-HT_{1A} receptors located on the cell body and dendrites of 5-HT neurons in the raphe nuclei function as presynaptic autoreceptors to inhibit their own activities. On the other hand, postsynaptic 5-HT_{1A} receptors are located on postsynaptic membranes of neurons or dendrites, which are innervated by 5-HT neurons (29, 30). Numerous studies have shown that stimulation of 5-HT_{1A} receptors strongly inhibits neuronal firings in various brain regions, including the cortico-limbic regions related to the seizure induction, via coupling to G-protein–gated inwardly rectifying potassium channels (30 – 34). Thus, the inhibition of neuronal activities in the cortico-limbic regions may be involved in (±)8-OH-DPAT–induced inhibition of SWD in GRY rats. Although previous studies also showed that stimulation of 5-HT_{1A} and 5-HT_2 receptors cooperatively acts in several models of epilepsy (1, 5, 12 – 15) or other CNS diseases (35, 36), the mode (e.g., independent, additive, or synergistic) of interaction between 5-HT_{1A} and 5-HT_2 receptors in inhibiting SWD remains to be clarified.

It should be noted that the responses of absence seizures to (±)8-OH-DPAT and the 5-HT reuptake inhibitors in GRY rats were distinct from those obtained from WAG/Rij rats. Previous studies showed that (±)8-OH-DPAT increased the incidence of SWD in WAG/Rij rats, which was antagonized by 5-HT_{1A} antagonists (e.g., WAY-100635 and NAN-190) (16, 17). In these animals, the 5-HT reuptake inhibitors (i.e., fluoxetine and citalopram) slightly to moderately increased the SWD generation (17). These responses of the 5-HT reuptake inhibitors were considered to be the net effects of 5-HT_{1A}–mediated facilitation and 5-HT_{2C}–mediated inhibition on SWD generation, since WAY-100635 reduced and SB-242084 (5-HT_{2C} antagonist) enhanced the action of fluoxetine (17). However, these actions of 5-HT ligands against absence seizures have been studied only in WAG/Rij rats. On the other hand, both the 5-HT_{1A} agonist [i.e., (±)8-OH-DPAT] and the 5-HT reuptake inhibitors (i.e., fluoxetine and clomipramine), unlikely in WAG/Rij rats, inhibit the SWD generation in GRY rats, while the (±)DOI-induced inhibition of SWD mimicked the action of mCPP in WAG/Rij rats (17). Our results on the 5-HT_{1A} agonist and 5-HT reuptake inhibitor do not deny the previous findings in WAG/Rij rats, but alternatively bring up a possibility that 5-HT_{1A} receptors negatively regulate absence seizures in certain pathological conditions. Thus, both 5-HT_{1A} and 5-HT_2 receptors seem to at least partly participate in the inhibition of SWD by the 5-HT reuptake inhibitor in GRY rats, although other mechanisms including the potential involvement of other 5-HT receptor subtypes and other neurotransmitter sys-

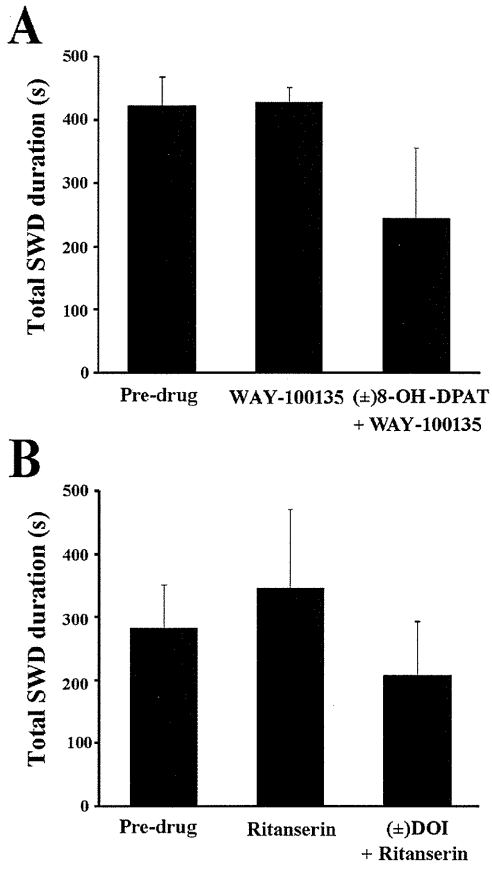

Fig. 4. Effects of WAY-100135 and ritanserin on 5-HT agonist–induced SWD inhibition. After a 15-min EEG recording (Pre-drug), animals were first treated with either WAY-100135 or ritanserin (each at 3 mg/kg) and 30 min later given 1 mg/kg of (±)8-OH-DPAT or (±)DOI, respectively. EEG was recorded for 15 min before and 15 – 30 min after the administration of (±)8-OH-DPAT or (±)DOI. A: Effects of WAY-100135 on (±)8-OH-DPAT–induced SWD inhibition. B: Effects of ritanserin on (±)DOI-induced SWD inhibition. Each column represents the mean ± S.E.M. of 4 or 6 GRY rats. No statistically significant difference was found among the treatments (one-way ANOVA and Tukey's test).

tems cannot be ruled out. The reasons for the differences in the 5-HT_{1A}–mediated responses between GRY and WAG/Rij rats are currently unknown. However, since 5-HT_{1A} receptors function as both postsynaptic and presynaptic receptors (11, 29, 30), differences in the functional balance of pre- and postsynaptic 5-HT_{1A} receptors, as well as those in the intrinsic activity of 5-HT neurons, between GRY and WAG/Rij rats may be involved. Indeed, WAG/Rij rats are known to exhibit not only absence seizures, but also depression (anhedonia)-like behaviors (e.g., increased immobility in a forced swim test and decreased sucrose intake) and altered sensitivity to antidepressants (37, 38). Further studies such as the determination of serotonergic activities (e.g., brain 5-HT contents and densities of 5-HT receptors or SERT) in these two strains are required to clarify the role of 5-HT_{1A} receptors in regulating absence seizures.

In conclusion, the present study demonstrated that both (±)8-OH-DPAT and (±)DOI significantly inhibited the incidence of SWD in GRY rats. The overall stimulation of the serotonergic system by 5-HT reuptake inhibitors, fluoxetine and clomipramine, also inhibited the SWD generation in GRY rats. Our results on the actions of (±)DOI are consistent with the previous findings in WAG/Rij rats, supporting the inhibitory function of 5-HT_2 receptors in the induction of absence seizures. On the other hand, since absence seizure responses in GRY rats to (±)8-OH-DPAT were distinct from those reported in WAG/Rij rats, the present study suggests a novel possibility that 5-HT_{1A} receptors negatively regulate absence seizures. Further clarification of the regulatory role of 5-HT_{1A} receptors in absence seizure generation is required.

Acknowledgments

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Scn1a missense mutation impairs $GABA_A$ receptor-mediated synaptic transmission in the rat hippocampus

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ABSTRACT

Mutations of the $Na_v1.1$ channel subunit SCN1A have been implicated in the pathogenesis of human febrile seizures (FS). We have recently developed hyperthermia-induced seizure-susceptible (Hiss) rat, a novel rat model of FS, which carries a missense mutation (N1417H) in *Scn1a* [1]. Here, we conducted electrophysiological studies to clarify the influences of the *Scn1a* mutation on the hippocampal synaptic transmission, specifically focusing on the GABAergic system. Hippocampal slices were prepared from Hiss or F344 (control) rats and maintained in artificial cerebrospinal fluid saturated with 95% O_2 and 5% CO_2 *in vitro*. Single neuron activity was recorded from CA1 pyramidal neurons and their responses to the test (unconditioned) or paired pulse (PP) stimulation of the Schaffer collateral/commissural fibers were evaluated. Hiss rats were first tested for pentylenetetrazole-induced seizures and confirmed to show high seizure susceptibility to the blockade of $GAGA_A$ receptors. The *Scn1a* mutation in Hiss rats did not directly affect spike generation (i.e., number of evoked spikes and firing threshold) of the CA1 pyramidal neurons elicited by the Schaffer collateral/commissural stimulation. However, $GABA_A$ receptor-mediated inhibition of pyramidal neurons by the PP stimulation was significantly disrupted in Hiss rats, yielding a significant increase in the number of PP-induced firings at PP intervals of 32–256 ms. The present study shows that the *Scn1a* missense mutation preferentially impairs $GABA_A$ receptor-mediated synaptic transmission without directly altering the excitability of the pyramidal neurons in the hippocampus, which may be linked to the pathogenesis of FS.

1. Introduction

Human febrile seizures (FS) are the most common type of seizures in childhood. Most FS are generally benign, but, about one-third of patients exhibit recurrent seizures and have a potential risk for secondary epilepsy (e.g., temporal lobe epilepsy) [2–5]. Although the pathophysiological mechanisms underlying FS are not fully elucidated, $Na_v1.1$ channels have been implicated in the etiology of FS [6–8]. Specifically, more than 200 mutations of the $Na_v1.1$ channel α subunit SCN1A have been reported in patients of generalized epilepsy with febrile seizures plus (GEFS+) and severe myoclonic epilepsy in infancy (SMEI) [6–8]. Furthermore, recent studies have shown that the truncated mutations of $Na_v1.1$ channels caused hypersusceptibility to hyperthermia-induced seizures in mice, which accompanied a marked reduction in sodium currents in the GABAergic neurons [9–11]. Nonetheless, due to the diversity of *Scn1a* mutations and/or the complexity in functional changes of $Na_v1.1$ channels in FS patients [6,8], the precise mechanisms underlying the pathogenesis of FS remain to be clarified.

We have recently developed a novel rat model which carries a missense mutation (N1417H) in the third pore-forming region of *Scn1a* using *N*-ethyl-*N*-nitrosourea mutagenesis [1]. Since these animals at a very young age (~5 week old) exhibited markedly high susceptibility to hyperthermia-induced seizures, we designated them hyperthermia-induced seizure-susceptible (Hiss) rats. Besides the vulnerability to hyperthermic seizures, Hiss rats were also very sensitive to seizures induced by pentylenetetrazole (PTZ, a blocker of $GABA_A$ receptors), suggesting that the *Scn1a* missense mutation impairs the GABAergic functions [1]. In addition, electrophysiological analyses using mutated $Na_v1.1$ channels or dissociated hippocampal neurons revealed that the N1417H missense mutation causes multiple changes in $Na_v1.1$ channel properties in GABA neurons, including a hyperpolarized shift in the voltage-dependency of $Na_v1.1$ inactivation, an increase in persistent leak currents and a reduced amplitude of evoked spikes [1]. However, influences of the *Scn1a* mutation on the GABAergic

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synaptic transmission and/or the hippocampal neural network remain to be clarified.

In the present study, therefore, we conducted electrophysiological studies in Hiss rats to clarify the effects of the *Scn1a* missense mutation on the hippocampal synaptic transmission, specifically focusing on the GABAergic inhibitory control of the CA1 pyramidal neurons. Our results show that the *Scn1a* missense mutation impairs $GABA_A$ receptor-mediated synaptic transmission in the hippocampus, supporting the notion that the *Scn1a* missense mutations play an important role in the pathogenesis of FS by disrupting the inhibitory GABAergic neurotransmissions.

2. Materials and methods

2.1. Animals

Hiss rats (F344-*Scn1a*$^{Kyo811/Kyo811}$) were obtained from the National BioResource Project for the Rat (NBRPR#0455) in Japan. As reported previously [1], Hiss rats carry homozygous *Scn1a*-$^{Kyo81/Kyo8111}$ alleles, which have the missense mutation N1417H located in the third pore-forming region of the *Scn1a* gene (Fig. 1A). Hiss rats were backcrossed more than 5 generations against F344/NSlc inbred background to eliminate mutations in chromosomal regions other than the *Scn1a* locus, and F344/NSlc (F344) rats were used as the control. The animals were kept in air-conditioned rooms under a 12-h light/dark cycle and allowed *ad libitum* access to food and water. The housing conditions and the animal care methods complied with NIH guide for the care and use of laboratory animals. The experimental protocols of this study were approved by the Experimental Animal Research Committee at Osaka University of Pharmaceutical Sciences.

2.2. Induction of PTZ-induced seizures

Male Hiss or F344 rats (8 weeks old) were used. Animals were cumulatively injected with an increasing dose of PTZ at 10, 20 or 30 mg/kg (i.p.) with a 30-min intervals. Immediately after each dosing of PTZ, animals were placed in an observation cage (28 × 45 × 20 cm) and the incidence of excitation behaviors and seizures were continuously monitored for 10 min using the following scores, (0) no change, (1) recurrent head twitches or wet dog shakes, (2) myoclonic jerk of forepaws and/or upper trunk, (3) recurrent and marked myoclonic jerk of forepaws and/or upper trunk, (4) clonic seizures, (5) marked clonic seizures with falling dawn.

2.3. Electrophysiology using hippocampal slices

Male Hiss or F344 rats (6–8 weeks old) were used. Experiments were carried out as reported previously with slight modifications [12–14]. After decapitation, the brain was immediately removed from the skull and chilled in ice-cold artificial cerebrospinal fluid (ACF) saturated with a gas mixture of 95% O_2 and 5% CO_2. A tissue block containing the dorsal hippocampus (−3.24 to −3.96 mm posterior to the bregma) [15] was then dissected out and cut into slices at a thickness of 400 μm using a Microslicer (Dosaka EM, DSK-3000, Kyoto, Japan) (Fig. 2A). The hippocampal slice was completely submerged in the recording chamber which was continuously perfused with ACF at a flow rate of about 1.5 ml/min. ACF contained (in millimolar): NaCl 116.4, KCl 5.4, $MgSO_4$ 1.3, NaH_2PO_4 0.92, $CaCl_2$ 2.5, $NaHCO_3$ 26.2, glucose 11.0) and was continuously bubbled with 95% O_2 and 5% CO_2. The temperature of the ACF was maintained at 29–30 °C.

Single neuron activity was extracellularly recorded from the CA1 pyramidal neurons using a glass microelectrode which was filled with 3 M NaCl and had an electrical resistance of 4–8 MΩ. For the stimulation of the Schaffer collateral/commissural fibers, a bipolar stimulating electrode was inserted into the stratum radiatum in the CA1 field (Fig. 2). Stimuli (0.1 ms duration) with various intensities were applied every 10 s to measure the threshold for spike generation of CA1 pyramidal neurons. To measure the PP-induced responses, the intensity of the stimulation was adjusted to about 120% of the threshold level and the PP stimulation was applied with various PP intervals ranging from 16 to 512 ms. The recorded signals were amplified (Microelectrode Amplifier MEZ-8301, Nihon Kohden, Tokyo, Japan), monitored and stored in a computer (PowerLab ML4/36, AD Instruments, Bella Vista, Australia). Ten to 20 successive responses of neurons to the Test (unconditioned single pulse) or PP stimulation were usually recorded to calculate the mean spike number and latency in each treatment. In the experiments using the receptor antagonists, either bicuculline (a selective $GABA_A$ antagonist, 10 μM), saclofen (a selective $GABA_B$ antagonist, 100 μM) or 2-amino-5-phosphonovaleric acid (APV: a selective NMDA antagonist, 30 μM) was dissolved in ACF and added to the perfusion medium. This concentration of each antagonist was reportedly sufficient to block the respective receptor *in vitro* [14,16]. The spike generation of pyramidal neurons by the Test or PP stimulation were recorded immediately before and 10 min after the drug application.

2.4. GAD67-immunofluorescence histochemistry

Experiments were carried out as reported previously with slight modifications [17]. Briefly, sections (4 μm) of 4% formaldehyde-fixed, paraffin-embedded brains from Hiss or F344 rats (6–8 weeks old) were deparaffinized, rehydrated and autoclaved for 10 min in 10 mM citrate buffer (pH 6.0). Endogenous peroxidase was quenched by incubation with 3% hydrogen peroxidase in PBS. After incubating the slides with blocking solution (10% normal rabbit serum) for 30 min at room temperature, sections were incubated with a mouse anti-GAD67 antibody (Santa Cruz Biotech., CA) in a humidified chamber for 12 h at 4 °C. The sections were then incubated with a TRITC conjugated rabbit anti-mouse IgG secondary antibody (Sigma–Aldrich, St. Louis, MO). Immunofluorescence images was taken with a confocal laser scanning microscope (LSM510 Ver.3.2, Carl Zeiss Japan, Tokyo, Japan) and processed with instrumental image software.

2.5. Drugs

The drugs used in this study were as follows: PTZ hydrochloride (Sigma–Aldrich), bicuculline hydrochloride (Sigma–Aldrich), saclofen (Sigma–Aldrich) and D,L-APV (Tocris, Bristol, UK). All other reagents were obtained from commercial sources. PTZ hydrochloride was dissolved in saline and intraperitoneally injected to the animals at a volume of 5 ml/kg.

2.6. Statistical analysis

Data are expressed as the mean ± SEM. Statistical significance of differences in the behavioral scores (two groups comparison) was performed using Mann–Whitney's *U*-test. Comparison of differences in the stimulus threshold and the number of spikes between two groups (Hiss and F344 rats) was determined by the Student's *t*-test, while the comparison among multiple groups was determined by one-way ANOVA followed by Tukey's *post hoc* multiple comparison test.

3. Results

3.1. Susceptibility to PTZ-induced seizures

To assess the seizure susceptibility to the $GABA_A$ receptor/Cl^- channel blocker PTZ, we treated the animals with an increasing dose of PTZ (10, 20 and 30 mg/kg, i.p.) in a cumulative fashion.

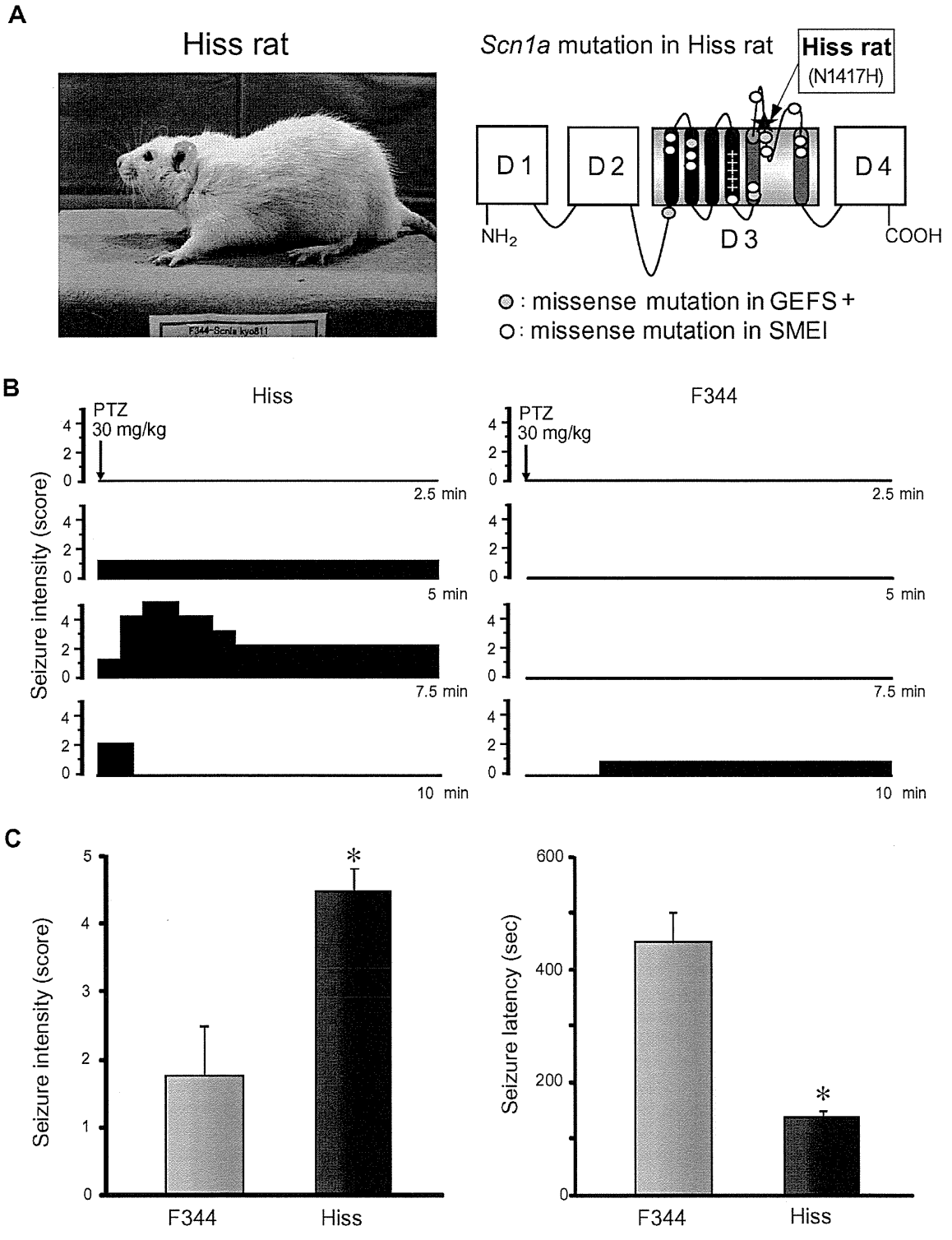

Fig. 1. Susceptibility of Hiss rats to pentylenetetrazole (PTZ)-induced seizures. (A) A photograph of a Hiss rat and a schematic drawing illustrating the location of the Scn1a missense (N1417H) mutation in Hiss rats. Scn1a consists of 4 homologous domains (D1–D4), each containing 6 transmembrane regions. The N1417H mutation is located in the pore-forming region of the third domain (D3). As a reference, missense mutation sites in D3 reported in GEFS + and SMEI are also shown (Meisler and Kearney, 2005). (B) A typical time-course of the PTZ-induced excitatory behaviors and seizures. Hiss or F344 rats were treated with an increasing dose of PTZ (10, 20 and 30 mg/kg, i.p.) in a cumulative fashion. At 30 mg/kg (i.p.), PTZ caused abnormal excitation and clonic seizures in Hiss rats while it usually induced only wet dog shakes (score 1) in F344 rats. (C) Comparison of the seizure intensity (seizure score) and the seizure latency (the time for seizure score 1) between Hiss and F344 rats. Each column represents the mean ± SEM of 5 rats. * $P < 0.05$; Significantly different from the control (F344) rats.

Neither Hiss nor F344 rats showed any abnormal behaviors following the treatments with 10 and 20 mg/kg PTZ. In the subsequent treatment with 30 mg/kg (i.p.), however, PTZ caused abnormal excitatory behaviors (e.g., head twitches and/or wet dog shakes) leading to marked myoclonic jerks and generalized clonic seizures in all Hiss examined ($N = 5$) (Fig. 1B). In contrast, PTZ-induced behaviors in F344 rats were very mild, in that PTZ (30 mg/kg, i.p.) induced only head twitches and/or wet dog shakes (score = 1)

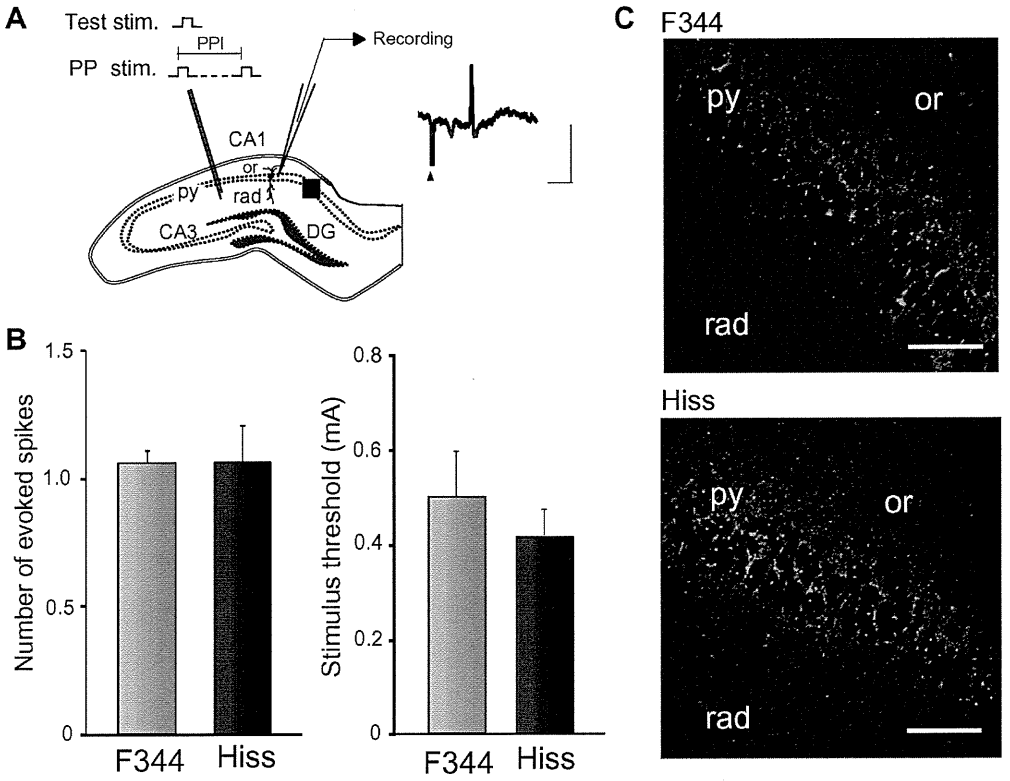

Fig. 2. Spike generation of hippocampal CA1 pyramidal neurons induced by stimulation of the Schaffer collateral/commissural fibers. (A) Schematic illustrations of the hippocampal slices and the recording of the spike generation in the CA1 field. A typical spike trace is also shown in the right panel. PPI: Paired pulse (PP) interval. or: stratum oriens, py: pyramidal layer, st: stratum radiatum. Calibration: 1 mV and 5 ms. (B) Comparison of the stimulus threshold and the spike number between Hiss and F344 rats. Each column represents the mean ± SEM of 9 or 13 neurons recorded in separate experiments. (C) Representative photograph illustrating GAD67-immunostaining of the CA1 field. The position of the photograph is shown as a closed square in schema A. GAD67-immunoreactivity (Red) is located around the cell body of the hippocampal CA1 pyramidal neurons similarly in Hiss and F344 rats. Calibration: 100 μm. (For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article.)

without causing any convulsive seizures in F344 rats, except for one which exhibited a transient (duration: <5 s) clonic seizure (score = 4) ($N = 5$). The seizure intensity (behavioral score) and latency (the time until score 1) in Hiss rats were significantly higher and shorter, respectively, than in F344 rats (Fig. 1C).

3.2. Electrophysiological responses of hippocampal pyramidal neurons

It is known that the hippocampal CA1 pyramidal neurons receive both $GABA_A$ receptor-inhibitory and NMDA receptor-mediated excitatory regulations upon PP stimulation [18,19]. We therefore evaluated the changes in hippocampal synaptic transmission in Hiss rats using the PP paradigm. Action potentials were recorded from CA1 pyramidal neurons and the test or PP stimulation was applied to the Schaffer collateral/commissural fibers in the stratum radiatum (Fig. 2A).

We first compared the threshold for spike generation in CA1 pyramidal neurons between Hiss and F344 rats. Stimulation of the stratum radiatum consistently elicited spike generation in CA1 pyramidal neurons with a mean spike latency of 8.64 ± 0.81 ($N = 9$) and 7.31 ± 0.81 ($N = 13$) msec in Hiss and F344 rats, respectively. The number of spikes evoked by the stimulation was nearly unit both in Hiss and F344 rats (Fig. 2B). In addition, the stimulus threshold for the spike generation was also equal between Hiss and F344 rats, suggesting that the synaptic neurotransmission from the Schaffer collateral/commissural fibers or the excitability of CA1 pyramidal neurons per se remained unaltered in Hiss rats (Fig. 2B).

After assessment of the stimulation threshold for spike generation, Test (unconditioned single pulse) or PP stimulation was delivered to the stratum radiatum at various PP intervals ranging from 16 to 512 ms in each neuron. Under these conditions, the PP stimulation (the 2nd stimulation) induced a slight facilitation of spike generation in F344 rats (Figs. 3 and 4), which was completely abolished in the presence of the NMDA antagonist APV (30 μM) (Fig. 4). However, the PP-induced facilitation of the spike generation was markedly augmented in Hiss rats (Figs. 3 and 4). The number of PP-elicited spikes was significantly higher in Hiss rats than in F344 rats at the PP intervals between 32 and 256 ms. In addition, bath application of the $GABA_A$ antagonist bicuculline (10 μM) also increased the PP-elicited spike generation to an extent similar to that in Hiss rats, whereas the $GABA_B$ antagonist saclofen (100 μM) showed no effects (Fig. 4).

Using GAD67-immunofluorescence staining, we also confirmed the distribution of GABAergic nerve terminals in the CA1 field. As shown in Fig. 2C, the GABAergic nerve terminals were mainly located around the cell bodies (somata) of the CA1 pyramidal neurons, and no apparent differences were found in the intensity of GAD67-immunoreactivity between Hiss and F344 rats.

4. Discussion

Previous studies have shown that homozygous *Scn1a* (−/−) knockout mice developed motor ataxia and die very early after birth (age of 2–3 weeks) [9]. Heterozygous *Scn1a* (+/−) mice also showed sporadic death after the age of 3 weeks concomitantly with spontaneous seizure induction [10]. In addition, knock-in mice with insertion of a loss-of-function nonsense mutation of SMEI into the *Scn1a* gene also developed an ataxic gait, spontaneous seizures and premature death [20]. All these animals exhibited a marked reduction in sodium currents in inhibitory GABAergic

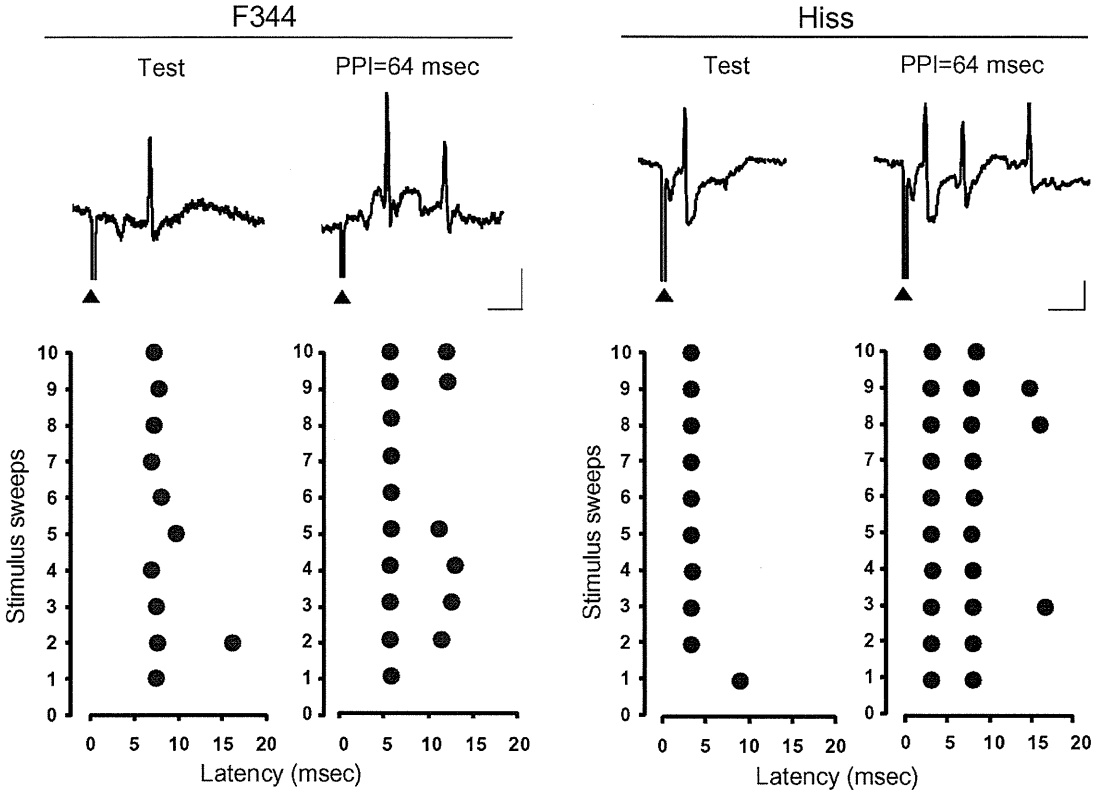

Fig. 3. Typical responses of hipocampal CA1 pyramidal neurons induced by the paired pulse (PP) stimulation. Test (unconditioned single pulse) or PP stimulation at a PP interval of 64 ms (PPI = 64 ms) was applied to the stratum radiatum, and the stimulus-evoked spike generation was compared between Hiss and F344 rats. The graph shows the poststimulus-latency plots of the evoked spikes, where each spike evoked by 10 successive stimulations was plotted according to its latency. Actual traces of the evoked spikes are shown on the top. Calibration: 1 mV, 5 ms.

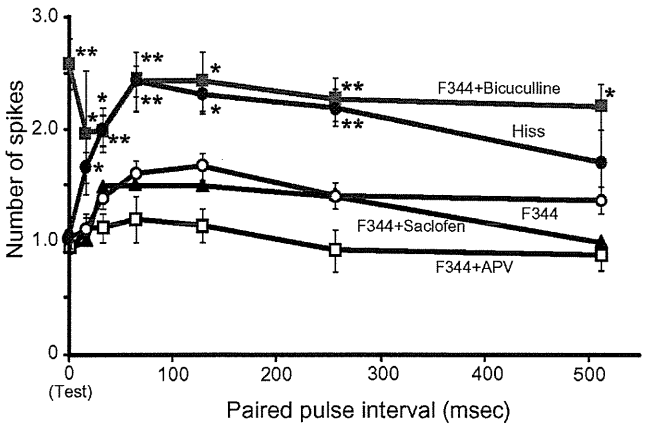

Fig. 4. Paired pulse (PP)-induced responses of hippocampal CA1 pyramidal neurons at various PP intervals in Hiss (closed circle) and F344 (open circle) rats. In the experiments using the receptor antagonists, either bicuculline (10 μM, closed square), saclofen (100 μM, closed triangle) or APV (30 μM, open square) was added to the perfusion medium, and the spike generation by the test (single pulse) or PP stimulation were recorded immediately before and 10 min after the drug application. Each column represents the mean ± SEM of 4–18 neurons recorded in separate experiments, except for saclofen ($N = 2$). $^{*}P < 0.05$, $^{**}P < 0.01$; Significantly different from F344 rats without treatment (F344).

interneurons, suggesting that the truncated mutations of *Scn1a* inhibit GABAergic activity to confer the seizure vulnerability. Nonetheless, it is known that the missense mutations of *Scn1a* gene reported in FS patients produce a variety changes in the channel functions such as gain-of-function changes (e.g., increased persistent leak current, depolarized shift in voltage-dependence of inactivation, hyperpolarized shift in voltage-dependence of activation), loss-of-function changes (e.g., hyperpolarized shift in voltage-dependence of inactivation and depolarized shift in voltage-dependence of activation) and mixed patterns of the above changes [6,8,21–25]. Due to the diversity of these functional alterations, influences of the *Scn1a* missense mutations on the GABAergic neural network or synaptic transmission remain to be clarified.

In the present study, we first confirmed that Hiss rats at an adult age continue to exhibit high susceptibility to PTZ-induced seizures. Electrophysiological evaluations using hippocampal slices from Hiss rats revealed that responses of the CA1 pyramidal neurons to the stimulation of the stratum radiatum were normal, suggesting that either the synaptic transmission from the Schaffer collateral/commissural fibers or the excitability of pyramidal neurons per se remained unaltered with the N1417H mutation. In contrast, the PP-induced facilitation of the firing of pyramidal neurons was markedly augmented in Hiss rats. In addition, a similar augmentation of the firing was also obtained in the presence of bicuculline (a selective $GABA_A$ receptor antagonist), but not saclofen (a selective $GABA_B$ receptor antagonist). Our results are consistent with previous findings [18] that the paired pulse facilitation of the field EPSP amplitude in the CA1 field was markedly enhanced by the targeted disruption of $GABA_A$ receptors, indicating that the CA1 pyramidal neurons receive $GABA_A$ receptor-mediated inhibition control with PP stimulation. It is therefore conceivable that the N1417H mutation in Hiss rats impairs $GABA_A$ receptor-mediated inhibitory synaptic transmission without significantly altering the excitability of the pyramidal neurons in the hippocampus. In addition, since GABAergic nerve terminals labeled by GAD67-immunostaining in the CA1 field were similarly observed both in Hiss and F344 rats, the disrupted GABAergic transmission in Hiss rats may not result from altered innervation density of GABAergic terminals, but possibly reflect their reduced activities. The prefer-