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Research article

A gain-of-function study of amelioration of pentylenetetrazole-induced seizures by endogenous prostaglandin D_2

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ABSTRACT

We previously showed that knockout mice of hematopoietic prostaglandin (PG) D synthase (H-PGDS) produce less PGD_2 to exacerbate pentylenetetrazole (PTZ)-induced seizures. Here, we adopted a gain-of-function strategy and used transgenic mice that over-express human H-PGDS enzyme, to elucidate the role of overproduction of endogenous PGD_2 in PTZ-induced seizures. H-PGDS-transgenic mice showed the elevated level of a urinary metabolite of PGD_2, tetranor-PGDM, 3.3- and 2.8-fold higher than the wild-type littermates under the basal condition and after the PTZ administration, respectively, without significantly changing the urinary concentration of a PGE_2-metabolite, tetranor-PGE_2. The intensity of PTZ-induced seizures was decreased in H-PGDS-transgenic mice as evident by the increased seizure onset latency, and a decrease in total duration of generalized tonic-clonic seizures and a total number of EEG seizure spikes during the postictal period (84 s, 17 s, and 5.3/min, respectively), as compared to wild-type mice (53 s, 24 s, and 12.6/min, respectively). These results indicate that overproduction of endogenous PGD_2 decreased PTZ-induces seizures.

1. Introduction

Epilepsy is a chronic disease characterized by spontaneous recurrent seizures that affect more than 50 million people across the world and is observed across all age groups with varying levels of severity. Inflammatory markers such as prostaglandins (PGs) are released whenever there is injury or trauma to protect the integrity of the biological system. PGD_2 is a major prostanoid produced in the central nervous system (CNS) of mice, rats, and humans, and functions as a neuromodulator for sleep-wake regulation and neuroinflammation [7,12]. Nevertheless, the role of PGE_2 in the pathogenesis of seizures cannot be ignored because EP_2, a PGE_2 receptor, agonist showed anticonvulsant effects [9].

PGD_2 is synthesized by 2 different types of PGD synthases (PGDS), *i.e.*, lipocalin-type PGDS and hematopoietic PGDS (H-PGDS). It elicits its action through binding to DP_1 receptors (DP_1R) or chemo-attractant receptor CRTH2 (DP_2R) [12]. Since H-PGDS derives PGD_2 production during neuronal injury and pathological conditions, henceforth, we focused on H-PGDS enzyme. It is also now well accepted that PGD_2 produced by the H-PGDS has been implicated in neuroinflammation [11] and is associated with the activation of microglia and astrocytes in a mouse model of demyelination [7]. We previously showed that deletion of the H-PGDS gene resulted in the decreased endogenous PGD_2 production that resulted in enhanced pentylenetetrazol (PTZ)-induced seizures, and concluded that H-PGDS-derived PGD_2 acts *via* DP_1Rs to suppress PTZ-induced seizures [4]. This loss of function study indicated that the H-PGDS deletion results in high-intensity seizures, inferring its role in seizure inhibition and suggest that the gain of function of H-PGDS may be an effective therapy for seizure inhibition. We hypothesized that over-production of endogenous PGD_2 will be effective in seizure suppression. Hence, by using human H-PGDS over-expressing transgenic (H-PGDS Tg) mice, we found that H-PGDS Tg mice produce more PGD_2 that eventually suppressed PTZ-induced seizures.

2. Materials and methods

Human H-PGDS over-expressing mice (an S-55 line) and their wild-type littermates of FVB background [3] were reared and maintained at Oriental Bioservice (Kyoto, Japan), until use. For behavioral studies, mice, weighing 25-35 g (13-18 weeks), were housed in an insulated sound-proofed recording room maintained at an ambient temperature of 24 ± 0.5 °C with a relative humidity of 60 ± 5% on an

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automatically controlled 12-h light/dark cycle (light on at 0800 h). They had free access to food and water. All experiments were performed in accordance with the guidelines laid down for the proper use and maintenance of animals. The experimental protocols were approved by the Animal Care Committee of International Institute of Integrative Sleep Medicine, University of Tsukuba (approval no. 16086), and every effort was made to minimize the number of animals used as well as any pain and discomfort.

Surgeries were performed under anesthesia using pentobarbital (50 mg/kg, intraperitoneally). Under aseptic conditions, mice were chronically implanted with electroencephalogram (EEG) and electromyogram (EMG) electrodes for polysomnography, as previously described [4–6]. After 8-10 days of postoperative recovery, the mice were placed in experimental cages for a 4-day habituation/acclimatization period and connected with counterbalanced recording leads. All mice were subjected to video-EEG/EMG recordings on 2 consecutive days during which the vehicle and the drug were administered. On day-1, the mice were treated with vehicle (saline, 10 ml/kg, i.p.), and on day-2, with PTZ (Sigma Aldrich; 50 mg/kg, i.p.).

Cortical EEG and EMG signals were amplified and filtered (EEG, 0.5-30 Hz; EMG, 20-200 Hz), then digitized at a sampling rate of 128 Hz and recorded/analyzed by using SleepSign software (Kissei Comtec, Nagano, Japan). The data analysis was performed as per the details given in Kaushik et al., 2014 [4]. Briefly, the latency to seizure onset was defined as the time from injection to the appearance of the first EEG seizure spike, and the duration of generalized-tonic-clonic seizures (GTCS) were calculated based on video-EEG/EMG recordings. Postictal EEG depression is characterized by low-amplitude, slow-wave EEG signal that occurs following behavioral seizures and, thus, the duration of this depression was calculated as the time between termination of behavioral seizures and reappearance of EEG signal with an amplitude exceeding 100 μV. Postictal EEG signal was contaminated with electrographic seizure (EEG seizure spikes) characterized by sharp, low frequency (4 Hz) and high amplitude (more than twice the baseline) EEG waves which appeared similar to slow-wave activity. The EEG signals exceeding twice the baseline amplitude were scored as EEG seizure spikes and counted during behavioral seizures and during 1-h following postictal EEG depression. EEG seizure spikes were detected and counted by using the peak analysis function of Origin Lab v8.5 Pro software (Data Analysis and Graphing Software, USA).

Stable PGD_2 metabolite in urine, 11,15-dioxo-9-hydroxy-2,3,4,5-tetranorprostan-1,20-dioic acid (tetranor-PGDM), was analyzed by mass spectrometry as described previously [8]. Briefly, urine sample of pre- and post-PTZ induced seizure were collected for 12 h from wild-type mice and H-PGDS Tg mice. The urine samples were diluted with 0.5 ml of water and acidified by HCl (final pH ~ 3), and 50 μl (5 ng) of deuterium-labeled tetranor PGDM and tetranor-PGEM (9,15-dioxo-11α-hydroxy-,2,3,4,5-etranorprostan-1,20-dioic acid) (Cayman Chemical, Ann Arbor, MI) were added as an internal standard. The mixtures were purified by solid-phase extraction using Sep-Pak Plus C18 cartridges (Waters, Milford, MA). The cartridges were activated with 5 ml of ethanol and equilibrated with 5 ml of water. The urine sample was applied to the cartridge, which was washed with 5 ml of 5% (v/v) acetonitrile followed by 5 ml of n-hexane. The analyte and internal standard were eluted from the cartridge with 5 ml of ethyl acetate. The eluate was collected and dried *in vacuo*. The resulting residue was reconstituted in 100 μl of 10% (v/v) acetonitrile. The sample solution containing tetranor PGDM and tetranor PGEM was then introduced into an API3000 LC–MS/MS system (Applied Biosystems, Foster City, CA) equipped with an electrospray (Turbospray) interface. The HPLC column was a 150 × 2.1-mm i.d. Inertsil ODS-3 (GL Sciences, Tokyo, Japan). LC separation was carried out using a mobile phase consisting of 0.01% (v/v) acetic acid (solvent A) and acetonitrile (solvent B). The LC–MS/MS was operated in the negative ion mode. The urinary tetranor PGDM was measured in the selected reaction monitoring mode. The transitions monitored were *m/z* 327-143 for the endogenous material and *m/z* 333-149 for the internal standard. The creatinine concentration was measured by the assay kit (Wako Pure Chemical, Osaka, Japan).

All data were expressed as the mean ± SEM. The data were compared using a paired t-test, with each animal serving as its own control for comparing before and after PTZ injection data. PGD_2 and PGE_2 metabolite data were compared using Two-Way ANOVA followed by Bonferroni post-hoc comparisions. In all cases, $p \leq 0.05$ was considered significant. Statistical analysis was performed by using IBM SPSS Statistics (Version 22).

3. Results

We examined the PGD_2 levels in human H-PGDS Tg and wild-type mice after saline and PTZ administration. Since PGD_2 is metabolized very quickly we estimated its stable metabolite, tetranor-PGDM, in the urine after administration of saline (10 ml/kg, i.p.) or PTZ (50 mg/kg, i.p.; n = 8-9 each group). H-PGDS Tg mice showed 3.3-fold higher basal levels of urinary tetranor-PGDM (19.57 ± 2.15 ng/mg Cre; $p = 0.05$) as compared to their wild-type littermates (6.02 ± 0.49 ng/mg Cre). PTZ-induced seizures further increased the urinary tetranor-PDGM, however, the seizure-induced increase in tetranor-PGDM was only 2-fold in H-PGDS Tg mice (54.22 ± 8.40 ng/mg Cre) as compared to the increase in their wild-type littermates (27.90 ± 3.47 ng/mg Cre; $p = 0.000$; Fig. 1A). Whereas the wild-type mice showed a 4.6-fold increase in tetranor-PGDM after PTZ-induced seizures (from 6.02 ± 0.49 to

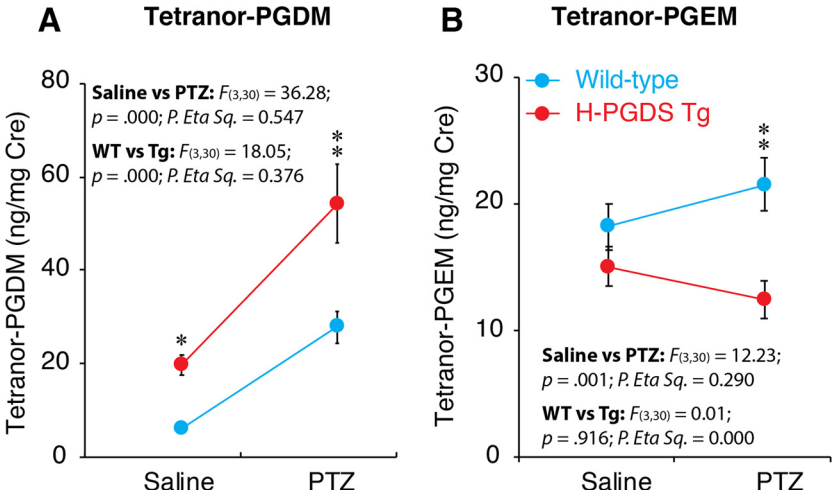

Fig. 1. Human H-PGDS over-expressing (H-PGDS Tg) mice produce more PGD_2 as compared to their wild-type littermates. Tetranor-PGDM **(A)** and tertanor-PGEM **(B)** changes in H-PGDS Tg (Tg) and wild-type (WT) mice. Urine samples were collected 12-h after saline or PTZ injection (i.p.) from wild-type and H-PGDS Tg mice and plotted. Data presented as the mean ± SEM (n = 8-9). Two-way ANOVA was used to determine the interaction between mouse-type and treatment-type and Bonferroni post-hoc test was used for multiple comparisons. *p ≤ 0.05, **p ≤ 0.01, wild-type *vs* H-PGDS Tg mice. P Eta Sq.: Partial Eta Squared.

27.90 ± 3.47 ng/mg Cre; $p = 0.002$), H-PGDS Tg mice showed a 2.8-fold increase (from 19.57 ± 2.15 to 54.22 ± 8.40 ng/mg Cre; $p = 0.000$) after PTZ-induced seizures as compared to their respective basal levels. Factorial analysis revealed that both, the genotype of the mice (*wild-type vs H-PGDS Tg:* $F_{(3,30)} = 18.05$; $p = 0.000$; *Partial Eta Squared 0.376*), and the treatment types (*saline vs PTZ:* $F_{(3,30)} = 36.28$; $p = 0.000$; *Partial Eta Squared 0.547)* have a significant effect on tetranor-PGDM. It suggests that H-PGDS Tg mice produce excess PGD_2 under both basal and stimulated conditions. We also determined changes in the urinary content of a PGE_2 metabolite, tetranor-PGEM, after saline and PTZ administration. No significant changes in tetranor-PGEM were observed between H-PGDS Tg and wild-type mice, except for a decrease in seizure-induced tetranor-PGEM in H-PGDS Tg mice (12.04 ± 1.48 ng/mg Cre) as compared to their wild-type littermates (21.52 ± 2.09 ng/mg Cre; $p = 0.002$) (Fig. 1B). Factorial analysis showed that there was significant interaction between wild-type and H-PGDS Tg mice (*wild-type vs H-PGDS Tg:* $F_{(3,30)} = 12.23$; $p = 0.001$; *Partial Eta Squared 0.290*), however, treatment type showed no interaction (*saline vs PTZ:* $F_{(3,30)} = 0.011$; $p = 0.916$; *Partial Eta Squared 0.000),* suggesting that although genotype of mice may seem to affect PGE_2 production, treatment per se had no effect on PGE_2 production.

Video analysis showed that administration of PTZ (50 mg/kg, i.p.) in both wild-type and in H-PGDS Tg mice, induced seizure behaviors such as rigid posture, myoclonic jerks with brief twitching, rearing and falling that eventually developed into GTCS, consisted of a train EEG spike wave complex which is a measure of the seizure intensity. Simultaneous EEG/EMG recording as an objective metric for evaluation and quantification of the seizure severity revealed that the latency of seizure onset, the time between the PTZ injection and the appearance of the first seizure spike, was significantly increased in H-PGDS Tg mice (84.0 ± 4.3 s) as compared to wild-type mice (53.3 ± 6.8 s; $p = 0.004$; $t = -3.785$; $df = 10$) (Fig. 2A). GTCS lasted for a 16.8 ± 2.5 s in H-PGDS Tg mice, significantly shorter compared to wild-type mice (24.3 ± 2.9; $p = 0.038$; $t = 2.395$; $df = 10$) (Fig. 2B). On the other hand, the duration of behavioral seizures (133.3 ± 18.4 and 136.2 ± 43.3; $p = 0.44$; $t = 0.804$; $df = 10$) and postictal EEG depression (117.2 ± 20.2 and 124.2 ± 14.6 s; $p = 0.784$; $t = 0.281$; $df = 10$) remain unchanged between H-PGDS Tg and wild-type mice (n = 6 in each group), respectively (Fig. 2C, D).

Postictal EEG was composed of sharp delimiting, high-amplitude EEG spikes exceeding baseline by two-fold, in the form of solitary spikes or polyspikes namely EEG seizure spikes. Representative EEG traces showed that whereas the EEG seizure spikes appeared for longer duration in wild-type mice, the H-PGDS Tg mice exhibit the EEG spikes for the much shorter duration (Fig. 3A). The seizure spike count during 1-h after postictal EEG depression in H-PGDS Tg mice (5.3 ± 1.6/min) decreased significantly compared to wild-type mice (12.6 ± 1.7/min; $p = 0.011$; $t = 3.136$; $df = 10$) (Fig. 3B), whereas the number of EEG seizure spikes during behavioral seizures were not significantly changed (189.2 ± 46.8 and 147.4 ± 46.2/min; $p = 0.540$; $t = 0.635$; $df = 10$) (Fig. 3C). Taken together, our data clearly showed that over-expression of the human H-PGDS enzyme in mice resulted in an excessive PGD_2 production that eventually decreased the intensity of the PTZ-induced seizures. The EEG/EMG and video data were analyzed for 2-h following PTZ administration. During this period, we did not observe any sleep in either wild-type or H-PGDS Tg mice (data not shown).

4. Discussion

In the present study, we used H-PGDS Tg mice, those having over-expression of the human H-PGDS enzyme, to examine whether these mice have increased endogenous PGD_2 production and resistance to PTZ-induced seizures. Our results clearly showed that H-PGDS Tg mice had enhanced endogenous PGD_2 production and had reduced susceptibility to chemically-induced seizures compared to their wild-type littermates, as characterized by an increase in latency to seizure onset and a decrease in the duration of GTCS and electrographic seizures after postictal EEG depression. We chose H-PGDS Tg mice based on our previous study which reported that most of PGD_2 produced after PTZ-induced seizures were derived *via* H-PGDS enzyme [4]. Our current findings are in good agreement with our previous study whereby, deletion of H-PGDS gene resulted in high seizure susceptibility in a PTZ-induced seizure model mouse [4]. Current findings are also in close proximity to studies where microinjections of exogenous PGD_2 or its analog into the brain of mice or rats inhibit seizures [1,2]. Moreover, PGD_2 has long been considered to be an important mediator of the inflammation because non-steroidal anti-inflammatory drugs which inhibit the formation of PGs aggravates chemically-induced seizures [7,10]. Santos et al. [9] recently reported that EP_2 receptor agonist PGE_2 attenuates PTZ- and pilocarpine-induced seizures, suggesting an anti-seizure role of PGE_2. However, no change in levels of PGE_2 metabolite, tetranor-PGEM, in our study following PTZ-induced seizures, clearly substantiates our claim that PGD_2 over-production is responsible for observed results instead of PGE_2 or other prostanoids such as $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and thromboxane B_2 [4]. Further, the observed low levels of tetranor-PGEM in H-PGDS Tg mice as compared to wild-type mice could be in part due to the fact that both PGD_2 and PGE_2 are derived from a common precursor, PGH_2, and over-expression of H-PGDS may result in utilization of available pool of PGH_2 [12]. It is well-known fact

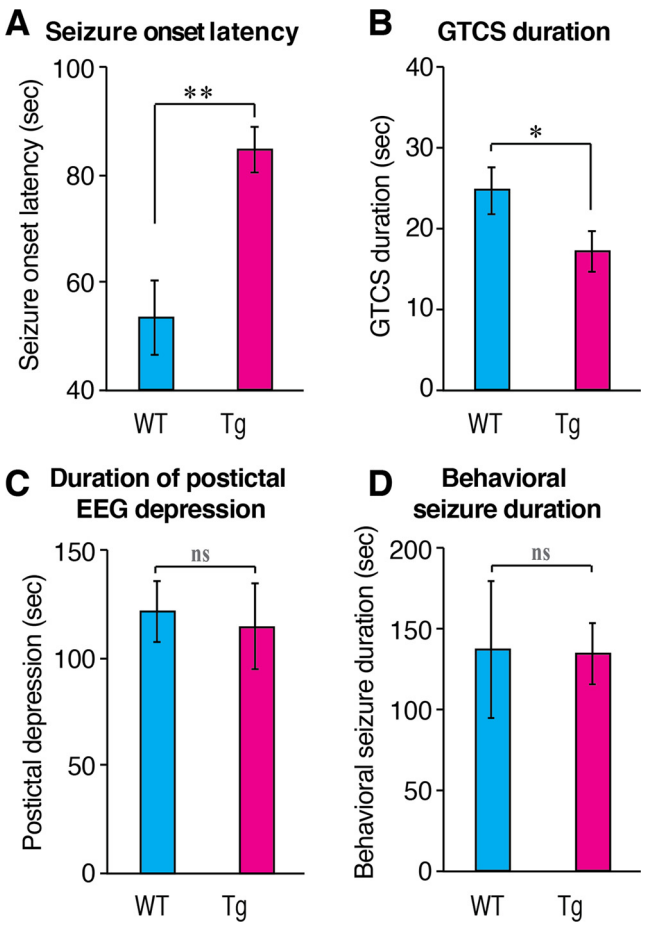

Fig. 2. Human H-PGDS over-expressing (H-PGDS Tg) mice showed low-intensity seizures compared to their wild-type littermates. (A–D), Represents changes in seizure onset latency (A), duration of GTCS (B), duration of postictal EEG depression (C) and duration of behavioral seizures (D) in H-PGDS Tg (magenta color bars) and wild-type (cyan color bars). Data are presented as the mean ± SEM (n = 6). *p ≤ 0.05, **p ≤ 0.01 compared with WT mice, using an independent sample t-test. WT: wild-type; Tg: H-PGDS Tg; ns: not significant.

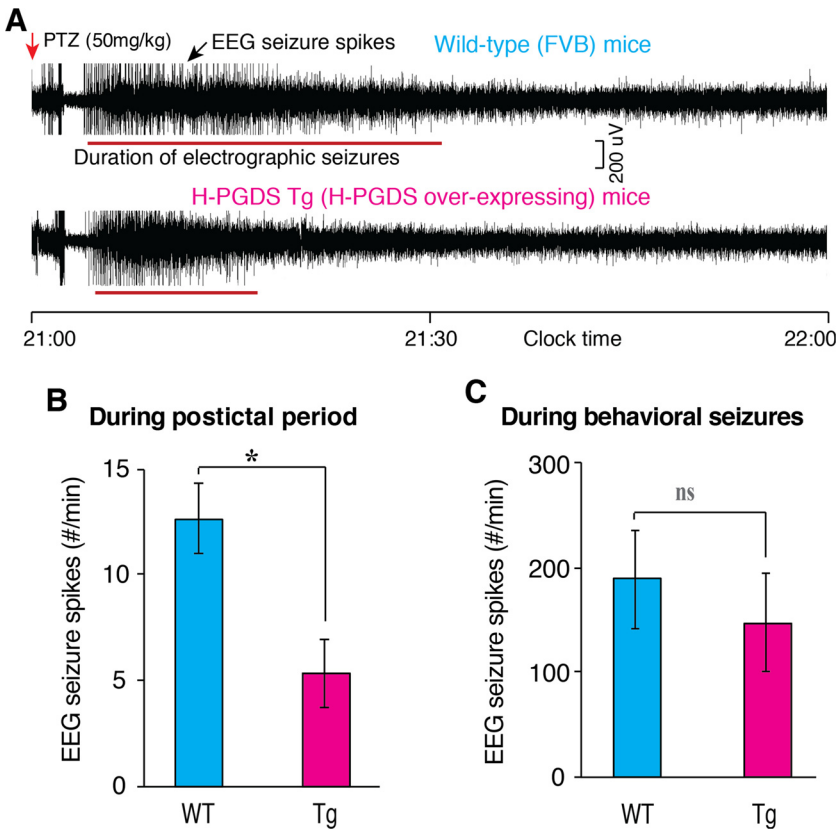

Fig. 3. Human H-PGDS over-expressing (H-PGDS Tg) mice showed a decrease in the electrographic seizures (EEG seizure spikes). (A), Representative traces from EEG recordings of a wild-type (upper) and an H-PGDS Tg (lower) mouse after PTZ injection (50 mg/kg, i.p.). **(B, C),** Shows the changes in EEG seizure spikes during the postictal period (B) and during behavioral seizures (C) in H-PGDS Tg (magenta color bars) and wild-type (cyan color bars). Data are presented as the mean ± SEM (n = 6). *p ≤ 0.05, **p ≤ 0.01 compared with WT mice, using an independent sample t-test. WT: wild-type; Tg: H-PGDS Tg; ns: not significant.

that seizures and/or epilepsy resulted in prostanoid production in the brain [4]. Hence, instead of measuring prostanoids in the brain, we estimated urinary metabolites of PGD_2 and PGE_2 (tetranor-PGDM and tetranor-PGEM) due to fact that PGD_2 and PGE_2 are very unstable in tissue, organs and/or bloodstream while their metabolites are highly stable in urine. No sleep after PTZ injection in this study, unlike our previous report [4], is surprising. However, the disparity in observed results can be attributed in part to the difference in the genetic background of the animals used. In the previous study, mice used were on C57BL/6 background, while in current study mice were developed from FVB mice. Although we believe that the observed seizure inhibition was resulted due to over-production of PGD_2, a role of H-PGDS over-expression on the PTZ pharmacokinetics can not be ruled out, because altered PTZ metabolization and/or BBB permeability may affect the seizure intensity and/or prostanoid production.

The current study, together with previous reports [1,2,4], provided strong evidence that endogenous PGD_2 effectively lowers the intensity of PTZ-induced seizures. Epilepsy is one of the major cause of brain injury and H-PGDS is one of the major prostanoid-producing enzymes during inflammation in the brain. In our previous study with loss of function of the H-PGDS enzyme and in the current study by using a gain of function of H-PGDS enzyme, we clearly demonstrated that the H-PGDS is crucial for the seizure suppression [4]. Therefore, the H-PGDS inducers or activators may be of therapeutic use for the treatment of people suffering from epilepsy.

Conflict of interest statement

All the authors have read and approved the manuscript and declare that there exists no conflict of interest.

Author contribution

M.K.K., K.A. and Y.U. designed research; M.K.K. and K.A. performed experiments; M.K.K., Y.C. and R.S. analyzed data; M.K.K. and Y.U. wrote the paper.

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