The type 1 cannabinoid receptor positive allosteric modulators GAT591 and GAT593 reduce spike-and-wave discharges in Genetic Absence Epilepsy Rats from Strasbourg

Dan L. McElroy a,1, Andrew J. Roebuck a,b,1, Quentin Greba a,1, Sumanta Garai c, Asher L. Brandt d, Orhan Yilmaz d, Stuart M. Cain e, Terrance P. Snutch e, Ganesh A. Thakur c, Robert B. Laprairie a,d,1, John G. Howland a,**,2

a Department of Anatomy, Physiology, and Pharmacology, University of Saskatchewan, Saskatoon, SK S7N 5E5, Canada
b School of Liberal Arts, Yukon University, Whitehorse, YT Y1A 5K4, Canada
c Department of Pharmaceutical Sciences, Northeastern University, Boston, MA 02115, United States
d Michael Smith Laboratories and Djavad Mowafaghian Centre for Brain Health, University of British Columbia, Vancouver, BC V6T 1Z4, Canada
e Department of Pharmacology, College of Medicine, Dalhousie University, Halifax, NS B3H 4R2, Canada

** Correspondence to: College of Pharmacy and Nutrition, University of Saskatchewan, 3B36 - 104 Clinic Place, Saskatoon, SK S7N 5E5, Canada.
* Correspondence to: Department of Anatomy, Physiology, and Pharmacology, University of Saskatchewan, GD30.7, Health Sciences Building, 107 Wiggins Road, Saskatoon, SK S7N 5E5, Canada.

A R T I C L E   I N F O

Keywords:
Endocannabinoid system
Childhood absence epilepsy
GAERS
Type 1 cannabinoid receptor
Positive allosteric modulator
Electroencephalogram

A B S T R A C T

Childhood absence epilepsy (CAE) is a non-convulsive seizure disorder primarily in children characterized by absence seizures. Absence seizures consist of 2.5–5 Hz spike-and-wave discharges (SWDs) detectable using electroencephalography (EEG). Current drug treatments are only partially effective and adverse side effects have spurred research into alternative treatment approaches. Recent research shows that positive allosteric modulation of the type-1 cannabinoid receptor (CB1R) reduces the frequency and duration of SWDs in Genetic Absence Epilepsy Rats from Strasbourg (GAERS), a model that recapitulates the SWDs in CAE. Here, we tested additional CB1R ago-PAMs, GAT591 and GAT593, for their potential in alleviating SWD activity in GAERS. In vitro experiments confirm that GAT591 and GAT593 exhibit increased potency and selectivity in cell cultures and behave as CB1R allosteric agonists and PAMs. To assess drug effects on SWDs, bilateral electrodes were surgically implanted in the somatosensory cortices of male GAERS and EEGs recorded for 4 h following systemic administration of GAT591 or GAT593 (1.0, 3.0 and 10.0 mg/kg). Both GAT591 and GAT593 dose-dependently reduced total SWD duration during the recording period. The greatest effect on SWD activity was observed at 10.0 mg/kg doses, with GAT591 and GAT593 reducing seizure duration by 36% and 34% respectively. Taken together, these results support the continued investigation of CB1R PAMs as a potential therapeutic to alleviate SWDs in absence epilepsy.

1. Introduction

Childhood absence epilepsy (CAE) develops in children and teenagers approximately 4–13 years (Vidaurre et al., 2009) or older (Reichsoellner et al., 2010), and is distinct from traditional “psychomotor” seizures (Brigo et al., 2018). Absence seizures may occur hundreds of times per day in patients and are associated with characteristic spike and wave discharges (SWDs; 2.5–5 Hz) detectable using

Abbreviations: 2-AG, 2-arachidonoylglycerol; AEA, anandamide; ago-PAM, allosteric agonist and positive allosteric modulator; CAE, childhood absence epilepsy; CB1R, cannabinoid type-1 receptor; ECS, endocannabinoid system; EEG, electroencephalography; GAERS, Genetic Absence Epilepsy Rats from Strasbourg; LFP, local field potential; PAM, positive allosteric modulator; SWD, spike and wave discharge; THC, Δ 9-tetrahydrocannabinol.

1 DLM, AJR, and QG contributed equally to this work.
2 ORCID: 0000-0003-3326-7118

https://doi.org/10.1016/j.ibneur.2022.01.006
Received 4 September 2021; Received in revised form 19 January 2022; Accepted 21 January 2022
Available online 24 January 2022
2021 by Elsevier Ltd on behalf of International Brain Research Organization. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
electroencephalography (EEG) (Brigo et al., 2018). Voltage-gated ion channels play fundamental roles in propagating neuronal activity associated with the generation of SWDs (Crunelli et al., 2020). Specifically, SWDs appear to have a genetic etiology including T-type calcium (Ca2+) channel mutations that underlie aberrant burst-firings observed in CAE (Budde and Pape, 2009; Cain and Sutich, 2013; Tringham et al., 2012; Vidaurre et al., 2009). Multi-target drugs approved to treat SWDs such as ethosuximide and valproic acid are effective in approximately 2/3 of patients; however, their use is associated with adverse effects including drowsiness, disorientation, nausea, and vomiting (Brigo et al., 2018; Crunelli et al., 2020). Considering the limited efficacy and side effects associated with current CAE treatment options, new pharmacological approaches are desirable.

Genetic Absence Epilepsy Rats from Strasbourg (GAERS) are a well-characterized animal model for CAE and recapitulate many behavioral comorbidities observed in human CAE patients (Crunelli et al., 2020; Depaulis et al., 2016; Henbid et al. 2017; Marks et al. 2016b). The GAERS model also maintains a high level of predictive validity when assessing antiepileptic drug efficacy for clinical use (Crunelli et al., 2020; Depaulis et al., 2016; Desai et al. 2013). GAERS express spontaneous SWDs associated with abnormal burst-firing in the thalamocortical network, including deep layers 5/6 of the somatosensory cortex, and arise from a spontaneously-occurring point mutation (GAC) in the Cav3.2 voltage-gated T-type Ca2+ channel gene, 

\[ \text{Cacna1h} \] (Polack et al., 2007; Powell et al., 2009). The pharmacological blockade of T-type Ca2+ channels effectively reduces the frequency and duration of SWDs in GAERS (Tringham et al. 2012) supporting GAERS as a viable preclinical model to investigate CAE therapeutics.

Cannabis has played a role in medicine (and recreation) for thousands of years, including medical use for ailments such as epilepsy (Ligresti et al., 2016). The endocannabinoid system (ECS) plays a fundamental role in modulating central nervous system activity and abnormalities (e.g., reduced receptors and endogenous cannabinoids) have been associated with the pathophysiology of epilepsy (Perucca, 2017; Rosenberg et al., 2017). The ECS includes 2 well-characterized G protein-coupled receptors (GPCR), the type 1 and 2 cannabinoid receptors (CB1R, CB2R) (Di Marzo et al., 2004; Ligresti et al., 2016). CB1R is primarily expressed on presynaptic terminals throughout the central nervous system and activated by the endogenous cannabinoids anandamide (AEA) and 2-arachidonoylglycerol (2-AG). Agonist-bound CB1R receptors (CB1R, CB2R) (Di Marzo et al., 2004; Ligresti et al., 2016). The endocannabinoid system (ECS) plays a fundamental role in modulating central nervous system activity and abnormalities (e.g., reduced receptors and endogenous cannabinoids) have been associated with the pathophysiology of epilepsy (Perucca, 2017; Rosenberg et al., 2017). The ECS includes 2 well-characterized G protein-coupled receptors (GPCR), the type 1 and 2 cannabinoid receptors (CB1R, CB2R) (Di Marzo et al., 2004; Ligresti et al., 2016). CB1R is primarily expressed on presynaptic terminals throughout the central nervous system and activated by the endogenous cannabinoids anandamide (AEA) and 2-arachidonoylglycerol (2-AG). Agonist-bound CB1R inhibits cAMP production, activates inwardly rectifying K+ channels via Goi/o and inhibits calcium influx via secondary activation of voltage-gated calcium channels, thereby impairing presynaptic neurotransmitter release (Chemin et al., 2001; Chevaleyre and Castillo, 2003; Ligresti et al., 2016; Mechoulam and Parker, 2013).

Compounds targeting the ECS have generally shown limited efficacy in reducing SWDs, although findings are variable (Crunelli et al., 2020; Rosenberg et al., 2017). For example, CB1R agonists both increase and decrease SWDs in animal models, along with issues regarding tolerance, desensitization, and possible psychoactive effects (Alavardashvili and Laprairie, 2016; Perescis et al., 2020; Roebuck et al., 2020b, 2021; Rosenberg et al., 2017). These observations have prompted further research into alternative pharmacological approaches to target CB1Rs. The limited success of cannabinoid receptor agonism has prompted the investigation of more subtle approaches to modulate the ECS. One approach includes using CB1R positive allosteric modulators (PAMs) to promote receptor signaling without direct agonism. PAMs bind to an allosteric site on the receptor to augment the potency and/or efficacy of the orthosteric ligand (e.g., 2-AG, AEA) but lack intrinsic efficacy alone. In contrast, ago-PAMs bind to an allosteric site on their receptor, augment the potency and/or efficacy of the orthosteric ligand, and can produce receptor activation even in the absence of an orthosteric ligand or agonist. Previously, we demonstrated that the pure PAM GAT229 and the racemic mixture GAT211 – containing both a PAM and ago-PAM – reduced SWDs in the GAERS model (Roebuck et al., 2021).

Beyond work in GAERS, Slivicki et al. (2020) demonstrated GAT211 enhances morphine antinoiception. Recently, two novel GAT compounds have been developed with increased potency and selectivity at CB1R in vitro: GAT591 and GAT593 (Garai et al., 2020). These compounds are fluorinated derivatives of GAT211 designed based on structure-activity studies of the CB1R allosteric binding site(s) that behave as ago-PAMs. In a follow-up to our previous study, here we report on the efficacy of GAT591 and GAT593 at reducing SWDs in GAERS. Given their selectivity and potencies, we predicted that acute administration of GAT591 or GAT593 would decrease the number and duration of SWDs using EEG in somatosensory cortex following systemic administration.

2. Experimental procedures

2.1. Materials

GAT591 and GAT593 were synthesized in the laboratory of Dr. Ganesh Thakur (Northeastern University, Boston, MA). The complete chemical synthesis of these compounds can be found in Garai et al. (2020). GAT591 and GAT593 are tri-fluorinated derivatives of GAT211 designed to act as ago-PAMs at CB1R having approximately 10-fold improved potency relative to GAT211 in vitro (Garai et al., 2020) (Fig. 1A). All other material suppliers are listed below.

2.2. Cell culture

Chinese hamster ovary (CHO)-K1 HitHunter (cAMP assay) or PathHunter (jarrestin2 assay) cells stably expressing human CB1R, were from DiscoveRx (Eurofins, Freemont CA) and cultured as described in earlier studies from our group (Zagzoog et al., 2021). Cells were maintained at 37°C, 5% CO2 in F-12/DMEM containing 1 mM L-glutamine, 10% FBS, and 1% Pen/Strep as well as 800 µg/mL gentamicin (HitHunter) or 800 µg/mL G418 and 300 µg/mL hygromycin B (PathHunter), as described previously (Zagzoog et al., 2021).

2.3. HitHunter cAMP assay

Inhibition of forskolin (FSK)-stimulated cAMP accumulation was quantified as described previously (Zagzoog et al., 2021). Cells (20,000 cells/well in low-volume 96 well plates) were incubated overnight in Opti-MEM containing 1% FBS at 37°C and 5% CO2. Opti-MEM media was removed and replaced with cell assay buffer (DiscoveRx) and cells were treated with 10 µM FSK and compounds for 90 min at 37°C. cAMP antibody solution and cAMP working detection solutions were added to cells (DiscoveRx), and cells were incubated for 60 min at room temperature. cAMP solution A (DiscoveRx) was added, and cells were incubated for an additional 60 min at room temperature. Chemiluminescence was measured on a Cytation5 plate reader (top read, gain 200, integration time 10,000 ms).

2.4. PathHunter jarrestin2 assay

Jarrestin2 recruitment was quantified with the DiscoveRx PathHunter assay (Zagzoog et al., 2021). Twenty thousand cells/well in low-volume 96 well plates were incubated overnight in Opti-MEM containing 1% FBS at 37°C and 5% CO2. Cells were treated with compounds for 90 min at 37°C. Detection solution was added to cells (DiscoveRx), and cells were incubated for 60 min at room temperature. Chemiluminescence was measured on a Cytation5 plate reader (top read, gain 200, integration time 10,000 ms).

2.5. Subjects

Male GAERS (n = 16 total) were bred and housed as previously described (Marks et al. 2016a, 2016b). Briefly, rats were initially sourced from the University of Melbourne and the strain was established.
Bipolar electrodes were chronically implanted bilaterally in somatosensory cortex and once daily for 3 days after surgery for pain management. Injection of Anafen (5 mg/kg, i.p.) was administered immediately before surgery and once daily for 3 days after surgery for pain management. Briefly, surgeries were performed under isoflurane anesthesia. A single stainless steel screws (one also serving as a ground) and dental cement. Implants were secured with 3.2 mm bregma (Roebuck et al., 2020b). Implants were secured with 3.2 mm bregma (Roebuck et al., 2020b) and availability of age, strain, and litter matched animals. GAT591 (n = 7) and GAT593 (n = 9) were assessed in separate groups of rats each tested following 4 treatments (vehicle, 1.0, 3.0, and 10.0 mg/kg) and a baseline recording session. Behavioral experiments were conducted on well-handled adult animals unless otherwise stated and EEG data was scored by 2 researchers blind to treatment. Experiments were conducted in accordance with the standards of the Canadian Council on Animal Care and the University of Saskatchewan Animal Research Ethics Board.

2.6. Effects of GAT591 and 593 on spike-and-wave discharges in GAERS

2.6.1. Surgery

Construction and implantation of electrodes proceeded according to established protocols (Farrell et al., 2018; Roebuck et al., 2020b, 2021). Briefly, surgeries were performed under isoflurane anesthesia. A single injection of Anafen (5 mg/kg, i.p.) was administered immediately before surgery and once daily for 3 days after surgery for pain management. Bipolar electrodes were chronically implanted bilaterally in somatosensory cortex (A/P 0.6 mm, M/L 5.1 mm, D/V – 3.4 mm, relative to bregma (Roebuck et al., 2020b)). Implants were secured with 3.2 mm stainless steel screws (one also serving as a ground) and dental cement. Animals were allowed at least 1 week to recover before habituation and testing.

2.6.2. Treatment preparation

GAT591 and GAT593 were prepared for systemic injection (i.p.) in a solution of ethanol, Kolliphor (Sigma Aldrich), and saline, at a 1:1:18 ratio and injected at a volume of 5.0 mL/kg.

2.6.3. EEG recording and analysis

Local field potentials (LFP) were acquired by tethered EEG [Grass Technologies (Farrell et al. 2018; Roebuck et al. 2020b, 2021)]. LFP signals were amplified 5000x and digitized at 100 Hz. Recordings occurred in 2 clear Plexiglas boxes (32 cm × 32 cm). Treatments (vehicle, 1.0, 3.0, and 10.0 mg/kg GAT591 or GAT593) and baseline recordings were conducted in 4 h time blocks in a randomized order with a minimum 5-day washout between treatments. To allow adequate time for drug absorption, in addition to time needed to transfer and connect rats to the EEG tether, EEG recordings began approximately 20 min post treatment and continued for 4 h. Behavior was monitored, and animals were prevented from sleeping by a gentle rapping upon the chamber door as necessary. At the end the experiment, animals were perfused, and electrode placements were confirmed.

SWDs were identified using a MATLAB script (MathWorks) developed by Dr. Stuart Cain and Jeff LeDue at the University of British Columbia (Download: https://ninc.centreforbrainhealth.ca/sites/default/files/eeg.zip). SWDs were defined as a burst > 3x baseline amplitude with a frequency between 7 and 12 Hz, lasting > 0.5 s (Marks, 2016a, 2016b; Powell et al., 2009; Roebuck et al., 2020b, 2021). SWDs were analyzed semi-automatically with the script and manually confirmed. Files were coded and analyzed by two experimenters blind to experimental conditions. To account for individual variability, SWD data were reported as normalized values relative to untreated baseline. Normalization was calculated as the % change from pre-treatment baseline data.

2.7. Experimental design, data analysis, and statistical analysis

Data for HitHunter cAMP and PathHunter β-arrestin2 data are shown as % of maximal CP55,940 response (i.e., 100%). Estimates of EC50 and Emax were determined using non-linear regression with variable slope (3 parameters (GraphPad, Prism, v. 9.0). Values are presented as the mean ± the standard error of the mean (S.E.M.) or 95% confidence interval (CI), as indicated in tables and figure legends. In vivo GAT591 and GAT593 EEG experiments used a within-subjects design in which each...
rat received all treatments. Statistical analyses of normalized data were conducted by two-way analysis of variance (ANOVA) with factors of Treatment (vehicle, 1.0, 3.0, and 10.0 mg/kg GAT591 or GAT593) and Time (1, 2, 3 and 4 h post-treatment) and these results are presented in Figs. 2 and 3. Raw EEG data (i.e., not normalized to baseline) are presented in Supplementary Figs. 2 (GAT591) and 3 (GAT593). Dose-response data presented in Fig. 4 were normalized to baseline values within animals and data were fit to a non-linear regression [agonist] versus normalized response in order to estimate ED50 and Emax. Post-hoc testing and summary panels used Dunnett’s multiple comparison test with comparisons made versus vehicle. Statistics were calculated with GraphPad Prism version 9.0.1 and statistical significance was set at \( p \leq 0.05 \). Post hoc testing was not performed unless \( p \leq 0.05 \). All values are reported as mean ± S.E.M., unless otherwise stated. All analysis was performed on complete data sets and no outliers were removed.

3. Results

3.1. GAT591 and GAT593 are CB1R ago-PAMs

The allosteric ago-PAM and PAM activity of GAT591 and GAT593 were established in a previous publication from members of our group (Garai et al., 2020). Here, we sought to confirm and extend this previous finding. CHO-K1 hCB1R HiHunt (cAMP) and PathHunter (\( \beta \)-arrestin2) cells were treated with 0.1 nM – 10 \( \mu \)M CP55,940 (reference agonist), GAT211, GAT591, or GAT593; or 10 nM CP55,940 + 0.1 nM – 10 \( \mu \)M GAT211, GAT591, or GAT593. Fig. 1A and C represent determinations of agonism. Fig. 1B and D are determinations of positive allosteric modulation in which compounds are tested in the presence of 10 nM CP55,940. Positive allosteric modulation in these panels is indicated by the ability of the allosteric ligand to augment cAMP inhibition or \( \beta \)-arrestin2 recruitment above that which was observed with 10 nM CP55,940 alone.

Congruent with previous findings (Garai et al., 2020), GAT591 and GAT593 were more potent and efficacious \( \alpha \)-GMo agonists than they were agonists of \( \beta \)-arrestin2 recruitment at hCB1R (i.e. \( \alpha \)-GMo-selective), with a non-significant greater potency than the parent compound GAT211 in the cAMP inhibition assay (Fig. 1A; Table 1). Similar to previous findings in the presence of 100 nM CP55,940 (Garai et al., 2020), GAT591 and GAT593 behaved as \( \alpha \)-GMo-selective PAMs in the presence of 10 nM CP55,940 with greater potency than the parent compound GAT211 (non-overlapping 95% CI) (Fig. 1B; Table 1). GAT591, augmented \( \beta \)-arrestin2 recruitment in the presence of 10 nM CP55,940 to a greater extent than the parent compound GAT211 and GAT593 (Fig. 1D; Table 1). No statistical comparisons of potency or efficacy are being made within these panels as the relative potency and efficacy of GAT591 and GAT593 were determined in Garai et al. (2020).

3.2. GAT591 reduces total SWD duration in male GAERS

Representative EEG traces (30 min) from all treatments administered to a rat are depicted in Fig. 2A. Detailed statistics are presented in Supplementary Table 1. A 2-way ANOVA compared the effect of Treatment (vehicle, 1.0, 3.0 and 10.0 mg/kg GAT591) and Time (1, 2, 3 and 4 h post-treatment) on SWD activity in GAERS (Fig. 2). No main effects or interactions were observed for SWD incidence following treatment with GAT591 (Fig. 2B). There was a main effect of Treatment for total SWD duration and post-hoc testing revealed that the 3.0 and 10.0 mg/kg doses of GAT591 lowered SWD duration compared to vehicle (Fig. 2C1, C2). There were no main effects or interactions for average SWD duration (Fig. 2D). For average oscillatory frequency of SWDs, there was a main effect of Time and post-hoc testing revealed that average oscillatory frequency increased between hours 1 and 4 (Fig. 2E).

3.3. GAT593 reduces total SWD duration in male GAERS

Representative EEG traces (30 min) from all treatments administered to a rat are depicted in Fig. 3A. Detailed statistics are presented in Supplementary Table 2. A 2-way ANOVA compared the effect of Treatment (vehicle, 1.0, 3.0 and 10.0 mg/kg GAT593) and Time (1, 2, 3 and 4 h post-treatment) on SWD activity in GAERS (Fig. 3). There were no main effects or interactions for SWD incidence following treatment with GAT593 (Fig. 3B). There was a main effect of Treatment for total SWD duration, with post-hoc testing revealing a reduction in seizure duration in the 1.0 mg/kg and 10 mg/kg doses of GAT593 compared to vehicle (Fig. 3C1, C2). There were no main effects or interactions for average SWD duration or average oscillatory frequency (Fig. 3D-E). See the Supplementary Fig. 1B for complete 4 h traces for one rat from this experiment.

3.4. GAT591 and GAT593 attenuate SWD incidence and duration

Fig. 4 re-presents data shown in Figs. 2 and 3 as a function of GAT591 dose-response (Fig. 4A-D) and GAT593 dose-response (Fig. 4E-F) with estimates of potency shown in Supplementary Table 3. GAT591 reduced the number of SWDs at all time points and was most effective at the 10.0 mg/kg dose (Fig. 4A). GAT593 also reduced SWD incidence; however, the effect was more variable between time points (Fig. 4B). Both GAT591 and GAT593 dose-dependently reduced total SWD duration at each time point (Fig. 4B,F). Furthermore, GAT591 and GAT593 dose-dependently reduced average SWD durations at each time point; however, effects were most notable at earlier time points (Fig. 4C,G). Finally, no differences were observed in GAT591 or GAT593 treated rats, for SWD oscillatory frequency (Fig. 4D,H).

4. Discussion

Here, we demonstrated that acute treatment with the CB1R ago-PAMs GAT591 and GAT593 reduce SWDs in the GAERS model of CAE. First, we showed that GAT591 and GAT593 behave as CB1R ago-PAMs with increased efficacy than GAT211 (Fig. 1). Next, we showed that systemic administration of GAT591 (Fig. 2) and GAT593 (Fig. 3) reduce total SWD duration in male GAERS. Finally, we presented data showing GAT591 and GAT593 dose-dependent effects on SWD incidence, total SWD duration, average individual SWD duration, and SWD oscillatory frequency at each post-treatment time point (Fig. 4). Previously, we reported no sex differences in SWD activity in GAERS (Roebuck et al., 2021) and therefore investigated only males in this experiment. These results support previous experiments conducted with the CB1R ago-PAM GAT211 and PAM GAT229 (Roebuck et al., 2021), suggesting that CB1R PAMs should continue to be explored for therapeutic potential in treating absence seizures.

There is increasing interest in the development of biased ligands that can preferentially activate select signaling pathways for potential therapeutic benefit. Previously published work from our group has demonstrated both GAT591 and GAT593 are G protein-biased allosteric modulators of CB1R that promote G protein-dependent cAMP accumulation relative to \( \beta \)-arrestin2 recruitment (Garai et al., 2020). Based on previous in vitro cell culture evidence (Garai et al., 2020; Laprairie et al., 2019b) and in vivo evidence in animal models of pain (Slivicki et al., 2018, 2020) and Huntington’s disease (Laprairie et al., 2019a), G protein bias at CB1R may correlate with improved cell viability, delayed pathogenesis, and reduced tolerance. Therefore, increasing evidence supports a correlation between G protein bias and improved outcomes in pain and neurodegenerative disease. However, two ongoing challenges in the study of GPCR ligand bias, including CB1R, are the consistent quantification of bias across
Fig. 2. GAT591 reduced the duration of spike and wave discharges (SWDs) in male GAERS (n = 7). Male GAERS were injected (i.p.) with vehicle, 1.0, 3.0, and 10.0 mg/kg GAT591 and EEG was recorded for 4 h post-treatment and compared to baseline (BL) activity. Representative EEG traces (30 min) depict SWD activity from a single rat, beginning 1 h after treatments with GAT591 (A). No differences in SWD Incidence were observed for Treatment or Time factors (B). GAT591 lowered SWD duration at 3.0 and 10.0 mg/kg doses (C1, C2). No main effects were observed for average duration of SWDs (D). The last panel shows a main effect of time in which average oscillatory SWD frequency increased between hour 1 and 4. No effect of treatment was observed (p = 0.06) (E). See Suppl. Fig. 2 for raw data not normalized to the baseline recording session. * = p ≤ 0.05 (main effect).
Fig. 3. GAT593 reduced the duration of SWDs in male GAERS (n = 9). Male GAERS were injected (i.p.) with vehicle, 1.0, 3.0, and 10.0 mg/kg GAT593 and EEG was recorded for 4 h post-treatment and compared to baseline (BL) activity. Representative EEG traces (30 min) depict SWD activity from a single rat, beginning 1 h after treatments with GAT593 (A). No differences in SWD incidence were observed across treatments (p = 0.054) or time (B). GAT593 lowered SWD duration at 1.0 and 10.0 mg/kg doses (C1, C2). No main effects were observed for average duration of SWDs (D) nor averaged frequency of SWDs (E). See Suppl. Fig. 3 for raw data not normalized to the baseline recording session. * = p ≤ 0.05.
Fig. 4. Analysis of GAT591 (A-D) and GAT593 (E-F) dose-response changes in SWDs in male GAERS (n = 7 and 9, respectively). Male GAERS were injected (i.p.) with vehicle, 1.0, 3.0, and 10.0 mg/kg GAT591 or GAT593 and EEG was recorded for 4 h post-treatment. Data were normalized relative to baseline measurements within animal (i.e., 0%) for SWD incidence (A,D), total SWD duration (B,F), average individual SWD duration (C,G), and SWD frequency (D,H). ED$_{50}$ and E$_{max}$ values were estimated using a non-linear regression [agonist] versus normalized response (GraphPad v. 9). Data are mean ± S.E.M.
model systems and the assessment of bias in vivo (reviewed in Manning et al., 2021). One of the long-term goals of our research with CB1R is to build G protein bias into the structure-activity relationship of these compounds and directly test whether this bias mediates the spatial or temporal benefits of allosteric ligands.

GAT591 and GAT593 showed some efficacy at alleviating total ictal time in GAERS during a 4 h recording period (Figs. 2C, 3C); however, SWD incidence, average duration of SWDs, and average SWD oscillatory frequency remained largely unaffected by treatments. Behavioral and electrophysiological observations suggest that both drugs appear to begin reducing SWD events approximately 30 min after treatment, with a maximum effect occurring around 45 min and a sustained reduction until about 2.5–3 h post-treatment. Additional behavioral alterations were observed, including rats entering a catatonic-like restful state with eyes open and maintained awareness, which was more pronounced at the 10 mg/kg dose. In summary, the 10 mg/kg dose of GAT591 and GAT593 was most effective for a 2–2.5 h window beginning ~30 min post-treatment, with smaller doses resulting in delayed and attenuated efficacy by comparison. Previously we showed that 10.0 mg/kg doses of GAT211 and GAT229 reduced SWDs by 40% and 50% respectively (Roebuck et al., 2021). In the present study, SWDs were reduced by 36% (GAT591) and 34% (GAT593) at 10.0 mg/kg doses. It remains unclear why these more potent ago-PAMs did not show enhanced efficacy in reducing SWDs, when compared to the PAMs tested previously. Further investigations including characterization of pharmacokinetic parameters (e.g., metabolic stability, half-life, CNS penetrance, etc.) across the various compounds may provide insight. Other compounds, such as ZCZ011 (Mitjavila et al., 2018), which is a CB1R ago-PAM, could be tested in GAERS, although they would likely have similar efficacy to the ZCZ011 (Mitjavila et al., 2018), which is a CB1R ago-PAM, could be tested in GAERS, although they would likely have similar efficacy to the

| Agonism | Inhibition of FSK-stimulated cAMP | Emax (% ± SEM) | [β-arrestin2 recruitment | Emax (% ± SEM) |
|---------|-----------------------------------|----------------|---------------------------|----------------|
| CP55,940 | 11 (4.3–27) | 100 ± 6.3 | 1500 (1200–2600) | 100 ± 5.6 |
| GAT211  | 52 (17–210) | 83 ± 8.5 | 1200 (520–2600) | 61 ± 6.4 |
| GAT591  | 4.1 (0–18)  | 91 ± 4.9  | 1300 (710–2700) | 61 ± 5.1  |
| GAT593  | 45 (24–83)  | 105 ± 4.8 | >10,000               | 47 ± 5.3  |

Positive allosteric modulation (>10 nM CP55,940)

Inhibition of FSK-stimulated cAMP

| Emax (% ± SEM) | [β-arrestin2 recruitment | Emax (% ± SEM) |
|----------------|---------------------------|----------------|
| GAT211  | 810 (210–1700) | 102 ± 8.6 | >10,000               | 15 ± 0.56 |
| GAT591  | 13 (6–69)    | 89 ± 5.2  | >10,000               | 91 ± 11  |
| GAT593  | 11 (1.5–63)  | 86 ± 5.9  | >10,000               | 20 ± 1.7  |

Inhibition of FSK-stimulated cAMP

Although CB1R PAMs exhibit efficacy at reducing SWDs, other CB1R ligands (e.g., THC) enhance the presence of SWDs in GAERS (Roebuck et al., 2020b). Research suggests that SWDs in GAERS likely results from abnormal firing in thalamocortical circuits localized to the barrel field nuclei in somatosensory cortex and thalamic nuclei (David et al., 2008). Both the somatosensory cortex and thalamic nuclei display early synchronized firing during SWDs, suggesting these two regions are a possible origin of SWDs in GAERS and CAE (David et al., 2008, Lenkov et al., 2013). Furthermore, aberrant firing in thalamo-cortical circuits may be due to a lack of reliable “pacemaker” influence from GABAergic interneurons in the nucleus reticularis of the thalamus (David et al., 2008, Lenkov et al., 2013, Traub et al., 2005). These findings are pertinent to the present experiments for two reasons: (1) CB1R is highly expressed in thalamic and cortical regions (Roebuck et al., 2021); (2) CB1R agonists often produce a disinhibitory effect in cortical circuits via CB1R-mediated inhibition of GABAergic cells, resulting in disinhibition and glutamatergic hypereexcitability observed in psychiatric disorders such as schizophrenia and epilepsy (Alaverdashvili and Laprairie, 2018, Rosenberg et al., 2017, Sherif et al., 2018). Given that SWDs are associated with disinhibition in thalamocortical circuits, upregulation of endocannabinoid production and CB1R expression may also help reduce SWDs and absence seizures.

There are some limitations in the present study. First, a concern with cannabinoid-based treatments is that pro-epileptic effects have been consistently observed in studies using full CB1R agonists (approximately 3 h post-administration) (Citroaro et al., 2013, Peresics et al., 2020, Van Rijn et al., 2010). In our previous experiment with GAT211 and GAT229, some SWD activity may have been missed due to the relatively short, 2 h recording durations (Roebuck et al., 2021). To account for this, recording periods were increased to 4 h in the current experiments. However, later in the present recordings, we did not observe an increase in SWDs following treatment with GAT591 or GAT593. Second, the degree to which GAT591 and GAT593 promote internalization of CB1Rs has not yet been characterized and represents an intriguing area for future research. Similar compounds (e.g., CP 55,940; Org27569) have been shown to induce CB1R internalization and are mediated by the β-arrestin 2 pathway (Ahn et al., 2013, Dopart et al., 2018). Given that the current study includes repeated exposure to GAT591 or GAT593, the internalization of CB1Rs induced by prior treatments is a real concern. Future studies should assess how GAT591 and GAT593 alter CB1R internalization and assess whether this influences subsequent SWD activity. Third, a within subjects design was used to minimize the number of rats used. However, we cannot exclude the possibility that repeated treatments with a given drug altered the ECS of the GAERS. A final consideration is the promiscuity of endocannabinoids. AEA acts on a variety of receptors in the brain, including TRPV1, L- and T-type Ca2+ channels, as well as GABA receptors, which is independent of CB1R
expression (Chemin et al., 2001; Golovko et al., 2015; Roebuck et al., 2021; Van Der Stelt et al., 2005). Therefore, behavioral and electrophysiological effects following CB1R modulation cannot be attributed entirely to cannabinoid receptors. That said, previous findings show that GAT211 can reverse SWD enhancement by SR141716A (CB1R orthosteric antagonist/inverse agonist) (Laprairie et al., 2017), suggesting that reduced SWDs in GAERS are, at least in part, due to the cannabinoid type-1 receptor (Roebuck et al., 2021).

5. Conclusion

Observations here and in previous studies (Roebuck et al., 2021) support dysregulation of the ECS as a component of ictogenesis in GAERS. By augmenting CB1R activity via positive allosteric modulation, the prevalence of SWDs was reduced by as much as 50%. Therefore, behavioral and electrophysiological expression (Chemin et al., 2001; Golovko et al., 2015; Roebuck et al., 2021; Van Der Stelt et al., 2005). Therefore, behavioral and electroadministration, Funding acquisition.

Acknowledgements

Funding for the project was provided by a Saskatchewan Health Research Foundation (SHRF) Establishment Grant and a CIHR Partnership Grant with GlaxoSmithKline to RBL, and a CIHR Project Grant to JGH. This work was also supported by grant from National Institutes of Health (NIH) to GAT (EY024717). Additional grant funding was provided by CIHR to TPS (#10677) and a CURE Epilepsy Award to SMC.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jibnue.2022.01.006.

References

Ahn, K.H., Mahmud, M.M., Samala, S., Lu, D., Kendall, D.A., 2013. Profiling two indole-2-carboxamides for allosteric modulation of the CB1 receptor. J. Neurochem. 124 (5), 584–589.

Alvaredosdulv, M., Laprairie, R.B., 2018. The future of type 1 cannabinoid receptor allosteric ligands. Drug Metab. Rev. 50 (1), 14–25.

Brigo, F., Trinka, E., Lattanzi, S., Bragazi, N.L., Nardone, R., Martini, M., 2018. A brief history of typical absence seizures — Petit mal revisited. Epilepsy Behav. 80, 346–352.

Budde, T., Pipe, H.C., 2009. Absence seizures: thalamic neurons and networks related to absence epilepsy. Encyclopedia of Basic Epilepsy Research, pp. 22–28.

Cain, S.M., Snutch, T.P., 2013. T-type calcium channels in burst-firing, network synchrony, and epilepsy. Biochim. Biophys. Acta Biomembr. 1828 (7), 1572–1578.

Chin, J., Moncriel, A., Perez-Reyes, E., Nargeot, J., Lory, P., 2001. Direct inhibitory T-type calcium channel action by the endogenous cannabinoid anandamide. EMBO J. 20 (24), 7033–7040.

Chevaleyre, V., Castillo, P.E., 2003. Heterosynaptic LTD of hippocampal GABAergic synapses: a novel role of endocannabinoids in regulating excitability. Neuropharmacology 69, 115–126.

Cruz, P., Pellegrini-Lagakos, T., 2015. The role of cannabinoids in generalized idiopathic epilepsies. J. Neurosci. Methods 260, 159–174.

Dezi, G., Ozturk, E., Stanic, D., Powell, K.L., Blumenfeld, H., O’Brien, T.J., Jones, N.C., 2013. Ethosuximide reduces epileptogenesis and behavioral comorbidity in the GAERS model of genetic generalized epilepsy. Epilepsia 54 (4), 615–643.

Di Marzo, V., Bifulco, M., De Petrocellis, L., 2004. The endocannabinoid system and its therapeutic exploitation. Nat. Rev. Drug Discov. 3 (9), 771–784.

Dopart, R., Lu, D., Lichtman, A.H., Kendall, D.A., 2018. Allosteric modulators of cannabinoid receptor 1: developing compounds for improved specificity. Drug Metab. Rev. 50 (1), 3–13.

Farrell, J.S., Greba, Q., Snutch, T.P., Howland, J.G., Teskey, G.C., 2018. Fast oxygen dynamics as a potential biomarker for epilepsy. Sci. Rep. 8 (1), 1–7.

Torrance P. Credti

Dan L. McElroy: Conceptualization, Formal analysis, Data curation, Writing – original draft, Writing – review & editing, Visualization. Andrew J. Roebuck: Conceptualization, Methodology, Formal analysis, Data curation, Writing – original draft, Writing – review & editing, Visualization. Quentin Greba: Conceptualization, Methodology, Investigation, Formal analysis, Data curation. Sumanta Garai: Methodology, Resources. Asher L. Brandt: Methodology, Investigation, Formal analysis. Orhan Yilmaz: Methodology, Investigation, Formal analysis. Stuart M. Cain: Methodology, Software, Formal analysis, Resources. Terrance P. Snutch: Methodology, Software, Resources, Supervision, Project administration, Funding acquisition. Ganesh A. Thakur: Conceptualization, Methodology, Resources, Supervision, Writing – review & editing, Project administration, Funding acquisition. Robert B. Laprairie: Conceptualization, Methodology, Resources, Visualization, Supervision, Writing – review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

None.
