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

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ABSTRACT

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, Δ9-tetrahydrocannabinol.

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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 (Ca^{2+}) channel mutations that underlie aberrant burst-firings observed in CAE (Budde and Pape, 2009; Cain and Snutch, 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; Dezsi 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 (GΔC) in the Cav3.2 voltage-gated T-type Ca^{2+} channel gene, *Cacna1h* (Polack et al., 2007; Powell et al., 2009). The pharmacological blockade of T-type Ca^{2+} 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 inhibits cAMP production, activates inwardly rectifying K^+ channels via $G\alpha_{i/o}$ 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 (Alaverdashvili and Laprairie, 2018; 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 their receptor to augment the potency and/or the 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 antinociception. 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 (βarrestin2 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% CO_2 in F-12/DMEM containing 1 mM L-glutamine, 10% FBS, and 1% Pen/Strep as well as 800 μg/mL geneticin (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% CO_2. 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 βarrestin2 assay

βarrestin2 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% CO_2. 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

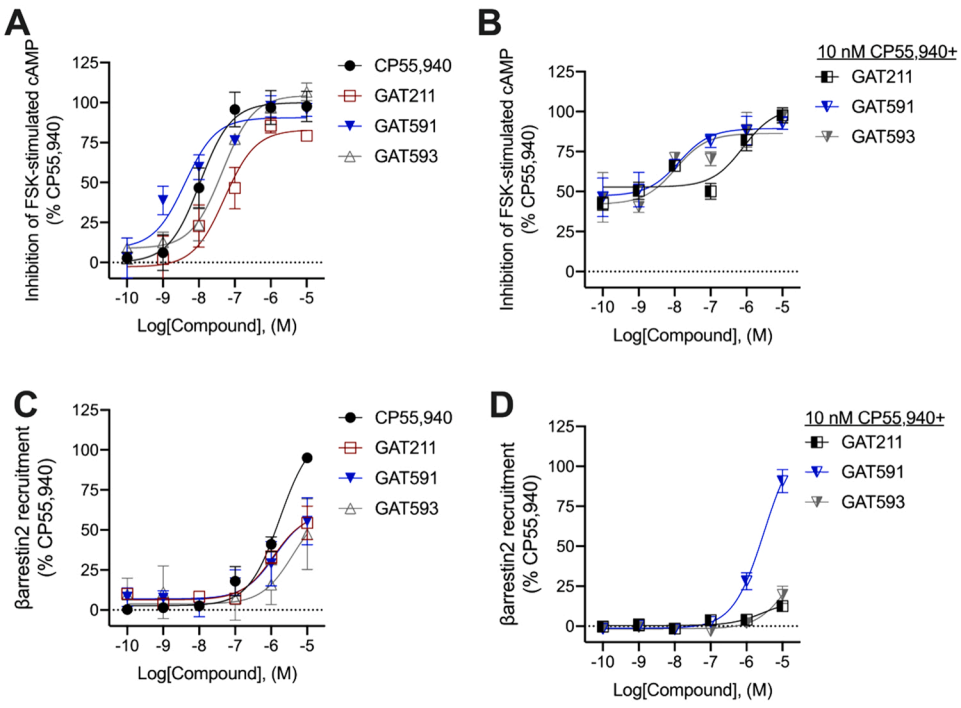

Fig. 1. CB1R-dependent inhibition of FSK-stimulated cAMP and βarrestin2 recruitment. CHO cells stably-expressing hCB1R were treated with (A,B) 10 μM FSK and 0.10 nM – 10 μM compounds ± 10 nM CP55,940 for 90 min, and inhibition of cAMP was measured; or (C,D) 0.10 nM – 10 μM compounds ± 10 nM CP55,940 for 90 min and βarrestin2 recruitment was measured. Data are expressed as % CP55,940 response. Data were fit to a nonlinear regression (3 parameter model, GraphPad v. 9) (see Table 1). Data are mean ± S.E.M. $n \geq 6$ independent experiments performed in triplicate.

in Canada in 2010 (Powell et al., 2014). The Snutch Laboratory at the University of British Columbia (snutchlab.msl.ubc.ca) provided our lab with weanlings in 2013 (Marks et al. 2016a, 2016b). Animals were maintained on 12 h light/dark cycle with lights on at 0700. Animals had access to food and water ad libitum except during testing. All experiments were conducted in adult, age matched, GAERS (5–8 months). Group sizes were determined based on previous experiments conducted in our lab (Marks et al. 2016b, 2019; Roebuck et al. 2020a, 2021) 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 are 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 βarrestin2 data are shown as % of maximal CP55,940 response (i.e., 100%). Estimates of EC_{50} and E_{max} 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 ED_{50} and E_{max}. *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 HitHunter (cAMP) and PathHunter (βarrestin2) cells were treated with 0.1 nM – 10 μM CP55,940 (reference agonist), GAT211, GAT591, or GAT593; or 10 nM CP55,940 + 0.1 nM - 10 μM 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 βarrestin2 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 $G\alpha_{i/o}$ agonists than they were agonists of βarrestin2 recruitment at hCB1R (i.e. $G\alpha_{i/o}$-selective), with a non-significant greater potency than the parent compound GAT211 in the cAMP inhibition assay (Fig. 1A,C; Table 1). Similar to previous findings in the presence of 100 nM CP55,940 (Garai et al., 2020), GAT591 and GAT593 behaved as $G\alpha_{i/o}$-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 βarrestin2 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). See the Supplementary Fig. 1A for complete 4 h traces for one rat from this experiment.

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 $G\alpha_{i/o}$-dependent inhibition of cAMP accumulation relative to βarrestin2 recruitment (Garai et al., 2020). Our present study re-affirms this earlier finding in that both GAT591 and GAT593 were demonstrably more potent as agonists and PAMs in the G protein-dependent cAMP inhibition assay compared to the βarrestin2 recruitment assay (Fig. 1). 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

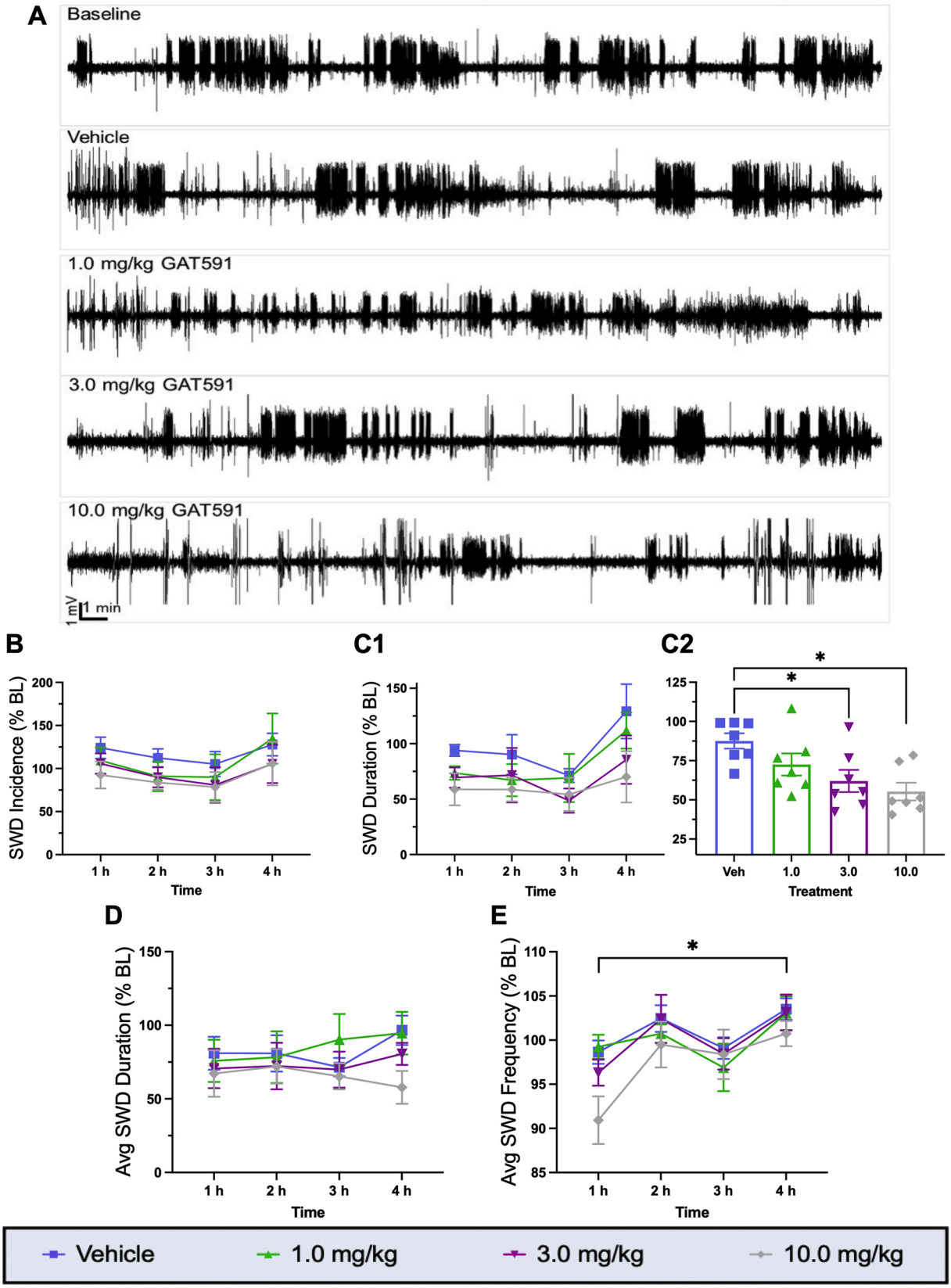

Fig. 2. GAT591 reduced the duration of spike and wave discharges (SWDs) in male GAERS (n = 7). Male GAERS were injected (*i.p.*) with vehicle, 1.0, 3.0, and 10.0 mg/kg GAT591 and EEG was recorded for 4 h post-treatment and compared to baseline (BL) activity. Representative EEG traces (30 min) depict 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. * = $p \leq 0.05$ (main effect).

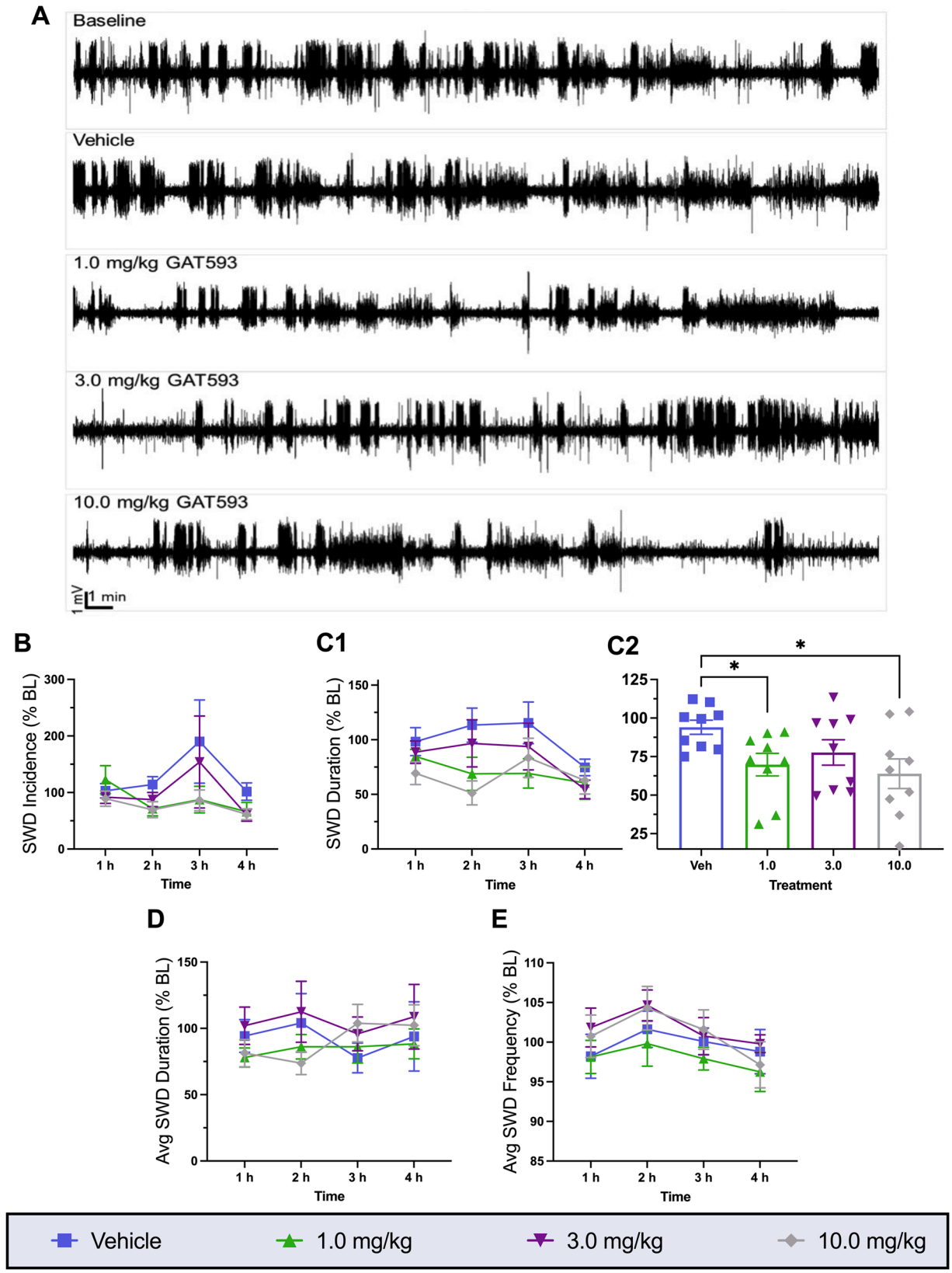

Fig. 3. GAT593 reduced the duration of SWDs in male GAERS (n = 9). Male GAERS were injected (*i.p.*) with vehicle, 1.0, 3.0, and 10.0 mg/kg GAT593 and EEG was recorded for 4 h post-treatment and compared to baseline (BL) activity. Representative EEG traces (30 min) depict SWD activity from a single rat, 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.* $= p \leq 0.05$.

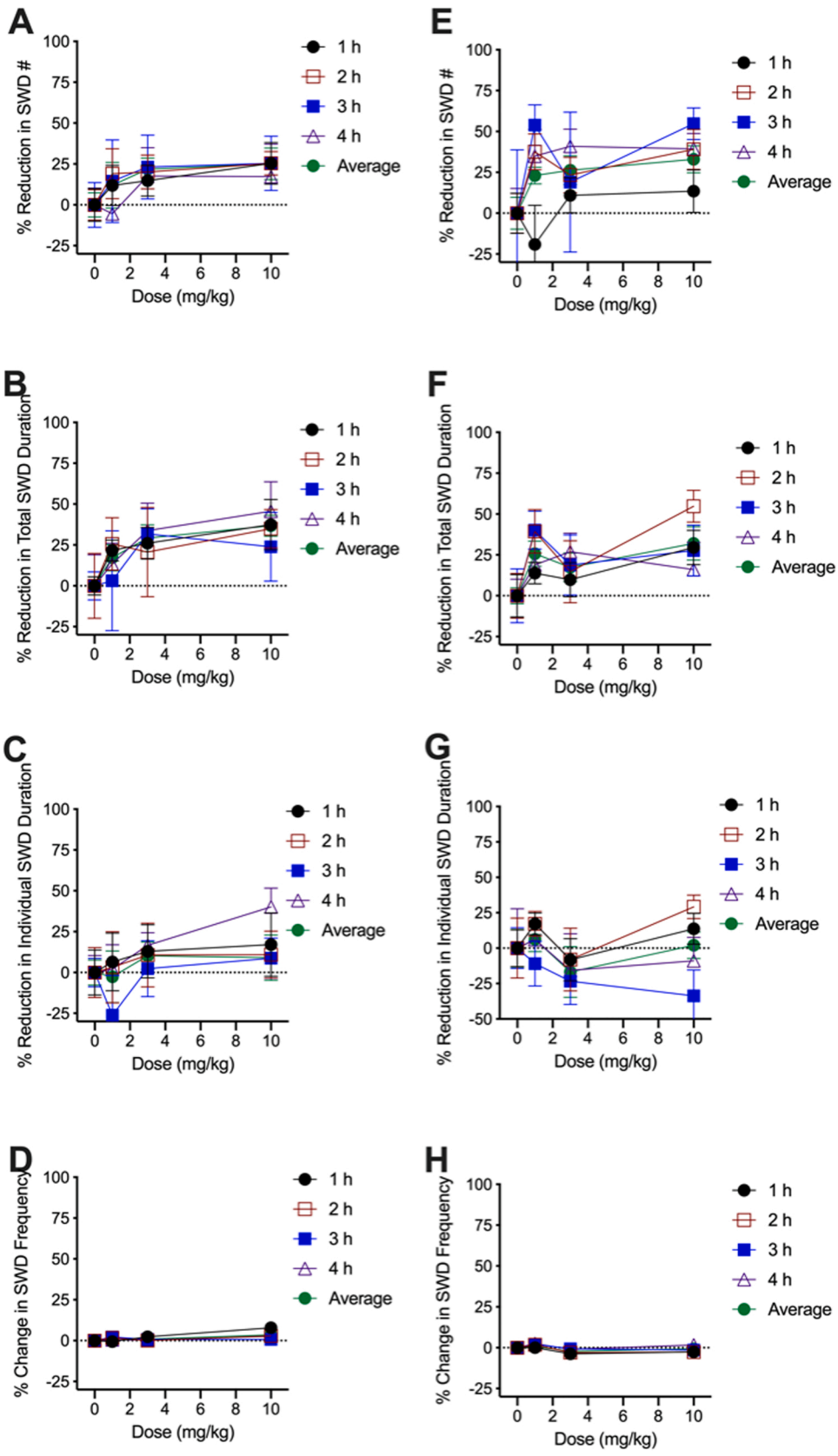

Fig. 4. Analysis of GAT591 (A-D) and GAT593 (E-F) dose-response changes in SWDs in male GAERS (n = 7 and 9, respectively). Male GAERS were injected (*i.p.*) with vehicle, 1.0, 3.0, and 10.0 mg/kg GAT591 or GAT593 and EEG was recorded for 4 h post-treatment. Data were normalized relative to baseline measurements 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.

Table 1
Modulation of CB1R by GAT591 and GAT593. CB1R-dependent inhibition of FSK-stimulated cAMP and βarrestin2 recruitment. CHO cells stably-expressing hCB1R were treated with 10 μM FSK and 0.10 nM – 10 μM compounds ± 10 nM CP55,940 for 90 min, and inhibition of cAMP was measured; or 0.10 nM – 10 μM compounds ± 10 nM CP55,940 for 90 min and βarrestin2 recruitment was measured. Data are expressed as EC_{50} (nM) with 95% confidence interval (CI) or E_{max} (% CP55,940 response) mean ± S.E.M. Data were fit to a nonlinear regression (3 parameter model, GraphPad v. 9). $n \geq 6$ independent experiments performed in triplicate. Data presented in Fig. 1.

	Agonism			
	Inhibition of FSK-stimulated cAMP		βarrestin2 recruitment	
	EC_{50} (nM) (95% CI)	E_{max} (%) ± SEM	EC_{50} (nM) (95% CI)	E_{max} (%) ± 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 (0.68–18)	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		βarrestin2 recruitment	
	EC_{50} (nM) (95% CI)	E_{max} (%) ± SEM	EC_{50} (nM) (95% CI)	E_{max} (%) ± SEM
GAT211	810 (210–1700)	102 ± 8.6	> 10,000	15 ± 0.56
GAT591	13 (1.6–69)	89 ± 5.2	> 10,000	91 ± 11
GAT593	11 (1.5–63)	86 ± 5.9	> 10,000	20 ± 1.7

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 doses. In summary, the 10 mg/kg dose of GAT591 and GAT591 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 ago-PAMs we have already tested (GAT211, GAT591, and GAT593). Further development of pure CB1R PAMs may prove valuable. Another future strategy could include a combinatorial approach (i.e., low-dose ethosuximide + GAT compound) with potential to reduce aversive side effects, in addition to capitalizing on each drug's ability to reduce SWDs in GAERS (Dezsi et al., 2013; Roebuck et al., 2021).

Currently, questions remain regarding the roles of the ECS in the generation and maintenance of SWDs. Roebuck and colleagues (2021) established the existence of ECS abnormalities in GAERS. For example, there is reduced expression of 2-AG and CB1Rs in the cortex of GAERS, which likely contributes to disinhibition of excitatory circuits throughout the cortex and the propagation of SWDs (Roebuck et al., 2021; Waagepetersen et al., 2003). Future experiments should continue to explore the association between the ECS and ictogenesis. Given that pure PAMs only increase receptor activation in the presence of ligands, it makes sense that their effects could be partial in the presence of reduced CB1R receptors and 2-AG in GAERS. Moreover, the modest reductions in SWD activity seen here, are in line with the altered ECS phenotypes reported in GAERS (Roebuck et al., 2021), suggesting that CB1R PAMs are limited by CB1R expression and 2-AG availability.

While 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 hyperexcitability 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) (Citraro et al., 2013; Perescis 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 Ca^{2+} 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%. Although ago-PAMs such as GAT591 and GAT593 did not eliminate SWDs, their use has not been shown to produce catalepsy, hypothermia, dependence, or tolerance (Garai et al., 2020) in other rodent models. Therefore, ago-PAMs represent a promising means of targeting CB1R devoid of undesirable on-target intoxicating or tolerance-inducing effects. In contrast to the ago-PAMs tested here, the CB1R agonist Δ^9-tetrahydrocannabinol (THC) increases SWDs in GAERS (Roebuck et al., 2020b). Ongoing studies are assessing the pharmacokinetic and pharmacodynamic effects of chronic ago-PAM treatment in the GAERS model of CAE. Long-term treatment with modulation of the ECS may produce effects that persist even after treatment cessation (Garai et al., 2021; Huntsman et al., 2019). We believe that further refinement of our lead ago-PAM compounds, to improve their drug-like properties and chronic efficacy across the lifespan, will lead to novel therapeutics for the treatment of CAE and other epilepsies.

Ethics

I have read and have abided by the statement of ethical standards for manuscripts submitted to Neuroscience.

CRediT authorship contribution statement

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.

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Appendix A. Supporting information

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

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