Blood-brain Barrier Damage in the Pentylenetetrazole Generalized Seizure Model Mice Using Gadolinium-enhanced Magnetic Resonance Imaging

Sonoko Danjo¹,²*, Junichi Danjo¹, Ichiro Ishikawa¹, Yugo Kadotomo¹ and Yu Nakamura¹

¹Department of Neuropsychiatry, Kagawa University School of Medicine, Miki, Kita, Kagawa 781-0793, Japan.
²Laboratory of Molecular and Cellular Neuroscience, Kagawa School of Pharmaceutical Sciences, Tokushima Bunri University, Sanuki, Kagawa 769-2193, Japan.

Authors’ contributions

This work was carried out in collaboration between all authors. Author SD designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors JD, II and YK managed the analyses of the study. Authors JD and YN managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Although epilepsy is a common disease, the pathological process has not been sufficiently elucidated. Previous studies have reported that blood-brain barrier (BBB) damage is involved in epileptogenesis. Our aim was to non-invasively and sequentially evaluate BBB damage in a mouse model of generalised seizure, using Gadolinium (Gd)-enhanced magnetic resonance imaging using T₁ weighting (GdET₁WI). In addition, we assessed whether or not valproate (VPA) could prevent BBB damage.

Methods: Mice were kindled with the daily intraperitoneal administration of 40 mg/kg pentylenetetrazole (PTZ). After each PTZ injection, convulsive behaviours were observed, and
seizures were scored from 0 to 5. Five consecutive scores of 4 or 5 were required to ensure kindling. We evaluated the changes in the BBB damage using the signal intensity (SI) ratio of GdET1WI. The SI was sequentially measured at baseline, score 1, score 3, PTZ-kindled, and 1-week post-kindled after PTZ withdrawal. In addition, the SI was measured in mice pretreated with VPA before PTZ injection.

**Results:** The SI values (means±standard error of the mean) at score 1, score 3, PTZ-kindled, and post-kindled increased to 0.70%±0.22%, 7.17%±1.86%, 7.43%±1.60%, and 6.82%±1.27%, respectively, compared to baseline. All values (except at score 1) were significantly higher than those at baseline (p-value < 0.05). We did not observe significant differences between score 3 or post-kindled and PTZ-kindled. In VPA-pretreated mice, the SI significantly increased to 8.77%±1.57% compared to baseline, although convulsions were fully controlled.

**Conclusion:** Our data suggest that BBB damage started before PTZ-induced kindling was acquired. The BBB damage was irreversible after PTZ-induced kindling. In addition, VPA prevented epileptic convulsive seizures but could not suppress BBB damage in PTZ-kindled mice.

**Keywords:** Blood-brain barrier; gadolinium-enhanced magnetic resonance imaging using T1 weighting; epileptogenesis; pentylenetetrazole; generalised seizure.

**ABBREVIATIONS**

**BBB:** Blood-brain barrier; **DC:** Diencephalon; **Gd:** Gadolinium; **GdET1WI:** Gadolinium-enhanced magnetic resonance imaging using T1 weighting; **Gd-HP-DO3A:** Gadolinium-1,4,7,10-tetraazacyclododecane; **MRI:** Magnetic resonance imaging; **PTZ:** Pentylenetetrazole; **ROI:** Regions of interest; **SI:** Signal intensities; **VPA:** Valproic acid.

**1. INTRODUCTION**

Although epilepsy is a common disease, affecting 1% of the world’s population, its cause has not been sufficiently elucidated [1]. In epilepsy research, it is very important to evaluate or examine the process of epileptogenesis. In epileptogenesis, excessive neuronal excitation occurs, excessively releasing the neurotransmitter glutamate and thereby causing neuronal cell death, leading to the further development and deterioration of epilepsy [2]. Elucidating the process of epileptogenesis and preventing its progress will lead to the fundamental treatment of epilepsy and bring benefits to many patients.

The blood-brain barrier (BBB) strictly regulates the migration of molecules through the blood in the brain to maintain homeostasis in the brain and is deeply involved in the pathophysiology of various central nervous diseases, including epilepsy [3,4,5,6]. Regarding partial seizures, it has been reported that nerve cell death along with the reconstruction and reorganisation of the neuron network progress in the epileptic focus, leading to the deterioration of epilepsy [2]. In the partial seizure model, it has been reported that inflammation associated with neuronal cell death induces weakening of the BBB, which enters a negative spiral and induces BBB disorders, resulting in the acquisition or exacerbation of convulsive seizures [7]. This means that BBB damage is an organic disorder at the macro level. However, regarding generalised seizures, there is no focus formation in principle, and BBB damage is, at first glance, not an organic disorder but rather a functional disorder. It has been reported that BBB damage occurs in the generalised seizure model; however, the relationship between the abnormal excitement of the glutamatergic neurons causing convulsions and BBB dysfunction is unknown, as are the factors causing changes in the BBB permeability [8,9].

We established a method for the noninvasive/spatial-temporal evaluation of changes in the BBB permeability using gadolinium(Gd)-enhanced magnetic resonance imaging (MRI) with T1 weighting (GdET1WI), which is an MRI method for rodents. Using GdET1WI, we confirmed the transient changes in the BBB permeability upon acute single administration of pentylenetetrazole (PTZ), which creates a mouse model of generalised seizure [10,11]. However, this merely confirmed the BBB hyperpermeability at the time of the appearance of generalised seizures, and when the changes in BBB permeability occur during epileptogenesis has not been sufficiently elucidated.

In the present study, we aimed to non-invasively and sequentially examine the changes in BBB...
permeability during epileptogenesis using PTZ-kindled mice. Furthermore, we also examined the effects of valproate (VPA), which is an antiepileptic drug for treating generalised seizures, on the changes in the BBB permeability.

2. EXPERIMENTAL PROCEDURES

2.1 Experimental Animals

The protocols for all animal experiments were approved by the Tokushima Bunri University Animal Care Committee according to the National Institutes of Health (USA) Animal Care and Use Protocol. All efforts were made to minimise the number of animals used and their suffering. Ten-week-old male ICR mice were purchased from Japan SLC (Shizuoka, Japan). All mice were maintained with laboratory chow and water available ad libitum on a 12-h light/dark cycle.

2.2 Schedule of PTZ Administration

The animals were placed in a plastic chamber (15×30×40 cm), and their behaviour was observed before and after PTZ administration. After the animals adopted a resting posture, they were injected with varying doses of PTZ (Sigma-Aldrich Corp.) Control mice received 0.2 ml/10 g saline, and non-kindled mice were acutely treated with subconvulsive doses (40 mg/kg) of PTZ. In the kindling experiments, the subconvulsive doses of PTZ injections were repeated once a day between 8:00 AM and 9:00 AM to produce the kindled mice. After each PTZ injection, the convulsive behaviours were observed for 30 min, and resultant convulsions were classified and scored according to a previous report [12] as follows: 0, normal; 1, immobilization, sniffing; 2, head nodding, facial and forelimb clonus (short myoclonic jerk); 3, continuous myoclonic jerk, tail rigidity; 4, generalized limbic seizures with kangaroo posture or violent convulsion; 5, continuous generalized seizures (tonic or clonic–tonic convulsions). In this study, “continuous myoclonic jerk with clonic convulsions” was given a score of 3, and “generalised seizures (tonic or clonic–tonic convulsions)” were assigned scores of 4 and 5.

2.3 Assessment of BBB Failure in Living Mice Using GdET\(_3\)WI

The MRI data were acquired using a 1.5-Tesla MRmini-SA (DS Pharma Biomedical Co., Ltd., Osaka, Japan), consisting of a solenoid MRI coil with a 30-mm inner diameter. One minute after the injection of Gd-HP-DO3A (gadoteridol, ProHances\(^{®}\); Bracco Diagnostics, Inc.), mice were anaesthetized with a 1.5%-2.0% isoflurane (160 mL/min, Escains; MERCK, USA)-oxygen mixture, and the head of the anaesthetized mouse was fixed firmly in a polycarbonate holder. MRI was performed under anaesthesia, and the body temperature measured using a rectal thermocouple was kept at 37.5±0.2 °C using a feedback-controlled warm-water blanket (Yamashita Tech System, Tokushima, Japan) connected to a rectal probe (Photon Control Inc., BC, Canada) during the scanning.

To investigate the BBB permeability, mice were bolus-injected via the tail vein with a mixture of 0.4 mmol/kg Gd-HP-DO3A as a nonionic Gd complex MRI contrast agent while awake. Gd-HP-DO3A does not cross the intact BBB and therefore does not accumulate in the normal brain parenchyma [10,11]. Five minutes after the intravenous (i.v.) injection of Gd-HP-DO3A, GdET\(_3\)WI was performed. Scans were acquired by a two-dimensional multi-slice spin echo sequence using the following parameters: TR (ms)/TE (ms)/flip angle (FA)=500/9/90°, field of view (FOV)=30×60 mm, matrix=128×256, voxel size=0.234×0.234×1.0 mm, number of excitations (NEX)=4, number of slices=11.

To evaluate the BBB permeability using GdET\(_3\)WI, the parenchyma of the diencephalon (DC) on a brain slice (bregma -1.70 to -2.06 mm) according to the mouse brain atlas [13] was defined as a region of interest (ROI) in the brain, avoiding the inclusion of major vascular structures, and the mean value of the signal intensity (SI) in the ROIs of four animals was determined. Each SI in the ROI was measured by GdET\(_3\)WI using the INTAGE Realia Professional software program (Cybernet Systems Co., Ltd., Tokyo, Japan). The same set of ROIs was applied for pre-contrast (without Gd-HP-DO3A) and post-contrast (with Gd-HP-DO3A) images in a given mouse, and the enhancement ratio was calculated as the ratio of the SI on post-contrast images to that on pre-contrast images as follows:

\[
\text{Enhancement ratio (\%) } = \frac{(\text{SI}_{w/o \text{Gd-HP-DO3A}} - \text{SI}_{w/o \text{Gd-HP-DO3A}})}{(\text{SI}_{w/o \text{Gd-HP-DO3A}})} \times 100
\]

2.4 Administration of VPA

VPA (Wako Pure Chem. Ltd., Japan) was dissolved in 0.5% (w/v) carboxymethyl cellulose
400 solution (Wako Pure Chem. Ltd.) and orally administered (p.o.; 400 mg/kg) at an injection volume of 0.05 ml/10 g of body weight 60 min before PTZ injection. The dose of VPA was selected based on the findings of previous reports [11].

2.5 Statistical Analyses

All data were expressed as the mean±standard error of the mean (SEM). The differences between the mean values for each group of SI values in the ROIs were analysed using a one-way analysis of variance (ANOVA) followed by Dunnett’s tests, and behavioural scores were analysed using the Kruskal–Wallis test. The significance level was established at p < 0.05.

3. RESULTS

3.1 Changes in the BBB Permeability in Kindled Mice

We investigated the changes in the BBB permeability during the generalised seizures using PTZ-kindled mice. Coronal GdET\textsubscript{1}WI in control mice showed that the SI of GdET\textsubscript{1}WI after bolus i.v. injection with Gd-HP-DO3A was not initially hyperintense in the brain parenchymal areas (Fig. 1a). This indicated that the Gd-HP-DO3A did not penetrate the parenchyma through the BBB in naïve mice.

We measured the SI when PTZ-kindled mice were in a non-convulsive state. When the mice were PTZ-kindled, the SI demonstrated a significant increase of 7.43±1.60% compared to the baseline (Fig. 1b and Table 1). GdET\textsubscript{1}WI was also performed one week following withdrawal (post-kindled). Regarding the post-kindled mice, as with PTZ-kindled mice, leakage of Gd-HP-DO3A in the brain parenchyma was confirmed. Regarding the post-kindled mice, the SI demonstrated a significant increase of 6.82±1.27% compared to the baseline (Fig. 1c and Table 1). No significant difference was observed in the SI between the post-kindled mice and the PTZ-kindled mice. These results suggested that the BBB damage was not changed for at least one week after being PTZ-kindled irreversibly.

3.2 Changes in the BBB Permeability during the Kindling Process

Next, we examined exactly when this irreversible BBB damage occurred during the PTZ-kindling process. During kindling, GdET\textsubscript{1}WI was performed when score-1 convulsions appeared (score-1) and when score-3 clonic convulsions appeared (score-3) (Fig. 2b and 2c). Regarding the SI values, increases of 0.70±0.22% and 7.17±1.86% were observed for scores 1 and 3, respectively (Fig. 2b, 2c and Table 2). Regarding score 3, a significant increase was observed compared to the baseline. Based on these results, we concluded that BBB damage began when clonic convulsions occurred.

3.3 Effects of VPA on Changes in the BBB Permeability

Next, we examined BBB damage when using VPA, a drug for treating general seizures. Using VPA, which is the first-line drug for treating generalised seizures, the effects on BBB damage were confirmed. The mice in which convulsions were induced using PTZ after kindling (PTZ-kindled with PTZ) and the mice in which VPA premedication was conducted and convulsions were induced using PTZ (PTZ-kindled with PTZ + VPA) were compared to those in the PTZ-kindled mice (Fig 3b, 3c and 3d). Regarding convulsive behaviour, tonic-clonic convulsions occurred in the PTZ-kindled with PTZ mice (Fig. 3). In addition, the PTZ-kindled with PTZ mice exhibited BBB damage with no significant difference in the SI value compared to the PTZ-kindled mice (Table 3). In contrast, the convulsive behaviour was completely suppressed in the PTZ-kindled with PTZ + VPA mice (Fig. 3). However, no significant difference was observed in the SI value compared to the PTZ-kindled mice (Table 3). Based on these results, we concluded that VPA suppresses the PTZ-induced convulsive behaviour but has no effect on the BBB damage that has already occurred and does not repair BBB damage.

4. DISCUSSION

Recently, neuroimaging by MRI has played an important role in both clinical situations and the evaluation of experimental epileptic animals. In this study, we examined the changes in the BBB permeability in epileptogenesis using GdET\textsubscript{1}WI. Because epileptogenesis can take a long time to develop, it is essential to recognise the initial change and examine the subsequent changes over time. Conventional BBB research methods have evaluated the BBB permeability and damage by observing whether or not intravenously injected pigments possessing BBB impermeability (Evan’s blue dye, horseradish...
peroxidase, etc.) leaked into the brain parenchyma. However, with these research methods, it is impossible to evaluate the changes in the BBB permeability over time for each individual animal. In contrast, molecular imaging using MRI contrast media is useful for conducting spatial-temporal analyses of BBB disorders and examining the intracerebral conditions [14,15].

GdET\_WI can semi-quantitatively observe the leakage of Gd into the brain parenchyma as an indicator of BBB disorders [10]. Evaluating the epileptic pathology by MRI is a very attractive technique, as it can be non-invasively and chronologically performed. MRI is useful as an examination method for evaluating the epileptic conditions as it allows the non-invasive observation of the morphological/functional physiological changes of the brain from the same individual.

4.1 BBB Damage will Persist Even after the Withdrawal of PTZ Following Kindling

Once kindled, BBB damage was observed even in a non-convulsive state irreversibly. We previously reported on the transient changes in the BBB permeability in acute PTZ single-injected mice [11]. In those mice, the BBB permeability was increased 15 minutes after PTZ administration. However, no marked changes in the BBB permeability were confirmed after 24 h, suggesting that the change in the BBB permeability of acute PTZ single-injected mice was transient and reversible.

The invasion of inflammatory cells due to BBB damage has been reported [9,16]. In animal models of temporal lobe epilepsy and status epilepticus, inflammatory reactions have been reported to significantly exacerbate the convulsive activity [17]. This study suggested that kindled mice may suffer the further deterioration of epileptic pathology due to the fact that the BBB is irreversibly damaged.

4.2 Changes in the BBB Appeared in the Event of a Non-convulsive State in the Middle of Kindling

In the kindling process, BBB hyperpermeability was confirmed 24 h after PTZ-induced convulsions in the stage of score-3. This suggested that BBB hyperpermeability was observed in the middle of PTZ kindling. BBB damage after kindling has been reported in previous studies. However, BBB hyperpermeability in the middle of kindling has not been reported as of yet. In this study, using an MRI apparatus for small animals, it was possible to evaluate the BBB permeability in the same individual over time. These results suggested that therapeutic intervention from before to during epileptogenesis is necessary.

**Fig. 1. The longitudinal monitoring of BBB failure following repeated injection of PTZ.**

Typical coronal GdET\_WI findings in control (a), PTZ-kindled (c), and post-kindled mice (d) 5 min after Gd-HP-DO3A (0.4 mmol/kg) injection. GdET\_WI produced pseudo-colored images that were reconstructed and superimposed over the T\_WI scans using an image processing software program (INTAGE Realia Professional software program). Inside the white lines is the brain parenchyma

**Fig. 2. The BBB permeability in the course of the PTZ-kindling mice**

Typical coronal GdET\_WI findings in control (a), score-1 (b), score-3 (c), and PTZ-kindled mice (d) 5 min after Gd-HP-DO3A (0.4 mmol/kg) injection. GdET\_WI produced pseudo-colored images that were reconstructed and superimposed over the T\_WI scans using an image processing software program (INTAGE Realia Professional software program)
Fig. 3. Effects of AEDs on PTZ-induced BBB failure

Typical coronal GdET1WI findings in VPA-pretreated mouse brains following injection with PTZ. (a) Control mouse with Gd-HP-D03A (0.4 mmol/kg), (b) PTZ-kindled mouse with Gd-HP-D03A (0.4 mmol/kg), (c) PTZ-kindled mouse with Gd-HP-D03A (0.4 mmol/kg)+PTZ (40 mg/kg), (d) PTZ-kindled mouse with Gd-HP-D03A (0.4 mmol/kg)+PTZ (40 mg/kg)+VPA (400 mg/kg). Behavioral changes were observed for 1 min after PTZ injection, and these mice were scored according on a 1–5 scale (see Section 2.2.)

Table 1. Changes in the enhancement ratio of BBB permeability after PTZ kindling

<table>
<thead>
<tr>
<th>Enhancement ratio</th>
<th>n</th>
<th>P (vs. control mice)</th>
</tr>
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<tbody>
<tr>
<td>Control mice</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PTZ-kindled mice</td>
<td>7.43 ± 1.60</td>
<td>4</td>
</tr>
<tr>
<td>post-kindled mice</td>
<td>6.82 ± 1.27</td>
<td>4</td>
</tr>
</tbody>
</table>

The longitudinal changes in the BBB permeability in control, PTZ-kindled, and post-kindled mice in DC. The values represent the enhancement ratio (%) in the ROIs of the DC after Gd-HP-D03A injection. The values represent the mean±SEM. The statistical significance was assessed using a one-way ANOVA followed by Dunnett’s tests. P values are compared with control mice.

Table 2. The enhancement ratio of the BBB permeability during PTZ kindling

<table>
<thead>
<tr>
<th>Enhancement ratio</th>
<th>n</th>
<th>P (vs. control mice)</th>
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</thead>
<tbody>
<tr>
<td>Control mice</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>score1 mice</td>
<td>0.70 ± 0.22</td>
<td>4</td>
</tr>
<tr>
<td>score3 mice</td>
<td>7.17 ± 1.86</td>
<td>4</td>
</tr>
<tr>
<td>PTZ-kindled mice</td>
<td>7.43 ± 1.60</td>
<td>4</td>
</tr>
</tbody>
</table>

The longitudinal changes in the BBB permeability in control, score-1, score-3, and PTZ-kindled mice in DC. The values represent the enhancement ratio (%) in the ROIs of the DC after Gd-HP-D03A injection. The values represent the mean±SEM. The statistical significance was assessed using a one-way ANOVA followed by Dunnett’s tests. P values are compared with control mice.

Table 3. The effects of VPA on the enhancement ratio of the BBB permeability in PTZ-kindled mice

<table>
<thead>
<tr>
<th>Enhancement ratio</th>
<th>n</th>
<th>P*</th>
<th>P**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control mice</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PTZ-kindled mice</td>
<td>7.43 ± 1.60</td>
<td>4</td>
<td>0.0135</td>
</tr>
<tr>
<td>PTZ-injected mice</td>
<td>10.15 ± 2.12</td>
<td>4</td>
<td>0.0015</td>
</tr>
<tr>
<td>VPA-pretreated</td>
<td>8.77 ± 1.57</td>
<td>4</td>
<td>0.0045</td>
</tr>
</tbody>
</table>

The changes in the BBB permeability in control, PTZ-kindled, PTZ-injected, and VPA-pretreated mice in DC. The values represent the enhancement ratio (%) in the ROIs of the DC 5 min after Gd-HP-D03A injection. The values represent the means±SEM. The statistical significance was assessed using a one-way ANOVA followed by Dunnett’s tests. P* values are compared with control mice and PTZ-kindled mice, respectively. Statistical significance was assessed using the Kruskal–Wallis test followed by Dunnett’s tests.
4.3 VPA Suppresses Convulsive Seizures but does not Repair BBB Damage

Regarding the effects of VPA premedication in kindled mice, while PTZ-induced convulsions were completely suppressed, BBB permeability remained elevated. These findings suggest that VPA, which is a drug used to treat general seizures, was effective against ictogenesis but not against epileptogenesis.

However, a previous report found that VPA improved the BBB damage following spinal cord injuries [18]. In addition, another report found that VPA prevented BBB damage following subarachnoid haemorrhaging and mitigated apoptosis of the nerve cells [19]. Furthermore, in our previous studies, VPA suppressed both convulsive seizures and BBB hyperpermeability in rapidly PTZ single-treated mice. However, in those studies, VPA was administered immediately after the onset, making it difficult to compare the outcomes with those under conditions of generalised epilepsy, which gradually causes BBB damage. Whether or not BBB damage can be prevented by administering VPA during epileptogenesis is an issue that should be explored in the future.

5. CONCLUSION

The present findings suggest that regulating BBB damage during the formation of generalised epilepsy suppresses the development of epilepsy and maybe a new therapeutic target for epilepsy.

CONSENT

It is not applicable.

ETHICAL APPROVAL

The protocols for all animal experiments were approved by the Tokushima Bunri University Animal Care Committee according to the National Institutes of Health (USA) Animal Care and Use Protocol. All efforts were made to minimise the number of animals used and their suffering.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES


