Prevention of status epilepticus-induced brain edema and neuronal cell loss by repeated treatment with high-dose levetiracetam

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\textbf{A B S T R A C T}

The management of status epilepticus (SE) is important to prevent mortality and the development of post-SE symptomatic epilepsy. Acquired epilepsy after an initial brain insult by SE can be experimentally reproduced in the murine model of SE induced by pilocarpine. In the present study, we evaluated the possibility of treatment with a high-dose of levetiracetam in this model. Repeated treatment with high-dose levetiracetam after termination of SE by diazepam significantly prevented the incidence of spontaneous recurrent seizures and mortality for at least 28 days. To determine the brain alterations after SE, magnetic resonance imaging was performed. Both $T_2$-weighted imaging and diffusion-weighted imaging showed changes in the limbic regions. These changes in the limbic regions demonstrated the development of cytotoxic edema three hours after SE, followed by the development of vasogenic edema two days after SE. In the pilocarpine-SE model, the incidence of spontaneous recurrent seizures after SE was strongly associated with neuronal damage within a few hours to days after SE by the development of vasogenic edema via the breakdown of the blood–brain barrier in the limbic regions. High-dose levetiracetam significantly suppressed the parameters in the limbic areas. These data indicate that repeated treatment with high-dose levetiracetam for at least two days after SE termination by diazepam is important for controlling the neuronal damage by preventing brain edema. Therefore, these findings suggest that early treatment with high-dose levetiracetam after SE termination by diazepam may protect against adverse sequelae via the inhibition of neurotoxicity induced by brain edema events.

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1. Introduction

Status epilepticus (SE) refers to neurologic emergencies that may lead to death or permanent neurologic injury. To avoid life-threatening injury, patients must be properly and quickly treated. Furthermore, SE causes 3–5% of symptomatic epilepsy (~35% of epileptic syndromes), thus SE patients are at a high risk of developing acquired epilepsy (Hesdorffer et al., 1998; Temkin, 2003; Jacobs et al., 2009). The management of SE is important to prevent mortality and the development of post-SE symptomatic epilepsy. Seizures must be treated as soon as possible and benzodiazepines (lorazepam or diazepam) are typically administered as the first-line antiepileptic drugs (AEDs). However, when these drugs fail, the second-line AEDs (phenytoin; PHT, fosphenytoin; fosPHT, valproate; VPA, and midazolam) are administered in refractory SE prior to giving phenobarbital; PB (Manno, 2011). Various clinical trials have indicated that conventional AEDs (e.g., DZP, PB, VPA, or PHT) suppressed acute seizures, but thus far there has been no success at preventing the development of post-SE acquired epilepsy under various clinical trials (Temkin, 2001, 2003, 2009). Although the mechanisms underlying the development of acquired epilepsy as part of the epileptogenic process are not well understood, the lack of efficacy of the AEDs suggests that the biological mechanisms of the acquired epilepsy process may be quite different from that of the established epileptic brain (Pitkanen et al., 2009).

Levetiracetam ([S]α-ethyl-2-oxo-1-pyrrolidine acetamide) with broad-spectrum antiepileptic effects is an established second-generation AED that is widely used in patients with either generalized or partial epilepsy (Lyseg-Williamson, 2011). In addition, levetiracetam is one of currently available candidates as the second-line AED for SE (Manno, 2011) and as an anti-epileptogenic drug (Pearl et al., 2013; Klein et al., 2012). Animal studies have shown that levetiracetam possesses anticonvulsant activity and results in neuroprotective effects (Mazzarati et al., 2004; Zheng et al., 2010). In addition, levetiracetam has been considered for the treatment of pilocarpine (PILO)–SE due to its anti-epileptogenic effects in basic and clinical studies. Two phase II clinical trials for levetiracetam indicated the possibility that it may decrease the risk of acquired epilepsy or prevent the development of acquired epilepsy (Pearl et al., 2013; Klein et al., 2012). However, the previous evidence in SE-based animal models has been conflicting and whether levetiracetam can prevent or modify epileptogenesis remains controversial (Lösch et al., 1998; Glien et al., 2002; Klitgaard and Pitkanen, 2003; Stratton et al., 2003; Gibbs et al., 2006; Brandt et al., 2007).

Temporal lobe epilepsy (TLE) is the most frequent type (75%) of symptomatic partial epilepsies that originate from the limbic regions (e.g., hippocampus and amygdala) after an initial brain insult, such as SE, stroke, and traumatic brain injury (TBI). Additionally, it is also one of the most refractory forms of epilepsy with approximately 30% of patients being unresponsive to AEDs (Engel, 1996; Kwan and Brodie, 2004). In this present study, we used a PILO-induced SE mice as a model of TLE to determine the effects of repeated administration of high-dose levetiracetam after the termination of SE by DZP. We observed that repeated high-dose levetiracetam prevented the development of brain edema in the limbic regions at the initial period of post-SE, and the incidence of spontaneous recurrent seizures.

2. Results

2.1. The mortality after SE and the incidence of spontaneous recurrent seizures following treatment with levetiracetam

Out of 155 PILO-injected mice, 62 mice (40%) died during SE and 20 mice (13%) did not develop SE. A total of 73 mice (54% of survivors) developed SE and survived, and 22 mice (20% of survivors) died by 28 days after SE. The average interval between PILO injection and the onset of SE (first convulsive seizure) was 12.31±2.50 (n=73). During SE after PILO injection, the mortality rate was 40% (n=62 of 155), and 54% of the mice (n=73 of 135) successfully developed SE. The average duration of SE (five convulsive seizures) until DZP injection was 33.47±10.45” (n=73). The duration of the latent period was approximately seven days (± 4 days, n=35) after SE with five convulsive seizures. To control the post-SE condition of the animals, SE was terminated by DZP after five convulsive seizures over a period of 90 min. The timing of spontaneous recurrent seizures (SRS) occurrence, frequency of SRS and structural changes of the brain are influenced by differences between species and strains (Shibley and Smith, 2002; Curia et al., 2008). Moreover, we observed that the incidence of SRS after SE in ICR mice varied by the number of convulsive SE in our experiments. When fewer than five convulsive SE were induced by PILO for 90 min, no SRS was observed for 28 days after SE (n=20). The mortality during the 28 days after SE was 40% (n=14 of 35) without levetiracetam and 38% (5 of 13) with 250 mg/kg of levetiracetam, but was only 12% (n=3 of 25) following treatment with 500 mg/kg levetiracetam (Fig. 1A). The incidence of convulsive seizures and nonconvulsive seizures during SRS was 76% (n=16 of 21) and 100% (n=21 of 21), respectively, during the 28 days after SE, but was only 5% (n=2 of 22) and 18% (n=4 of 22), respectively, following treatment with 500 mg/kg levetiracetam (Fig. 1B and C). However, 250 mg/kg levetiracetam did not reduce the incidence of convulsive seizures 62.5% (n=5 of 8) and nonconvulsive seizures 87.5% (n=7 of 8) as SRS after SE. In addition, most of mice after SE were very sensitive to touch and displayed aggressive behaviors; however, the behaviors were rare in high-dose levetiracetam (500 mg/kg)-treated mice, similar to naïve animals.

2.2. MRI changes were induced by high-dose levetiracetam

In the MRI study, the SI of T1WI and T2 values was measured in epileptogenic brains during the latent period after SE termination by DZP. Two days, but not three hours post-SE, T2 values were increased in the hippocampus, amygdala and piriform cortex (Fig. 2A), and increased T2 values were found in the same regions (Table 1). On the other hand, increased SI of DWI were observed in the hippocampus and amygdala and piriform cortex at three hours and two days after SE in comparison to the findings in naïve animals (Fig. 2A). The ADC was significantly decreased in the limbic regions at three hours, and was increased at two days...
after SE termination by DZP (Table 1). These changes in the limbic regions were observed to reach a maximum around two days after SE. However, these changes in the limbic regions after SE were not observed in the levetiracetam (500 mg/kg)-treated mice (Fig. 2B). Therefore, it is conceivable that the initial phase of post-SE was due to cytotoxic edema where the BBB was still intact, while by two days of post-SE, vasogenic edema may have developed due to BBB failure. These results suggested that high-dose levetiracetam treatment prevented the development of brain edema in the limbic regions after SE.

2.3. High-dose levetiracetam protected against BBB failure after SE

Vasogenic edema is generally characterized by an increase in the interstitial space fluid volume due to the loss of BBB integrity. In order to evaluate the loss of BBB integrity in vivo, the non-invasive nature of Gd-enhanced MRI using T1WI (GdET1WI) was employed. The coronal GdET1WI in control mice showed that the GdET1WI after the bolus intravenous injection with Gd-HP-DO3A was not initially hyperintense in the brain parenchymal areas (Fig. 3, pre-SE). This indicated that the Gd-HP-DO3A did not penetrate into the parenchyma through the BBB in naive mice. Hyperintensities of GdET1WI were observed in the parenchyma of the limbic regions two days after SE without levetiracetam (Fig. 3, post-SE 2d w/o). These findings indicate that the failure of the BBB can be successfully and specifically detected by GdET1WI. This BBB failure was dramatically inhibited by multiple treatments with high-dose levetiracetam after SE (Fig. 3, post-SE 2d w/LEV). These observations suggested that the leakage of Gd-HP-DO3A occurred in the brain parenchyma via the BBB of restricted limbic areas after the termination of SE, and the process of BBB failure was prevented by high-dose levetiracetam.

2.4. The increased brain water content after SE was inhibited by high-dose levetiracetam

In order to confirm the effects of levetiracetam on brain edema, especially vasogenic edema, the development of brain edema was assessed by measuring the water content in various brain regions at three hours and one, two and seven days after SE. The post-SE findings included a significant increase in the water content of the limbic regions, hippocampus, amygdala and piriform cortex at both two and seven days after SE (Fig. 4), but not other regions (data not shown). The increased brain water content was significantly reduced by treatment with high-dose levetiracetam (500 mg/kg) for two days starting 60 min after DZP injection (Fig. 4). These results indicated that the high-dose levetiracetam significantly inhibited the SE-induced brain edema, although it is hard to detect cytotoxic edema in an ex vivo brain via the water content method.

2.5. High-dose levetiracetam prevented neuronal cell loss after SE

To test whether high-dose levetiracetam shows neuroprotective effects after SE, mice were sacrificed three hours, two days and seven days after SE with or without levetiracetam treatment, and were evaluated by a histological examination using cresyl violet staining (Fig. 5A). No dead neurons were detected by cresyl violet staining in the naive animals. After SE, cresyl violet staining revealed neuronal cell death in the CA1, CA3 and DG areas at two days after SE. These findings indicated that high-dose levetiracetam (500 mg/kg) significantly reduced the number of neuronal cells lost in the CA1 and CA3 during the early phase of the latent period (Fig. 5B). Therefore, the neuropathological data suggested that the treatment with high-dose levetiracetam has a neuroprotective effect against SE-induced neuronal cell loss in this initial period of post-SE.

3. Discussion

A key result of the present study was that the early repeated treatment of high-dose levetiracetam after SE termination by DZP prevented the incidence of SRS as post-SE acquired...
epilepsy, apparently by protecting against brain edema and neuronal cell loss. The post-SE acquired epilepsy as symptomatic epilepsy conceptually occurs with the onset of epileptic seizures after a brain insult (e.g. stroke, TBI, SE, etc.), following a latent period without seizures. This process between the brain insult and occurrence of acquired epilepsy is termed epileptogenesis. The ideal target for preventing acquired epilepsy is an appropriate therapeutic drug that stops the epileptogenic process. However, classical AEDs (e.g. DZP, PB, VPA or PHT) did not prevent the development of acquired epilepsy after a prolonged febrile seizure, brain tumor, craniotomy, stroke, or TBI in human clinical trials (Temkin, 2001, 2003, 2009). Therefore, the lack of efficacy of older AEDs suggests that the biological mechanisms of epileptogenesis may be quite different from those of the seizures in the established epileptic brain. However, the mechanisms underlying this epileptogenesis are poorly understood (Pitkanen et al., 2009).

Levetiracetam, one of the newer AEDs, has a unique mechanism of action, wide therapeutic spectrum and a favorable pharmacokinetic profile, and in addition, may prevent or modify the development of acquired epilepsy (Löscher et al., 1998; Glien et al., 2002; Klitgaard and Pitkanen, 2003; Stratton et al., 2003). On the other hand, in animal studies, some reports showed that levetiracetam did not prevent acquired epilepsy when administered in post-SE models of TLE (Gibbs et al., 2006; Brandt et al., 2007). These conflicting findings regarding the effects of levetiracetam in post-SE models were likely caused by the differences in the study design (e.g. dose, timing and duration of intervention, and kind of animal models including species and strains; Dudek et al., 2008). In the present study, we evaluated the effect of high-dose levetiracetam in PILO-SE TLE model mice by performing longitudinal MRI studies, in addition to conventional studies of survival and brain histology. The timing of levetiracetam administration after SE was also found to be important,

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Fig. 2 – The influence of high-dose levetiracetam (LEV) on the brain edema, as determined on coronal T2WI and DWI after PILO-induced SE in mice. (A) Typical MR images at the level of the hippocampus (HP) and amygdala and piriform cortex (AG/PC) in representative PILO-SE mice at three hours, two days and seven days after PILO-induced SE and before PILO injection (pre-SE) in mice. The increased SI in the T2WI and DWI represents edema in the HP and AG/PC. Limbic regions show the HP (arrow head) and AG/PC (arrow). (B) Typical MR images in representative high-dose levetiracetam (500 mg/kg)-treated PILO-SE mice at three hours, two days and seven days after PILO-induced SE (post-SE) and pre-SE in mice. Limbic regions show the HP (arrow head) and AG/PC (arrow).
Gd-HP-DO3A represent Gd-HP-DO3A leaking through the brain in a pilot study (data not shown). In clinical cases, patients with effective in terms of its anti-epileptogenic effects in this model levetiracetam was administered two days after SE, it was not.

Fig. 3 - High-dose levetiracetam prevents the loss of BBB integrity after PILO-induced SE in mice. Typical coronal GdET1WI in pre- and post-SE mice following treatment with or without high-dose (500 mg/kg) levetiracetam. Top panels: a; Gd-HP-D03A in pre-SE, b; Gd-HP-D03A in post-SE (3 h), c; Gd-HP-D03A in post-SE (2d). GdET1 signals (blue) represent Gd-HP-D03A leaking through the brain parenchyma. Bottom panels: d; Gd-HP-D03A in pre-SE, e; Gd-HP-D03A+500 mg/kg levetiracetam in post-SE (3 h), f; Gd-HP-D03A+500 mg/kg levetiracetam in post-SE (2 d).

Table 1 - The effects of high-dose LEV on the MRI signal abnormalities at different times after the SE in the hippocampus, amygdala/piriform cortex and cerebral cortex.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Time</th>
<th>HP</th>
<th>AG/PC</th>
<th>CX</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>w/o</td>
<td>w/LEV</td>
<td>w/o</td>
<td>w/LEV</td>
</tr>
<tr>
<td>T2 SI</td>
<td>Pre-SE</td>
<td>648.4 ± 32.1</td>
<td>663.2 ± 22.7</td>
<td>644.7 ± 34.0</td>
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<td></td>
<td>Post-SE 3 h</td>
<td>666.6 ± 42.2</td>
<td>670.5 ± 30.2</td>
<td>702.5 ± 49.9</td>
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<td></td>
<td>Post-SE 2d</td>
<td>914.5 ± 51.6</td>
<td>861.3 ± 59.8</td>
<td>672.8 ± 72.9</td>
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<tr>
<td></td>
<td>Post-SE 7d</td>
<td>672.8 ± 61.5</td>
<td>660.2 ± 58.4</td>
<td>726.8 ± 73.9</td>
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<tr>
<td>T2 (ms)</td>
<td>Pre-SE</td>
<td>44.0 ± 7.8</td>
<td>46.7 ± 5.6</td>
<td>40.4 ± 4.5</td>
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<tr>
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<td>Post-SE 3 h</td>
<td>45.1 ± 7.6</td>
<td>47.6 ± 7.1</td>
<td>46.4 ± 4.9</td>
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<td></td>
<td>Post-SE 2d</td>
<td>72.0 ± 9.3</td>
<td>78.0 ± 11.0</td>
<td>49.0 ± 10.1</td>
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<td>Post-SE 7d</td>
<td>61.9 ± 4.1</td>
<td>55.1 ± 10.8</td>
<td>44.5 ± 5.4</td>
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<tr>
<td>DW SI</td>
<td>Pre-SE</td>
<td>357.1 ± 44.9</td>
<td>412.3 ± 40.4</td>
<td>391.2 ± 18.6</td>
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<td>Post-SE 3 h</td>
<td>487.6 ± 45.3</td>
<td>499.9 ± 35.8</td>
<td>421.5 ± 41.9</td>
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<tr>
<td></td>
<td>Post-SE 2d</td>
<td>506.0 ± 58.2</td>
<td>598.7 ± 58.0</td>
<td>429.7 ± 55.5</td>
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<tr>
<td>ADC (×10⁻³ mm²/s)</td>
<td>Pre-SE</td>
<td>0.79 ± 0.11</td>
<td>0.72 ± 0.10</td>
<td>0.73 ± 0.22</td>
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<td>Post-SE 3 h</td>
<td>2.50 ± 0.10</td>
<td>0.70 ± 0.10</td>
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<td>Post-SE 2d</td>
<td>1.00 ± 0.14</td>
<td>0.67 ± 0.08</td>
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<td>Post-SE 7d</td>
<td>0.76 ± 0.24</td>
<td>0.85 ± 0.08</td>
<td>0.74 ± 0.13</td>
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</table>

The mean SI of T2WI (T2 SI) and DWI (DW SI), T2 values (ms) and ADC (×10⁻³ mm²/s) ± S.D. in the HP, AG/PC and CX for control animals (pre-SE) and animals at three hours (post-SE 3 h), two days (post-SE 2d) and seven days (post-SE 7d) after SE. n=6 mice of each group. (ANOVA with Dunnett’s text).

with earlier administration being more effective, because when levetiracetam was administered two days after SE, it was not effective in terms of its anti-epileptogenic effects in this model in a pilot study (data not shown). In clinical cases, patients with SE are rapidly treated with intravenous levetiracetam (2000–3000 mg for 15 min) for termination of SE (Trinka, 2011; Deshpande and DeLorenzo, 2014). This suggests that this rapid treatment with levetiracetam probably decreases or prevents the acute molecular and cellular alterations resulting from SE. DZP is commonly used to treat SE, especially in Japan (Societas Neurologica Japonica, 2010). In the PILO-SE model, convulsive SE was terminated by DZP, but nonconvulsive SE was not. To attenuate nonconvulsive SE after DZP treatment, the addition of continuous levetiracetam treatment was found to be effective (Mazarati et al., 2004). A recent report indicated that interictal spikes with ripples during post-SE occurred at significantly lower rates following levetiracetam+DZP treatment compared to DZP-only controls (Lévesque et al., 2015). Under this condition, repeated treatment of high-dose levetiracetam (500 mg/kg), but not 250 mg/kg of levetiracetam, significantly inhibited the incidence of mortality, spontaneous recurrent seizures and brain edema for 28 days after SE (Figs. 1, 2 and 4, Table 1). It is difficult to explain this effect, because the cause of death is not clear and was defined as ‘sudden unexpected death in epilepsy’ (SUDEP). Although the mechanisms of SUDEP remain largely unknown and evidence- based preventative measures are lacking, SUDEP has been attributed to cardiac, respiratory and arousal abnormalities during and after seizures. Potential cellular mechanisms involve the serotonergic system, adenosine release during seizures and autonomic changes (Massey et al., 2014). The brain edema caused by initial brain insults (e.g. TBI, stroke, SE, etc.) is one of the major factors leading to mortality, and to the development of acquired epilepsy (Manley et al., 2000; Donkin and Vink, 2010; Ifland et al., 2014). The brain edema is classified into two different categories; cytotoxic and vasogenic edema, and the mechanisms of onset are different for the two categories (Klatzo, 1987). The mechanisms underlying the development of cytotoxic edema soon after convulsive SE termination by DZP may include energy depletion, and/or the
excessive release of glutamate and subsequent cell damage (Liang et al., 2007; Righini et al., 1994; Wang et al., 1996). Cell damage due to cytotoxic edema is induced by changes in the osmotic balance between the intracellular components and extracellular fluid in the neurons, astrocytes and endothelial cells constituting the neurovascular unit (Nag et al., 2009). Vasogenic edema is subsequent to the cell damage of BBB composed of cell types, especially astrocytes and endothelial cells. These findings were consistent with the fact that GdET1WI showed that BBB failure occurred after SE in this model (Fig. 3). We confirmed that there was significant pyramidal neuron loss in the hippocampus two days after SE, although we did not determine whether the cell loss was caused by apoptosis in this model (Fig. 5). Therefore, the delayed onset of vasogenic edema in this model suggests that time is needed for the loss of BBB integrity, which appears to result from prior cytotoxic edema. This sequential process of brain edema formation is important to lead to cell death during the latent period.

High-dose levetiracetam treatment protected against the development of both cytotoxic and vasogenic edema (Figs. 2 and 4, Table 1). Although it is not known how levetiracetam induces these protective effects, one possibility is that repeated high-dose levetiracetam may be associated with the prevention of SE-induced cytotoxic edema via energy depletion, which would consequently lead to the protection of BBB integrity. The development of brain damage following TBI and stroke may be similar mechanisms that lead to the formation of brain edema by SE (Donkin and Vink, 2010; Manley et al., 2000). Therefore, brain edema may be one of the major factors involved in the incidence of acquired epilepsy, and repeated high-dose levetiracetam may suppress the incidence of acquired epilepsy after these brain insults. Further prospective evaluations are needed to elucidate the mechanisms of action underlying the repeated high-dose levetiracetam treatment for reducing brain edema and other disturbances in water balance. Despite its clinical importance, the molecular mechanisms of brain water accumulation and clearance by the cerebrovascular and parenchymal homeostatic mechanisms during epileptogenesis remain poorly understood. The present findings indicated that the development of brain edema is critical in the initial process during the development of acquired epilepsy (spontaneous recurrent seizures) after SE.

4. Conclusions

In conclusion, we found that repeated treatment with high-dose levetiracetam exerted anti-SE-induced brain edema effect, and a novel mechanism of action of levetiracetam normalized the abnormalities in the water balance following SE. Brain edema associated with changes in the water environment is a common and critical finding after SE. The timing of MRI acquisition following SE is an important factor that determines whether the T2 and DWI changes show a pattern of cytotoxic or vasogenic edema. Although little is known regarding the mechanism underlying the anti-epileptic action of LEV, the ability of LEV to prevent brain damage and the development of seizures following SE makes it an important agent for the treatment of SE. Clarifying the effects of repeated high-dose levetiracetam on the balance between the damage and recovery processes during the early phase of post-SE should open new avenues for understanding the mechanism of the treatment of SE and acquired epilepsy and may be a key for planning possible treatments.

5. Experimental procedure

5.1. Experimental animals

The protocols for all animal experiments were approved by the Tokushima Bunri University Animal Care Committees.
and were performed according to the National Institutes of Health (USA) Animal Care and Use Protocol. All efforts were made to minimize the number of animals used and their suffering. One hundred fifty five male, eight-week-old ICR mice were purchased from Japan SLC (Shizuoka, Japan). All mice were maintained with laboratory chow and water ad libitum on a 12-h light/dark cycle. The utilized animals were euthanized using saturated KCl.

5.2. PILO-induced SE model and seizure assessment

The PILO-induced SE model is a well-studied animal SE-based acquired epilepsy model of TLE (Turski et al., 1989; Cavalheiro et al., 1996). To minimize the peripheral cholinergic side effects of PILO, ICR (CD-1) mice (9–10-week old), weighing 35–45 g, were injected with methyl scopolamine (1 mg/kg, intraperitoneally (i.p.) in 0.9% NaCl, Sigma Aldrich, St Louis, MO, USA) 30 min prior to PILO. A single dose of PILO was administered (290–310 mg/kg, i.p. in 0.9% NaCl, Sigma Aldrich). The animals were placed in a plastic chamber (15 × 15 × 30 cm) and their behavior was observed before and after PILO injection. The control mice received 0.1 ml/10 g saline injections. Status epilepticus (SE) was defined by the incidence of five generalized convulsive seizures (convulsive seizures; myoclonic jerking consisting of a whole body jerk with or without irregular, bilateral forelimb movements; with the kangaroo posture; modified Racine scale; 3–5), and continuous or intermittent non-convulsive seizures (nonconvulsive seizures; immobilization and, facial, vibrissal and forelimb clonus (short myoclonic jerk) with stiffened and extended tail; modified Racine scale; 1 and 2) for maximal 90 min (Racine, 1972). To terminate the SE, all mice were injected with diazepam (DZP; Cercine®, Takeda Pharmaceutical Ltd, 5 mg/kg, i.p.), and repeated if needed to suppress convulsions. The post-SE care of the animals included oral rehydration therapy to increase the number of SE survivors; all mice were fed moistened rodent chow in a petri dish inside the cage and were orally administrated a rehydration solution (OS-1®; Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan) at 2 ml a day for at least two days after SE to help replenish fluids, and thereafter when needed. Seizure monitoring began one day after SE. Mice were placed in individual blocks in a plastic chamber and video-recorded for nine hours (between 8:30 and 17:30) each day, every day, for 28 days after SE. The incidence of convulsive seizures and nonconvulsive seizures as spontaneous recurrent seizures were observed, but we did not determine the frequency of seizures during the monitoring. Seizure monitoring and the recordings were manually reviewed by well-trained investigators who were blinded to the experimental groups. Out of 155 PILO-injected mice, 62 mice (40%) died during SE, and 20 mice (13%) did not develop SE. Seventy-three mice (54% of survivors) developed SE and survived, while 22 mice (30% of survivors) died during 28 days after SE, with or without levetiracetam treatment.

5.3. Administration of levetiracetam

The high-doses of levetiracetam (LKT Labs, Inc. MN, USA) used in this study were 250 and 500 mg/kg. Levetiracetam dissolved in distilled water was orally administered at an injection volume of 0.1 ml/10 g of body weight 30 min after DZP injection, and thereafter twice a day (at 8:30 and 17:30) for 28 days.
5.4. **In vivo magnetic resonance imaging (MRI)**

The 73 mice who developed SE were anesthetized with a 1.5–1.8% isoflurane (Escaïn®, 160 mL/min: MERCK, USA)-oxygen mixture. During the MRI measurements, the body temperature was measured using a rectal thermocouple and it was kept constant at 37 ± 0.2 °C with a feedback-controlled warm-water blanket (Yamashita Technology System, Tokushima, Japan) connected to the rectal probe (Photon Control Inc. Burnaby, BC, Canada). The MRI data were acquired using an MRmini-SA (DS Pharma Biomedical, Osaka, Japan), consisting of a 1.5-Tesla permanent magnet made of Nd-Fe-B material, a compact computer-controlled console and a solenoid MRI coil with a 30-mm inner diameter. The head of an anesthetized mouse was fixed firmly on a polycarbonate holder (NEOMAX ENG, Takasaki, Japan). We obtained coronal MR images in all SE survivors using a 2D spin-echo (SE) multi-slice (MS) T1-weighted imaging (T1WI) sequence, FOV = 20 × 40 mm², matrix = 128 × 256, voxel size = 0.234 × 0.234 × 1.0 mm and a slice thickness of 1.0 mm for all 11 contiguous coronal MR images. The typical T1WI parameters were TR (ms)/TE (ms) = 500/9, NEX = 8. After T1WI were acquired, T2WI-weighted images (T2WI) and diffusion-weighted images (DWI) were obtained with the following parameters: the T2WI parameters were TR (ms)/TE (ms) = 2500/69, NEX = 4, and the DWI parameters were TR (ms)/TE (ms) = 2500/69, b value = 850 s/mm², NEX = 4. To measure the T2 relaxation time and apparent diffusion coefficient (ADC) from T2WI and DWI, respectively, three regions (the cerebral cortex and limbic regions (hippocampus, amygdala and piriform cortex) (Fig. 2) on a brain slice (bregma –1.70 to –2.06 mm) according to mouse brain atlas (Paxions and Franklin, 2012) were defined as regions of interest (ROI) in the brain, and the mean value of the signal intensity (SI) in the three ROIs of six animals treated with or without levetiracetam at each time point was determined using the INTAGE Realia Professional software program (Cybernet Systems Co. Ltd., Tokyo, Japan). To eliminate the non-brain regions from the MR images, the non-brain regions were removed from each MRI image (Figs. 2 and 3). In order to investigate the BBB permeability after SE, all SE survivors were bolus injected via a femoral vein with 0.4 mmol/kg Gd-HP-DO3A (gadoteridol, ProHance®, Bracco Diagnostic, Inc.) as a nonionic gadolinium complex MRI contrast agent. It is well known that Gd-HP-DO3A does not cross the intact BBB and therefore, does not accumulate in the normal brain parenchyma (Roberts and Noseworthy, 2005; Ichikawa and Itoh, 2011; Danjo et al., 2013). Therefore, any accumulation indicates an increase in BBB permeability.

5.5. **Brain water content**

The brain was rapidly removed and dissected out into six regions; the cerebral cortex, hippocampus, amygdala/piriform cortex, caudate putamen, diencephalon (including thalamus and hypothalamus), pons/medulla and cerebellum (Watanabe et al., 2013). The wet weight of each brain region was measured, these were then placed in an oven (100 °C) for 24 h and then re-weighed (dry weight). The percentage of brain water content was calculated as [(wet weight – dry weight)/wet weight of brain tissue × 100%] (Hama et al., 2012). Comparisons between vehicle- and levetiracetam-treated mice were made at three hours, two days and seven days after SE (n=10 mice for all groups).

5.6. **Histological and stereological analysis**

For the histological analyses, mice were deeply anesthetized and euthanized with sodium pentobarbital (50 mg/kg, Sigma) and perfused transcardially with heparinized 0.1 M phosphate-buffered saline (PBS), followed by 4% paraformaldehyde (PFA) in 0.1 M PBS, pH 7.4. After perfusion, brains were removed and post-fixed overnight in 4% buffered PFA at 4 °C and then were cryoprotected in 30% sucrose. Serial frozen sections (10 μm) were cut on a sliding Cryostat (Leica, CM3050 S) and mounted onto slides, and then were dried overnight. The sections were stained with 0.5% Cresyl violet acetate, rinsed in distilled water, dehydrated, differentiated and coverslipped with Permount™ Mounting Medium (Fisher Scientific Inc., Tokyo). A quantitative analysis of hippocampal cell loss in cresyl violet-stained sections were performed by an observer blinded using unbiased stereological methods. Nissl-stained neurons in 10 square counting frames (25 × 25 μm) in CA1, CA3 and the dentate gyrus were counted in both the right and left dorsal hippocampi, and the numbers for each side were averaged into single values in each animal (n=5 for all groups).

5.7. **Statistical analysis**

All data are expressed as the means ± standard deviation (SD). The differences between the mean values for each group regarding mortality, the incidence of spontaneous recurrent seizures, the water content and MRI parameters were analyzed using the one-way ANOVA followed by Dunnet’s test. A p-value less than 0.05 was considered to be statistically significant.

**Conflicts of interest**

None declared.

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