Pharmacological profiling of conventional antiseizure medicines against seizures and kindling acquisition in female mice

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Abstract

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Rationale: Historical ASM discovery has primarily relied on demonstration of efficacy in clinically validated acute seizure models evoked in male, wild-type rodents (e.g., maximal electroshock, subcutaneous pentylenetetrazol, or kindling). However, little work has been done to establish ASM response profiles in female rodents with acute or chronic seizures. Therefore, this thesis aimed to build on our previously established female C57BL/6J studies (Lehmann & Barker-Haliski 2023) to fill this knowledge gap and to define the acute median effective dose (ED_{50}) of 4 FDA-approved ASMs (levetiracetam (LEV), carbamazepine (CBZ), valproic acid (VPA), and phenobarbital (PB)) in female outbred CF-1 mice using the 6 Hz 32 mA model of focal seizures. Further, no compound has yet been found to prevent the development of epilepsy so we propose a moderate-throughput anti-epileptogenesis drug screening paradigm for early compound profiling via repeated administration of anticonvulsant doses of VPA to assess the potential to prevent the formation of chronic hyperexcitable neuronal network or modify the severity of behavioral comorbidities of epilepsy in male versus female mice using the corneal kindled mouse (CKM) model of chronic seizures.

Methods: Female outbred CF-1 mice were used to quantify of an ED_{50} of candidate ASMs (n=8 mice/dose) in a 6 Hz 32 mA test. Motor impairment was assessed via performance on a fixed speed rotarod. To establish a moderate-throughput, early-stage disease modification screening paradigm, male (n=40) and female (n=45) CF-1 mice underwent 60 Hz corneal kindling, a model of chronic network hyperexcitability, for 3-4 weeks in the presence or absence of twice daily VPA (150 mg/kg, intraperitoneal – i.p.) or vehicle administration. Mice were monitored twice daily for the presence and severity of evoked behavioral seizures until all animals met the kindled seizure endpoint. Following the kindling acquisition period, all CKM were challenged in an open field test to quantify the extent to which ASM administration during the kindling process modified anxiety-like behavioral comorbidities of epilepsy.

Results: The calculated ED_{50} [and 95% confidence intervals] in female CF-1 mice for VPA (100 mg/kg [60.5-139]), CBZ (14.0 mg/kg [8.77-23.1]), LEV (14.3 mg/kg [0.591-36.0]) and PB (18.3 mg/kg [6.8-30.4]) were consistent with our previously reported values in the 6 Hz test in inbred female C57BL/6J mice (Lehmann and Barker-Haliski 2023). Notably, CBZ was poorly tolerated in CF-1 female mice: 87.5% of female mice were impaired on a rotarod at the highest dose tested. No other motor impairment was noted. In both male and female corneal kindling acquisition studies, VPA prevented kindled seizure presentation. The number of CKM reaching the fully kindled state was lower in both female (2/10) and male (2/11) mice than in VEH-treated female (8/10) and male (14/15) mice (male: X^2 value = 18.57, $p < 0.001$; female: X^2 value = 12.77, $p < 0.001$). However, repeated VPA administration did not significantly impact CKM activity in the center of an open field versus the activity of VEH-treated mice,

suggesting no disease modifying effect of VPA administration on the anxiety-like behavioral comorbidity of epilepsy.

Conclusion: This study establishes the anticonvulsant properties of four prototypical ASMs in wild-type outbred female CF-1 mice to inform future preclinical epilepsy studies using novel investigational compounds. This work also establishes the feasibility of a disease-modification drug screening paradigm for epilepsy in male and female mice using the corneal kindled mouse model and open field test of anxiety-like behaviors, a known clinical comorbidity of epilepsy. Altogether, this work offers a strategy on which to rapidly assess the anticonvulsant and disease modifying potential of candidate investigational treatments for the symptomatic management, and possibly prevention, of epilepsy.

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Introduction

Epilepsy is a chronic neurological disorder characterized by recurrent, unprovoked seizures that can affect male and female individuals across all age groups. It is one of the most common neurological conditions with over 50 million people impacted worldwide (("Epilepsy," 2024; Fiest et al., 2017; Hauser & Beghi, 2008). Despite its prevalence, approximately 40–50% of epilepsy cases are idiopathic, meaning their underlying pathophysiological cause remains unknown (J. S. Huff & Fountain, 2011). Behaviorally, seizures result from abnormal, excessive, or synchronous neuronal activity in the brain, often due to an imbalance between excitatory and inhibitory signaling (S. J. Huff & Murr, 2023). Currently, most approved antiseizure medications (ASMs) treat the seizure symptoms of epilepsy, rather than addressing the underlying pathophysiology of epilepsy, or modifying the burden of behavioral comorbidities of the disease. There are more than 30 FDA-approved ASMs available to help manage seizure symptoms (Gunasekera, Sirven, & Feyissa, 2023; Stephen & Brodie, 2011). These ASMs primarily function by modulating ion channels, enhancing inhibitory neurotransmission (e.g., via gamma-aminobutyric acid (GABA)), or suppressing excitatory neurotransmission (e.g., glutamate) (Gunasekera et al., 2023; Hakami, 2021). While these available therapies can be effective for many patients, approximately 30% of individuals with epilepsy continue to experience seizures despite trying different available treatment—an issue known as drug-resistant or refractory epilepsy (Kwan et al., 2010; Kwan & Brodie, 2004). This unmet clinical challenge underscores the urgent need for continued efforts to identify novel therapeutic targets and disease-modifying treatments that go beyond symptomatic seizure control to ultimately address the root mechanisms of epileptogenesis.

Epilepsy is one of the few neurological disorders that has seen notable success in translating investigational compounds from preclinical studies into clinically approved treatments. The preclinical use of a small number of predictive rodent models has been instrumental to over 50 years of progress

(M. Barker-Haliski & Hawkins, 2024), enabling researchers to establish the efficacy and safety of investigational ASMs prior to advancing candidate compounds into human clinical trials (Simonato et al., 2014). However, despite these advancements, no compound has yet been identified to effectively prevent the onset of epilepsy in at risk individuals (Loscher & Schmidt, 2011). In addition, historical ASM discovery has primarily relied on demonstration of efficacy in clinically validated acute seizure models (e.g. maximal electroshock (MES), subcutaneous pentylenetetrazol (scPTZ), or kindling) exclusively evoked in male, wild-type rodents (M. Barker-Haliski & Steve White, 2020). Very little work has been made publicly available to similarly establish the efficacy and tolerability profile to prototype ASMs in female mice using these same seizure tests. This is particularly problematic because women with epilepsy face additional challenges in therapeutic management, including hormonal fluctuations that can exacerbate seizure frequency (O'Connor & Zupanc, 2009) and women are more likely than men to experience adverse drug reactions and side effects of medications because drug dosages are more frequently based on clinical trials conducted in men (Zucker & Prendergast, 2020). Additionally, sex hormones are known to influence drug metabolism through modulation of enzyme activity and transporter expression (Kashuba & Nafziger, 1998). These mechanisms remain poorly understood, thus necessitating more female data. The National Institute of Health (NIH) implemented a policy in 2014 requiring the inclusion of both sexes as a biological variable (Clayton & Collins, 2014), however, many established ASMs still lack robust sex-specific efficacy and tolerability data in established and clinically validated rodent seizure and epilepsy models.

Acute seizure models, such as the 6 Hz psychomotor seizure test and the MES test, are widely employed in initial identification of investigational compounds and have demonstrated strong predictive validity for identifying clinically effective compounds and those with novel mechanisms of action (M. Barker-Haliski & Hawkins, 2024). For this reason, the present study utilized the 6 Hz model, which can be

induced using a range of current intensities. Prior studies have shown that stimulus at 22 mA and 32 mA localized neuronal activation to the amygdala and piriform cortex (Barton, Klein, Wolf, & White, 2001). Therefore, in this present study we applied the 6 Hz 32 mA stimulus intensity to provide a high-throughput screening model of focal onset seizures that provides reasonable ASM differentiation capacity (Barton et al., 2001). Moreover, we sought to apply a moderate-throughput screening model of chronic focal seizures and thus selected the corneal kindled mouse (CKM) model, which represents another valuable tool for early-stage antiseizure drug discovery (Matagne & Klitgaard, 1998). CKM mimics key features of human acquired temporal lobe epilepsy (TLE), such as chronic seizures and behavioral comorbidities like anxiety and is used to evaluate investigational ASMs by repetitive electrical stimulation of the brain to mimic the progressive development of epileptogenesis (Goddard, McIntyre, & Leech, 1969; Potschka & Löscher, 1999). Thus, our present study implemented two well-characterized mouse models of acute and chronic focal seizures that have been routinely implemented in male mice for ASM discovery.

The aim of this study was to address the gap in available anticonvulsant activity profiles of FDA-approved ASMs in female rodents. This study first established the acute median effective dose (ED_{50}) for 4 compounds in the 6 Hz 32 mA test of acute focal seizures in female outbred CF-1 mice: (levetiracetam (LEV), a neurotransmitter modulator through interference of proper vesicle formation (Contreras-García et al., 2022), carbamazepine (CBZ), a sodium channel blocker (Pratt et al., 2012), valproic acid (VPA), a broad spectrum ASM (Ghodke-Puranik et al., 2013), and phenobarbital (PB), a GABA positive allosteric modulator (Pratt et al., 2012)). Then, we assessed the disease-modifying potential of repeated VPA administration in the corneal kindled mouse model to establish a moderate-throughput discovery platform for epilepsy prevention in male and female mice. Finally, to demonstrate the utility of our testing paradigm for future ASM discovery, we applied this ASM screening paradigm

to initially probe the pharmacokinetic and pharmacodynamic relationships of two candidate investigational drugs for the treatment and prevention of seizures in both males and female CF-1 mice. Altogether, this work demonstrates a useful conceptual framework on which to assess ASM activity in female rodents and uncover new, potentially impactful ASMs for the treatment of a variety of epilepsy patients.

Materials and Methods

Animals

Outbred adult female CF-1 mice (20-35 g) were obtained from Charles River (Wilmington, MA). All animal experimentation was approved by the University of Washington Institutional Animal Care and Use Committee and conformed to ARRIVE guidelines (Percie du Sert et al., 2020). Mice were housed in a temperature-controlled vivarium (n=5 mice/cage) on a 14:10 light/dark cycle with free access to food and water, except for periods of behavioral manipulation as previously reported (Meeker, Beckman, Knox, Treuting, & Barker-Haliski, 2019). Animals acclimated to the housing facility for a minimum of 5 days prior to experimental testing. On the testing day, mice were given a minimum of 1 hour to acclimate to the procedure room prior to all experimental manipulation.

Test Substances

Formulation for prototype antiseizure medicines (ASMs). Four FDA-approved prototype ASMs were selected to represent distinct molecular mechanisms of relevance to epilepsy. All ASMs were formulated in 0.5% methylcellulose (MC, Sigma-Aldrich, M0430). LEV (TCI chemicals; L0234), CBZ (Sigma Aldrich; C2024), and PB (Sigma Aldrich; P1636), were formulated as a suspension of 5 mg/mL or 10 mg/mL. VPA (Sigma Aldrich; P4543), was formulated as a solution of 50 mg/mL.

Drug Formulation for investigational ASMs. Both Compound A and Compound B were originally formulated in 5% polyethylene glycol 400 (PEG-400, Sigma-Aldrich; 8.07485s) and 5% Polysorbate 80 (Tween 80, VWR Chemicals; 0442-1L) in 0.5% MC. Following initial in vivo studies in the corneal kindling model, a new formulation was selected for further time-course testing in the 6 Hz seizure model that consisted of polyethylene glycol 200 (PEG-200, Sigma-Aldrich; 8074831000) suspended to 20 mg/mL and stirred until dissolved (for approximately 1 hour). Stock solution of 20% tocopherol polyethylene glycol succinate (TPGS, Millipore Sigma; 57668) in sterile saline (pharmaceutical grade) was added to this stock solution in a 3:1 (PEG200:20%TPGS) ratio to create a 15 mg/mL solution and then further diluted to 2.5 mg/mL (Supplemental Table 1). This optimized formulation was used for subsequent time course studies in the 6 Hz seizure model.

6 Hz Seizure Testing

6 Hz seizures were induced in groups of mice using an electroconvulsive stimulation unit (Grass S48 Stimulator). A drop of 0.5% tetracaine (0.5% solution w/v in 0.9% saline) was applied to both corneas immediately prior to the electrical stimulation to provide local anesthesia and to ensure electrical conductivity. Mice were challenged with a 32 mA equivalent current for 3 sec delivered through corneal electrodes to elicit a psychomotor seizure (Barton et al., 2001). The 6 Hz seizure is characterized by an initial momentary stun followed immediately by forelimb clonus, twitching of the vibrissae, and Straub tail (M. Barker-Haliski et al., 2018; Barton et al., 2001; Walrave et al., 2015). Animals not immediately displaying these behaviors post-stimulation (within 5-10 second) were considered “protected”. In our experience, animals demonstrate these behaviors immediately following the cessation of corneal stimulation, when they do occur. ED_{50} were estimated using the staircase estimation procedure as previously described by F.M. Wadley (Wadley, 1952).

Time course experiment. Mice were acutely administered 10 mg/kg of CMPD A or B via the i.p. route and tested with 6 Hz stimulation at timepoints of 0.25, 0.5, 1, 2, or 4 hours post-drug administration (n= 4 each timepoint) to determine the time-to-peak anticonvulsant effect (TPE) for each compound.

Median Effective Dose (ED_{50}) Quantification. Male and female mice were treated with escalating doses of LEV, CBZ, VPA, or PB via i.p. administration at doses bracketing the predetermined ED_{50} for each compound in female C57BL/6J mice (Lehmann & Barker-Haliski, 2023). Seizure stimulations were conducted post-i.p. administration by a blinded investigator at the previously determined TPE for each compound (1 hour (LEV), 0.25 hour (CBZ), 0.25 hour (VPA), or 0.5 hour (PB)), as earlier reported by M. L. Barker-Haliski, Vanegas, Mau, Underwood, & White (M. L. Barker-Haliski, Vanegas, Mau, Underwood, & White, 2016), and Lehmann & Barker-Haliski (Lehmann & Barker-Haliski, 2023).

Rotarod test of minimal motor impairment. Immediately prior to acute 6 Hz seizure testing, each mouse was evaluated for minimal motor impairment (MMI) using a fixed speed rotarod. Briefly, when a normal mouse is placed on a rod that rotates at a speed of 6 rpm, the animal can maintain its equilibrium for long periods of time (Dunham & Miya, 1957). Each time a mouse falls off the rotarod in the 60 second trial period, it is immediately returned to the rotarod until the 60 second trial period has elapsed. A mouse was considered “impaired” if it fell 3 or more times off this rod in the 1 min period. The extent of impairment (“impaired”/number of mice tested) at each dose was tabulated for all experimental groups.

Pharmacokinetic analysis of investigational ASM plasma and brain concentration following 6 Hz seizure testing

Terminal Tissue Collection for Bioanalysis. Terminal plasma and brain tissue were collected within 5 minutes of behavioral seizure testing of investigational ASMs in the acute 6 Hz time course test. Mice were euthanized by live decapitation and whole trunk blood collected using microtainer tubes pre-coated with K2EDTA anticoagulant (Becton, Dickenson & Company product # 365974), with plasma separated via centrifugation (1,000 rcf for 10 min) at 4ºC within 30 minutes of collection. Plasma was isolated and transferred into individual polypropylene tubes, frozen, and stored at -80ºC for high performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay. The brains were removed and then briefly rinsed with 1x PBS, pat-dried with a paper towel, and weighed. Each brain was flash frozen in an individual, clearly labeled vial overlaying dry ice, and stored at -80ºC for LC-MS/MS assay.

Brain Homogenization. Brain hemispheres were lightly thawed and cut on the sagittal plane. Each hemisphere was weighed and isolated into a 2 mL microcentrifuge tube with 2.4 mm metal beads (Fisher Scientific; 15-340-151). Using the Omni Bead Ruptor 24, brains are homogenized in 1:4 w/v water solution at the speed of 3.1 m/s for 60 sec (modified from (Liang et al., 2011). Brains were prepared for LC-MS/MS analysis using the same preparation described below for plasma.

Sample Preparation. Plasma and brain samples (10 µL) were spiked with 150 ng/mL internal standard (diazepam d-5; Cayman Chemical, 18548) and vortexed with methanol (HPLC Grade, ThermoSci, 022909.K7) and 0.1% formic acid (99%, LC/MS grade, Fisher Scientific, A117-05AMP) to precipitate proteins. The solution is separated from solid debris by centrifugation (16,000 rcf for 5 min) at room

temperature followed by supernatant collection (70 µL) into autosampler vials (Thermo Scientific, 6PRC11STS1X) for analysis.

Pharmacokinetic analysis. CMPD B concentrations in brain and plasma samples were assessed LC-MS/MS using a 1290 UHPLC (Agilent Technologies) in tandem with a 6460 triple quad mass spectrometer (Agilent Technologies). 5 µL of each sample was injected and analytes were separated with a 1.7 um Acquity UPLC BEH C18 Column (2.1 × 100 mm; Waters Corp, #186002352). For CMPD B, mobile phases of 0.1% formic acid in methanol and 0.1% formic acid in water were delivered at a flow rate of 0.30 mL/min over 12 min, with a stepwise adjustment of mobile phase concentration gradient as followed: 0–0.5 min, 5% B; 0.5–11.0 min, linear increase to 100% B; 11.0–13.0 min, hold at 100% B; 13.0–13.1 min, return to 5% B; and 13.1–15.0 min, re-equilibration at 5% B. Mass spectrometric detection was performed with an electrospray ionization (ESI) source operated in positive ion mode. The source parameters were as follows: capillary spray voltage, 3500 V; gas temperature, 325°C; gas flow, 6 L/min; nebulizer gas, 45 psi; and sheath gas flow, 11 L/min. Multiple reaction monitoring (MRM) was used to monitor the transitions m/z 319.2→ 274.2 and 319.2→ 234.1 for CMPD B and m/z 290.1 → 154.1 for the internal standard (diazepam d-5), with a collision energy of 30 V and a declustering potential of 70 V for CMPD B and 135 V for the internal standard. The calibration curve ranged from 0.72 to 173 ng/mL and was linear with $R^2 = 0.997$ for plasma samples and $R^2 = 0.996$ for brain samples (Supplemental Figure 2).

Mouse Model of Epileptogenesis

Corneal Kindling Model. Male and female CF-1 mice were administered of anticonvulsant doses of either VPA (50 mg/kg, n = 15 males, 10 females), CMPD A (10 mg/kg, n = 15 males; n = 12 females),

CMPD B (10 mg/kg, n = 10 females), or VEH (n = 15 males; n = 10 females) via the i.p. route. At the previously determined TPE, mice were stimulated with an electrical current of 60 Hz (sinusoidal pulse, 3.0 mA) for 3 sec via corneal electrodes (M. L. Barker-Haliski et al., 2017; M. L. Barker-Haliski et al., 2016; Rowley & White, 2010). Evoked behavioral seizures were scored based on a modified Racine scale originally developed for amygdala-kindled rats, (M. L. Barker-Haliski et al., 2017; M. L. Barker-Haliski et al., 2016; Rowley & White, 2010) wherein; 1 = jaw chomping and vibrissae twitching, 2 = head bobbing and Straub tail, 3 = unilateral forelimb clonus, 4 = bilateral forelimb clonus and hind-limb rearing, 5 = bilateral forelimb clonus and rearing followed by loss of righting reflex. Twice daily drug treatment and stimulations continued for each mouse for up to 40 sessions (~4 weeks) or until the mouse achieved the criterion of 5 consecutive Racine stage 5 seizures, whereby it was considered "fully kindled". Fully kindled mice were then stimulated once every other day until the conclusion of the behavioral monitoring period. During the last 7 sessions, mice underwent a treatment-free washout period in which stimulations would occur as normal, but no treatments were given to assess seizure presentation and kindling progression after sub-chronic ASM administration.

Post-corneal kindling open field behavioral study. Mice were then given a 3-day stimulation-free period following the corneal kindling acquisition period. Two days of open field (OF) testing was performed in a non-orientating clear Plexiglas unit (40 L x 40 W x 30 H cm) equipped with infrared beams and automated location tracking software to record the vertical and horizontal location of each mouse in real time and distinguish between perimeter and center regions of the field (Accuscan, Omnitech Electronics). Mice were randomized to the testing chambers by the automated recording software and chambers were cleaned with 30% isopropyl alcohol between each mouse. On the first OF testing day, mice were allowed to explore the OF chamber without drug administration for 10 min to assess the

effect of sub-chronic ASM administration during the kindling acquisition period on the presentation of anxiety-like behaviors (i.e., total distance traveled, distance traveled in the center, time spent in the center, and rearing episode count) after the kindling period concluded. On the second day, all kindled mice again received their initially administered investigational compound (VPA, CMPD A, CMPD B, or VEH) and returned to the OF chambers to quantify the extent of acute ASM administration on locomotor activity for 10 min (i.e., total distance traveled, resting episodes, and horizontal activity counts). Mice activities were recorded in the same testing chambers for each testing day, with recordings initiated 5 minutes before the TPE of each administered compound on the second experimental testing day (i.e. 10 min post-VPA administration, 55 min post-CMPD A administration, 25 min post-CMPD B administration, and 55 min post-VEH administration).

Statistics

The ED_{50} for each compound was calculated using computer-assisted log-probit linear regression analysis method (LITCHFIELD & WILCOXON, 1949; Wadley, 1952) and reported in mg/kg with a 95% confidence interval (CI). A χ^2 test was performed to assess statistical significance in the 6 Hz dose-response studies, while the 6 Hz time course study utilized the Fisher's exact test to assess association between protected vs. not protected from seizures. HPLC-MS/MS data was analyzed by two-way ANOVA to compare time-related increases in brain and plasma concentration as a function of route of administration (ROA; i.e., i.p. vs p.o.). Corneal kindling acquisition is represented as the percent of all mice enrolled in the study that achieved fully kindled state and analyzed using a Kaplan-Meier log-rank test (survival curve) to demonstrate kindling progression over time. A paired Wilcoxon t-test was performed on the average seizure scoring of the 7 sessions before drug washout and the final 7 sessions during drug washout period to determine significant changes during this testing window. Activity in the

OF chamber on each testing day was evaluated by one-way ANOVA, with Dunnett's post-hoc test. Data analysis was conducted with GraphPad Prism v. 10.2.1, with $p < 0.05$ considered statistically significant.

Results

Anticonvulsant activity of prototype ASMs is consistent between young female CF-1 mice and C57BL/6J mice in the 6 Hz model of focal onset seizure

There is limited published data available on the ED_{50} of conventional ASMs in young female mice, thus we aimed to establish the ED_{50} of several mechanistically distinct ASMs in outbred CF-1 female mice to compare to our previously defined 6 Hz ED_{50} values in female inbred C57BL/6J mice (Lehmann & Barker-Haliski, 2023). Using the staircase estimation procedure at the previously determined TPE for each compound, we estimated the ED_{50} of VPA (100 mg/kg, 95% Cl [60.5-139]; $p = 0.028$), CBZ (14.0 mg/kg, 95% Cl [8.77-23.1]; $p = 0.011$), LEV (14.3 mg/kg, 95% Cl [0.591-36.0]; $p = 0.163$) and PB (18.3 mg/kg, 95% Cl [6.8-30.4]; $p = 0.028$); doses that were consistent with our previously reported values in the 6 Hz test in age-matched female inbred C57BL/6J mice (Lehmann & Barker-Haliski, 2023) (Fig.1, B-E; Table 1). Of note, all compounds were well tolerated at the doses and time points tested in the CF-1 female mice, except for CBZ, which was poorly tolerated: 87.5% of female mice were impaired on a rotarod at the highest dose tested (40 mg/kg), which is similarly to what was previously observed by Lehmann and Barker-Haliski (2023), wherein 60% of female C57BL/6J mice were impaired at this same dose. However, unlike earlier reports that 37.5% of C57BL/6J females exhibited motor impairment with VPA 300 mg/kg treatment, this tolerability deficit was not seen in this present study with CF-1 females.

Formulation optimization for investigational ASMs improves anticonvulsant activity in vivo

The ED_{50} for CMPD A and CMPD B could not be defined as anticonvulsant activity was not achieved in more than 50-60% (Figure 1F-G) in mouse 6 Hz studies. New formulations were thus evaluated to potentially improve drug delivery to the brain and optimize anticonvulsant 6 Hz seizure test activity (Supplemental Table 1). All compound formulations appeared to be improved suspensions relative to the original formulation (Supplemental Table 1). Based on a limited *in vivo* activity study (Supplemental Table 2), Formulation #3 (1:3 PEG 200 / 20% TPGS) was selected for further quantitative anticonvulsant activity testing in CF-1 mice (Supplemental Table 3 & Supplemental Figure 1).
With the new formulation, we repeated the in vivo activity profiling in both male and female CF-1 mice via both i.p. and p.o. administration routes to define whether CMPD A or CMPD B was orally active (Figure 2A). Notably, CMPD A administration to CF-1 males was found to have a 2 hrs TPE (4/4 mice protected) with i.p. administration ($p = 0.087$), whereas the TPE was extended to 0.5-2hrs (2/4 protected at 0.5, 1, and 2 hrs) with p.o. administration ($p = 0.956$; Figure 2B). Female mice displayed an earlier TPE at 0.25-1hr (3/4 protected at 0.25, 0.5, and 1 hr) through i.p. injections ($p = 0.726$) and 1 hr (3/4 protected) with p.o. administration ($p = 0.286$; Figure 2C). CMPD B has a TPE at 1 hr (3/3 protected) with i.p. administration in male mice ($p = 0.006$) and 2 hrs (3/4 protected) with p.o. administration $p = 0.846$; Figure 2D). Female mice treated with CMPD B also showed earlier TPE with 4/4 mice protected following i.p. administration ($p = 0.068$) at 1 hr and 4/4 protected following p.o. administration ($p = 0.085$) at 0.25 hr (Figure 2E).

Optimized formulation improves CNS penetrance as demonstrated by onset of motor impairments in mice treated with investigational compounds prior to 6 Hz testing

The rotarod test assesses mouse motor coordination and balance following acute drug administration. With our initial formulation for CMPD A and B, not only did we fail to attain maximal anticonvulsant

activity in the 6 Hz test, but we also did not observe any adverse motor impairment effects in treated mice using the fixed speed rotarod test. However, our optimized formulation (Formulation #3) showed some improved CNS penetrance, as mice similarly treated with Formulation #3 demonstrated motor impairments at 10 mg/kg (1/4 mice were impaired at 0.5 hr in females).

CMPD B demonstrate route-dependent brain penetrance that correlates to in vivo anticonvulsant activity in male and female mice

To initially probe the pharmacodynamic and pharmacokinetic relationship of CMPD B for anti-convulsant activity , plasma and brain concentrations were measured at multiple time points (0.25, 0.5, 1, 2, and 4 hrs) following a single 10 mg/kg intraperitoneal (i.p.) or oral (p.o.) administration in male and female CF-1 mice following seizure testing (Figure 3). Comparisons were made using a two-way ANOVA analyzing the main effects of time, ROA, and their interaction with each other. In male and female CF-1 mice, i.p. administration resulted in highest drug concentrations in plasma (male: $F(4, 8) = 2.507$, $p = 0.125$; female: ($F(1, 2) = 60.11$, $p = 0.16$). However, male plasma concentrations show a significant interaction between time and ROA, which was not observed in brain samples or female data (male: $F(4, 8) = 8.616$, $p = 0.005$) (Figure 3A). Specifically, i.p. administration of CMPD B to male CF-1 mice resulted in a plasma concentration peak at around 1 hour (T_{max}), while a T_{max} was not observed with p.o. administration as concentrations continued to rise at the longest time point tested (4 hours). In brain tissues, male and female mice both demonstrated increases in drug exposures over the 0.25 hr to 4 hr time points, (male: $F(1, 3) = 27.79$, $p = 0.013$; female: $F(1, 3) = 10.47$, $p = 0.048$) and significant peak concentration differences between administration routes (male: $F(4, 12) = 25.2$, $p < 0.001$; female: ($F(3, 9) = 31.8$, $p < 0.001$) (Figure 3C-D). These data illustrate that CMPD B is highly brain penetrate by both the i.p. and p.o. routes of administration, and that exposure likely correlates with in vivo seizure

activity in both male and females (Figure 3E–F). We note that the experimental brain homogenate far surpassed our estimated calibration range (Supplementary Figure 2 B) so brain concentrations above 170 ng/mL are based on extrapolation beyond the calibration region.

Repeated valproic acid (VPA) administration prevented corneal kindled seizure presentation but did not modify kindled seizure acquisition

There were no apparent sex-related differences in kindling acquisition rates between sexes (Figure 4B-E). Further, repeated VPA administration significantly suppressed acute seizure presentation (versus VEH-treated mice, males: X^2 value = 18.560, $p < 0.001$; females: X^2 value = 12.770, $p < 0.001$); no mice reached kindling criterion during the initial 33 drug-treated stimulation sessions (Figure 4D-E). Relative to VEH-treated mice, CMPD A (males: X^2 value = 0.610, $p = 0.435$; females: X^2 value = 4.842, $p = 0.028$) and CMPD B-treated mice (X^2 value = 1.998, $p = 0.157$) displayed some delay in reaching kindling criterion. However, during the last 7 sessions of drug washout, mice previously treated with VPA and CMPD A attained higher seizure scores, matching the seizure severity score of VEH-treated mice (Figure 4F-G; (VPA; males: $p < 0.001$, females: $p = 0.004$) (CMPD A; males: $p = 0.031$, females: $p = 0.020$)). Further, conclusion of drug administration did not lead to sustained resistance to evoked seizure presentation. CMPD B-treated mice in this drug washout period did also have a significant increase in scoring during washout period but maintained a low percentage of mice from (Figure 4G; males: n/a, females: $p = 0.031$. Most notably, there were no significant adverse effects noted in either repeated treatment group.

Repeated administration of investigational antiseizure medicines exert sex-specific differences on presentation of anxiety-like behavioral comorbidity of epilepsy in corneal kindled CF-1 mice

On day 1 of post-kindling behavioral testing, anxiety-like behavior was assessed on a non-habituated OF task (Figure 5). Epilepsy is commonly clinically associated with neuropsychiatric deficits (Novak, Vizjak, & Rakusa, 2022; Vrinda, Arun, Srikumar, Kutty, & Shankaranarayana Rao, 2019), including increased anxiety, so we sought to determine whether either sub-chronic treatment paradigm could significantly modify the presentation of this behavioral comorbidity of disease. Specifically, if these drugs exert a disease modifying effect, anxiety-like behavioral outcomes would be significantly modified between investigational compound-treated and VEH-treated mice. However, we did not identify any substantial differences in OF behavior between experimental groups. In males, CMPD A-treated mice displayed significant decreases in time spent in the OF center ($F(2, 40) = 6.142$, $p = 0.005$; Figure 5C), suggesting an anxiogenic-like effect of repeated CMPD A administration. However, repeated CMPD A and VPA administration to male mice was also associated with decreased rearing behavior ($F(2, 40) = 9.171$, $p < 0.001$; Figure 5E), suggesting an anxiety-like phenotype associated with repeated administration of these compounds. Contrary to male mice results, there were only some modest increases in rearing behavior in female mice previously treated with VPA versus VEH mice ($F(3, 37) = 9.986$, $p < 0.001$; Figure 5L). No other differences were found in CMPD A and B-treated female mice compared to VEH-treated female mice (Figure 5H-N).

Acute administration of investigational antiseizure medicines exert sex-specific differences on locomotor activity of corneal kindled CF-1 mice

The habituated OF task allows for the evaluation of acute drug-induced effects on locomotion (M. L. Barker-Haliski et al., 2017; M. L. Barker-Haliski et al., 2016). Thus, we sought to assess whether fully kindled mice that were repeatedly administered VPA or the investigational compounds demonstrated any changes in OF exploration following acute administration of those same compounds following a 5

day washout-period (tested at the compound TPE). Mice received the matched treatment from the kindling period before being challenged for 10 min in the OF chamber to assess the acute effects of each investigational compound on locomotor behavior. Females displayed no significant changes (Figure 6H-N) except for an increase in rearing behaviors in VPA-treated mice ($F(3, 43) = 9.389$, $p < 0.001$; Figure 6L), but Dunnett's post-hoc tests revealed decreased horizontal activity ($F(2, 40) = 3.961$, $p = 0.027$; Figure 6D) and rearing episodes ($F(2, 40) = 17.200$, $p = 0.027$; Figure 6E) in VPA and CMPD A-treated male mice. Notably, more resting episodes were also observed in male mice acutely treated with CMPD A versus VEH-treated male mice ($F(2, 40) = 6.329$, $p = 0.004$; Figure 6G), overall indicating acute suppressive effects of anticonvulsant doses of this investigational compound on locomotor activity specifically in males.

Discussion

This study provides several critical findings of relevance to the treatment and prevention of seizures in people with epilepsy, with a particular focus on the impact of investigational compounds in females. First, our findings confirm that outbred male and female CF-1 mice exhibit similar anticonvulsant activity profiles in the 6 Hz focal seizure model in response to acute administration of LEV, CBZ, VPA, and PB. During the evaluation of two novel investigational ASMs, sufficient anticonvulsant activity was not detected to quantify ED_{50} values, so drug delivery strategies were re-evaluated to improve CNS penetrance and pharmacodynamic outcomes (i.e. seizure control). Formulation #3 displayed the most anticonvulsant activity in the 6 Hz 32 mA test for both compounds in our formulation trials (Supplemental Table 1-2) utilizing TPGS, a safe pharmaceutical additive known to enhance drug solubility, permeability, and stability (Kumbhar et al., 2022). These properties are essential components for increasing absorption and bioavailability of candidate compounds according to the Biopharmaceutics Classification System, a pharmaceutical development tool used for classifying drugs (Amidon,

Lennernäs, Shah, & Crison, 1995; Samineni, Chimakurthy, & Konidala, 2022). Second, we herein demonstrate the acute anticonvulsant activity of two novel investigational compounds in male and female CF-1 mice. Importantly, the time-related concentration curve of CMPD B exposure in brain and plasma of male and female CF-1 mice revealed higher accumulation of drug in the brain than in the plasma following both i.p. and p.o. administration routes (Figure 3E-F), values that corresponded to in vivo pharmacodynamic outcomes in the 6 Hz seizure test presently employed. However, we take caution in fully defining brain concentration trends as the amount detected in the brain homogenate exceed our calibration curve, so further optimization of the protocol will need to be assessed to validate the reliability of this outcome. Administration route also showed a significant impact on drug presence and data suggests that p.o. administrations resulted in delayed accumulation in the brain (Figure 3C-D). Further investigation is necessary to explore VEH effects independently on anticonvulsant activity and establish the ED_{50} of each investigational compound with the optimized formulation using the 6 Hz 32 mA seizure model. Finally, we validated a moderate throughput drug screening paradigm to uncover disease modifying treatments for the potential prevention of epilepsy using the CKM model evoked in mice paired to a behavioral comorbidity testing paradigm. In this CKM study for anti-epileptogenesis, repeated VPA administration during the kindling period was likely masking seizure symptoms rather than treating the underlying pathological processes of epilepsy, as evidenced by its increased seizure activity during the washout period of this model (Figure 4) and lack of improvement in open field testing compared to the VEH-treated mice (Figure 5). Although CMPD B has potential to be a disease modifying compound for epilepsy although CKM must be repeated in the new formulation (Formulation #3).

Animal seizure and epilepsy models have long been instrumental in the discovery and development of antiseizure medications, enabling the evaluation of therapeutic activity and tolerability prior to clinical

testing. However, the historical reliance on male rodents in preclinical research—often justified by the perceived complexity of female physiology—has resulted in limited understanding of sex-specific drug responses. Studies have suggested that both male and females generally responded similarly to ASMs although anecdotal feedback from patients indicate more adverse side effect may be seen in females (Giuliano et al., 2022), thus these discrepancies highlight the need for sex-based evaluation during the early stages of drug discovery before in-human clinical trials.

This study employed the CKM model to assess a disease modification screening paradigm for investigational drug discovery. Corneal kindling offers a controlled and precisely timed approach to model epileptogenesis by delivering repeated subthreshold stimuli to gradually elicit a permanent seizure-prone state. Although time-intensive, this method provides valuable insight into the progressive stages of epileptogenesis (Matagne & Klitgaard, 1998). Unexpectedly, our results revealed masked seizure symptoms during the treatment phase of kindling in VPA-treated mice; however, during the seven-day drug washout period, seizure suppression was lost, and animals rapidly progressed to stage 5 seizures (Figure 4B-C). Our protocol utilizes paired stimulation with treatment to track its effects during the development of epilepsy. This finding suggests that VPA may not confer disease-modifying effects, but rather offers symptomatic seizure control. Our finding contradicts with those of Silver et al. (Silver, Shin, & McNamara, 1991) who previously reported disease-modifying properties of VPA in a rat amygdala kindling model. Notably, their study utilized a shorter treatment window—nine days of paired stimulation and VPA exposure—followed by a 2 week rest period and then resumed kindling without drug treatment (Silver et al., 1991). In comparison, our extended treatment through the full initial acquisition phase may provide a more sensitive test of a compound's potential to interrupt any neurobiological changes occurring during epileptogenesis. Furthermore, the amygdala kindling model

involves procedural electrode implantation and direct stimuli application to the amygdala which requires single housed rats and overall, utilizes more resources. On the contrary, our epileptogenesis model makes use of a non-invasive approach with stimulation via corneal electrodes in mice, making CKM cost-effective and avoids the need for single housed animals that can negatively impact the rodent's mental well-being as they tend to thrive in social environments. All in all, as an early drug discovery tool, the corneal kindling model may provide an improved moderate-throughput screening approach to discern potential anti-epileptogenic treatments. We further tested the differentiation capacity of this experimental paradigm using 2 investigational compounds (Figure 4). Notably, CMPD B demonstrated sustained seizure suppression during the washout phase, suggesting potentially promising antiepileptogenic activity that is differentiated from the in vivo effect of VPA. It should be noted, however, that our disease modification studies did not include an optimized drug formulation paradigm thus future studies should evaluate the disease modifying potential of CMPD B using formulations optimized for IP dosing. There is a debate whether this model can effectively translate the underlying biology of human epilepsy (Bertram, 2007), but applications of current effective therapies for human TLE treatments have equally translated in rodent kindling (Loscher, 2016), possibly due to its crucial parallels in circuitry for limbic epilepsy (Bertram, 2007). Some limitations to this model may include an important distinction between the rodent kindling model and human epilepsy. In CKM, the seizure inducing stimulus is applied to an otherwise healthy brain. While the mice reach fully kindled state, some mild effects such as neuronal loss and axonal sprouting may occur because of the repeated stimulation whereas in humans, these structural changes may be the cause of seizures. Therefore, CKM assumes a relatively intact circuitry which could not be predicted in humans (Pitkänen & Halonen, 1998). Nonetheless, CKM remains a highly valuable rodent screening model in assessing disease modifying potential of investigational compounds.

Epilepsy is frequently associated with increased risk for anxiety disorders, which may stem from anticipatory stress, fear of seizure recurrence, social and functional limitations, sleep disruption, or neurobiological changes (Hingray, McGonigal, Kotwas, & Micoulaud-Franchi, 2019; Kanner, Carrazana, Munger Clary, Rabinowicz, & Faught, 2024; Munger Clary, 2022). CKM not only replicates seizure development but also reproduces behavioral comorbidities commonly observed in TLE (Hingray et al., 2019; Kanner et al., 2024; Munger Clary, 2022). Typically, studies have shown that in humans, females tend to have a higher prevalence to mood disorders while males are more impacted by neurological disorders related to neurodevelopment such as schizophrenia, autism, or Parkinson's disease (Pallier et al., 2022; Pinares-Garcia, Stratikopoulos, Zagato, Loke, & Lee, 2018). On the contrary, our main findings for anxiety-like behaviors measured through the open field test did not improve with any treatment relative to VEH-treated animals. Rather, male mice in all treatment groups exhibited increased anxiety-like behavior and impaired locomotor activity, whereas this effect was not observed in females. This could be due to bias in the OF task itself. It is important to note that most preclinical behavioral models of psychiatric illness, including those for anxiety, were historically developed and validated in male rodents. It has been established that in OF testing, females are likely to be more locomotive and spend more time in the OF center, which can be construed as being "less anxious" (Knight et al., 2021; Ramos, Berton, Mormède, & Chaouloff, 1997). This is hypothesized to be explained by organizational differences in neurocircuitry development (Biedermann et al., 2017), or sex hormones because females rodents have cyclic fluctuations of estrogen and progesterone, which have been reported to exert both anxiolytic and anxiogenic effects (Scholl, Afzal, Fox, Watt, & Forster, 2019). Thus, we take caution to not misinterpret the data without additional, sex-sensitive assays (Börchers, Krieger, Asker, Maric, & Skibicka, 2022). Further research is warranted to better understand

the underlying biological mechanisms that drive sex-specific behavioral responses and to refine behavioral models for more accurate cross-sex comparisons in epilepsy research.

In summary, this study highlights the importance for assessing the potential for sex-specific differences in ASM response in epilepsy drug discovery and provides a novel framework on which to rigorously evaluate the potential for investigational compounds to modify disease progression. These findings suggest that there are no marked differences in anticonvulsant activity in the ASMs presently tested in female mice using a well-established preclinical seizure model (the 6 Hz test). Further, we herein uncover two potentially novel investigational compounds that exert both anticonvulsant and disease-modifying potential in both male and female CF-1 mice, warranting further in vivo study in additional rodent seizure and epilepsy models such as the post-kainic acid rat model of acquired temporal lobe epilepsy (M. Barker-Haliski & Steve White, 2020). Altogether, this work aims to better reflect patient diversity and support the development of more inclusive and effective epilepsy therapies for people with epilepsy.

Figures and Tables

Table 1. Median anticonvulsant doses (ED_{50}; in mg/kg) of prototype ASMs in 6 Hz 32mA seizure test in mice following i.p. administration at time-of-peak effect of each compound. Data are presented as from outbred CF-1 and inbred C57BL/6J mice in both biological sexes. Data represented as median doses (mg/kg; i.p.) and 95% confidence intervals for each compound.

ASM (i.p. route in 0.5% methylcellulose)	**Time of Test post-administration** (hours)	**CF-1 Male** ED_{50} mg/kg and 95% CI; i.p. route (from Barton et al., 2001)	**C57BL/6J Female** ED_{50} mg/kg and 95% CI; i.p. route (from Lehmann & Barker-Haliski, 2023)	**CF-1 Female** ED_{50} mg/kg and 95% CI; i.p. route
LEV (TCI chemicals; L0234)	1	19.4 [9.90 - 36.0]	20.2 [7.85 - 36.6]	14.3 [0.591 - 36.0]
CBZ (Sigma Aldrich; C2024)	0.25	47.9 [a] [27.4–94.2]	7.91 [4.33 - 12.4]	14.0 [8.77 - 23.1]
VPA (Sigma Aldrich; P4543)	0.25	126 [94.5–152]	163 [122 – 212]	100 [60.5 - 139]
PB (Sigma Aldrich; P1636)	0.5	14.8 [8.90–23.9]	n/a	18.3 [6.8 - 30.4]

a - Additional data from the PANAChE database reports CBZ to have an ED_{50} of 23.69 mg/kg 95% Cl [18.28 - 26.25]

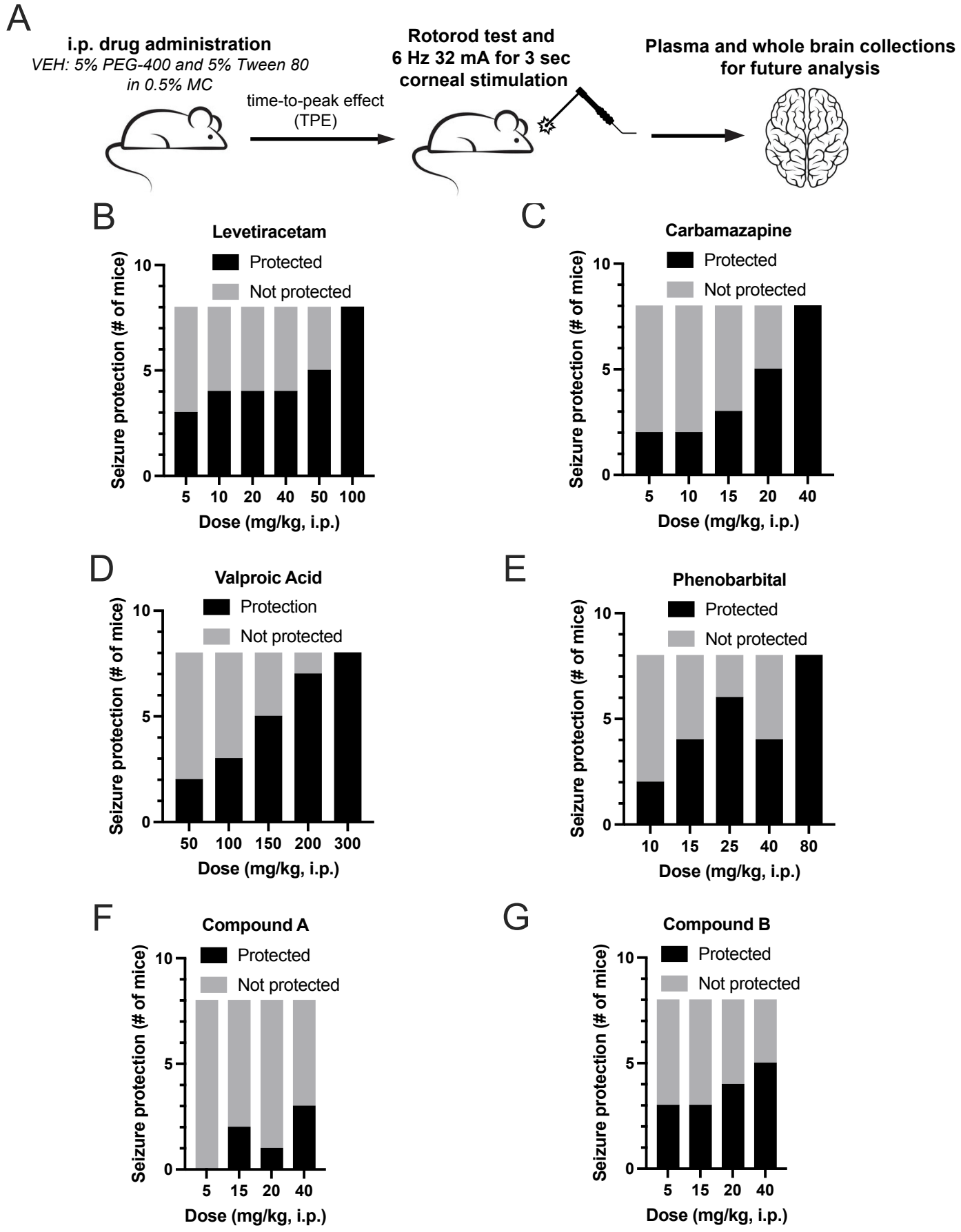

Figure 1. (A) Experimental study design for 6 Hz 32 mA seizure test to determine the median effective dose (ED50; the dose of an ASM that blocks seizures in 50% of animals tested) based on anticonvulsant efficacy in CF-1 female mice following i.p. administration of **(B)** levetiracetam (LEV), **(C)** carbamazepine (CBZ), **(D)** Valproic Acid (VPA), **(E)** Phenobarbital (PB), **(F)** investigational compound A (CMPD A), and **(G)** investigational compound B (CMPD B). Data represented as number of mice protected or not protected from seizure activity. n= 8 per dose.

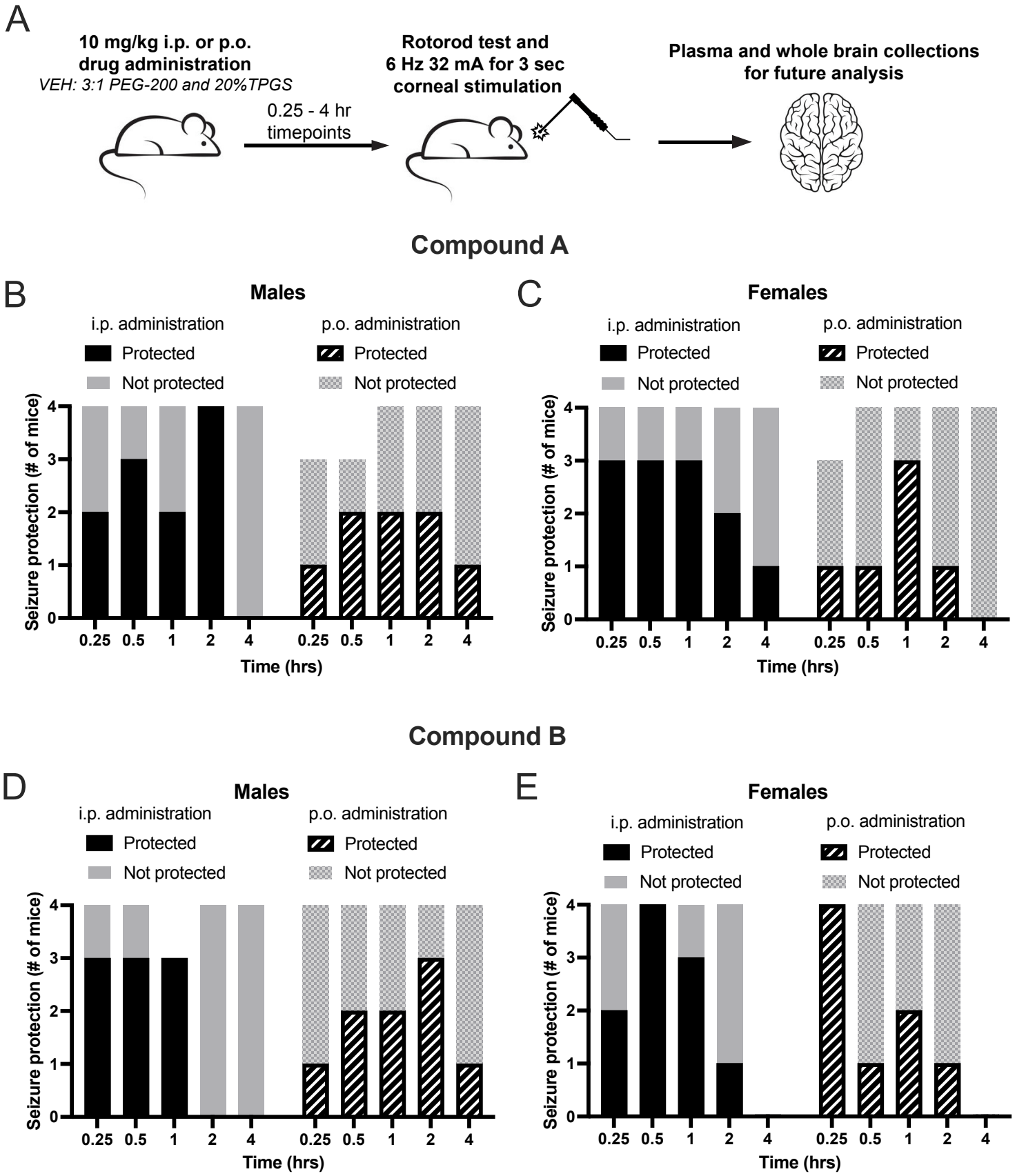

Figure 2. (A) Experimental study design for 6 Hz 32 mA seizure test to determine time-to-peak effect (TPE) based on anticonvulsant efficacy following a 10 mg/kg i.p. or p.o. administration. Testing done with **(B)** CMPD A with CF-1 males and **(C)** CF-1 females and with **(D)** CMPD B CF-1 males and **(E)** CF-1 females. Data represented as number of mice protected or not protected from seizure activity. Timepoints include 0.25 hr, 0.5 hr, 1 hr, 2 hr, and 4 hr. n= 3-4 per timepoint

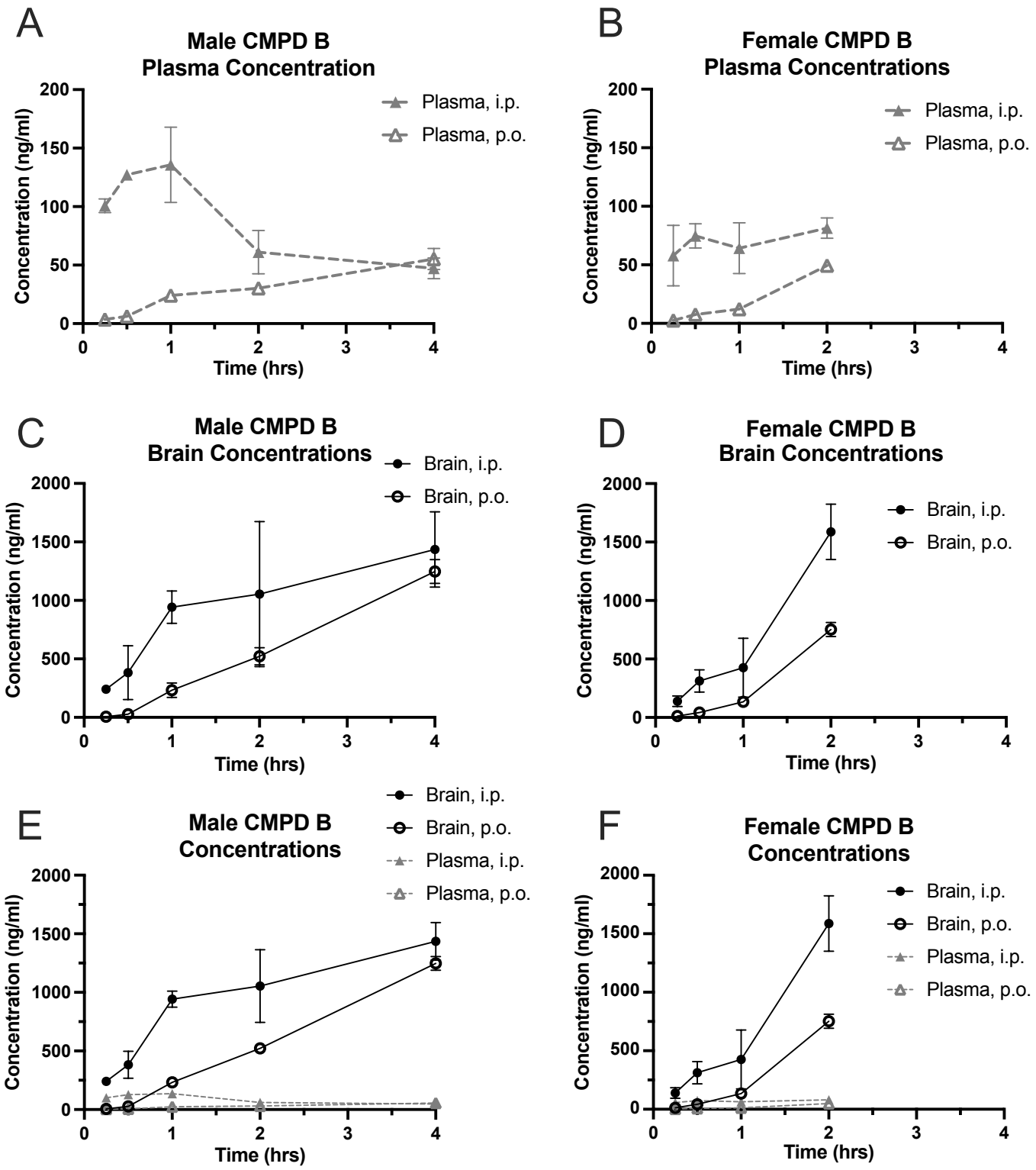

Figure 3. Plasma and brain concentration–time profiles of CMPD B in mice following a 10 mg/kg i.p. or p.o. administration. Drug concentrations were quantified using LC–MS/MS. Each data point represents the mean ± SEM (n = 3-4 animals per time point). Samples were collected immediately after 6 Hz time course study at 0.25, 0.5, 1, 2, and 4 hours after dose. **(A-B)** CMPD B concentrations in the plasma in male (main effect of time F (4, 8) = 2.507, p = 0.1251; ROA F (1, 2) = 66.38, *p = 0.0147; and interaction F (4, 8) = 8.616, **p = 0.005), and female mice (main effect of time F (3, 6) = 2.470, p = 0.159; ROA F (1, 2) = 60.11, *p = 0.016; and interaction F (3, 6) = 0.5285, p = 0.679). **(C-D)** CMPD B concentrations in the brain in male (main effect of time F (4, 12) = 25.20, ****p < 0.001; ROA F (1, 3) = 27.79, *p = 0.013; and interaction F (4, 10) = 1.685, p = 0.229, and female mice (main effect of time F (3, 9) = 31.80, ****p < 0.001; ROA F (1, 3) = 10.47, *p = 0.048; and interaction F (3, 4) = 3.107, p = 0.151). **(E-F)** Combined graph of male or female drug concentrations in plasma and brain extractions.

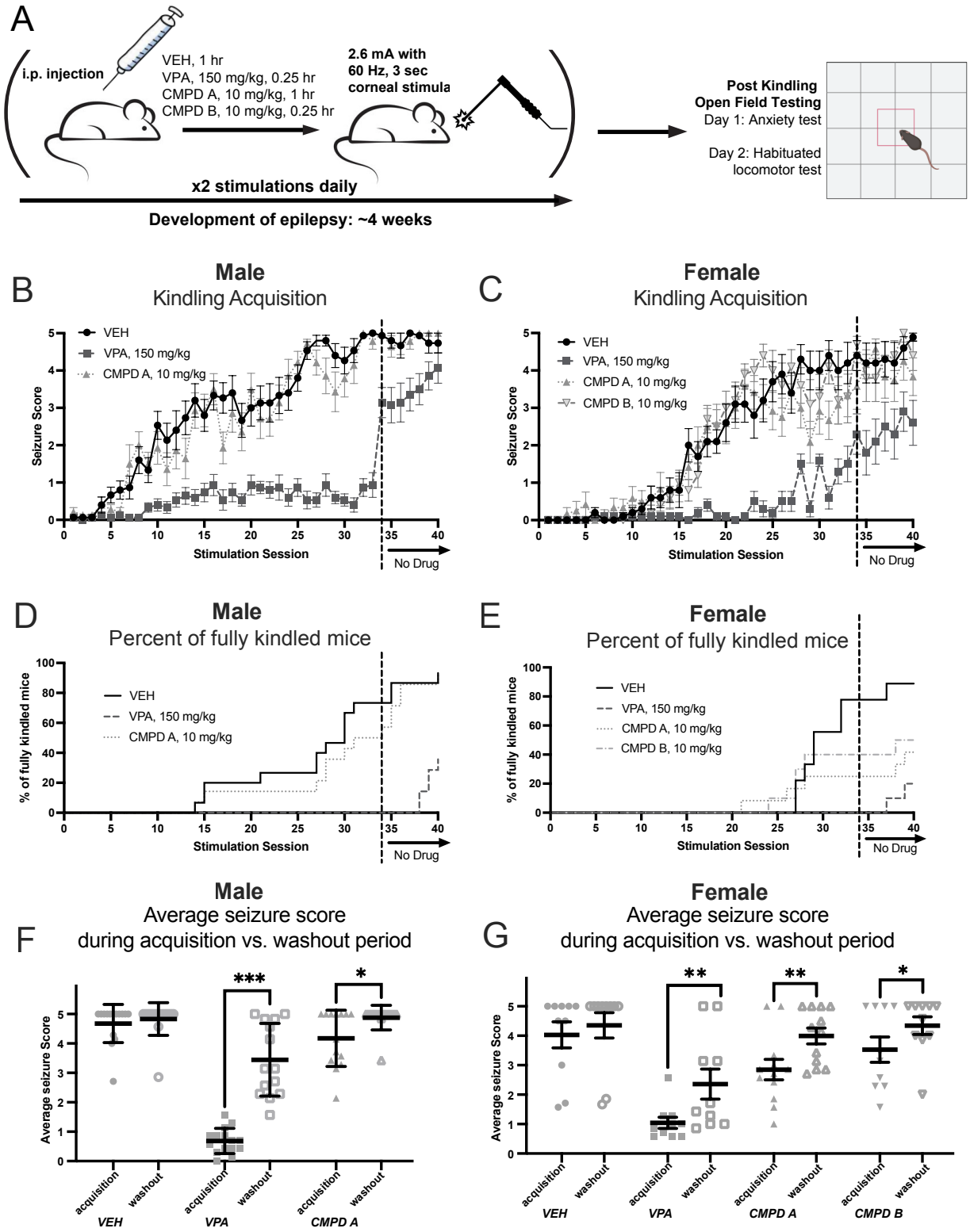

Figure 4. (A) Experimental study design for corneal kindling model of epileptogenesis. Animals received treatment or VEH and stimulations 2x a day. After 33 sessions, stimulations continue without treatment for the last 7 sessions as a "drug washout" period. After kindling, mice undergo 2 behavioral assessment days via open field testing. (Males: VEH n= 15, VPA n= 15, CMPD A n= 14; Females: VEH n= 10, VPA n= 10, CMPD A n= 12, CMPD B n= 10). **(B-C)** Male kindling acquisition in VEH, VPA, and CMPD A-treated mice and (C) Female kindling acquisition in VEH, VPA, CMPD A, and CMPD B-treated mice. **(D-E)** percent of fully kindled mice per treatment group at each stimulation session in male and female mice. Data presented as a Kaplan Meier survival curve. **(F-G)** Comparison between average seizure scoring during the last 7 sessions of kindling acquisition with treatment and 7 session drug washout period in each treatment group. Data presented as a mean ± SEM. **(F)** Males: ***p < 0.001 (VPA), *p = 0.031 (CMPD A) using paired Wilcoxon t-test. 1 mortality during washout period in VPA group. **(G)** Females: **p = 0.004 (VPA), **p = 0.020 (CMPD A), *p = 0.031 (CMPD B) using paired Wilcoxon t-test.

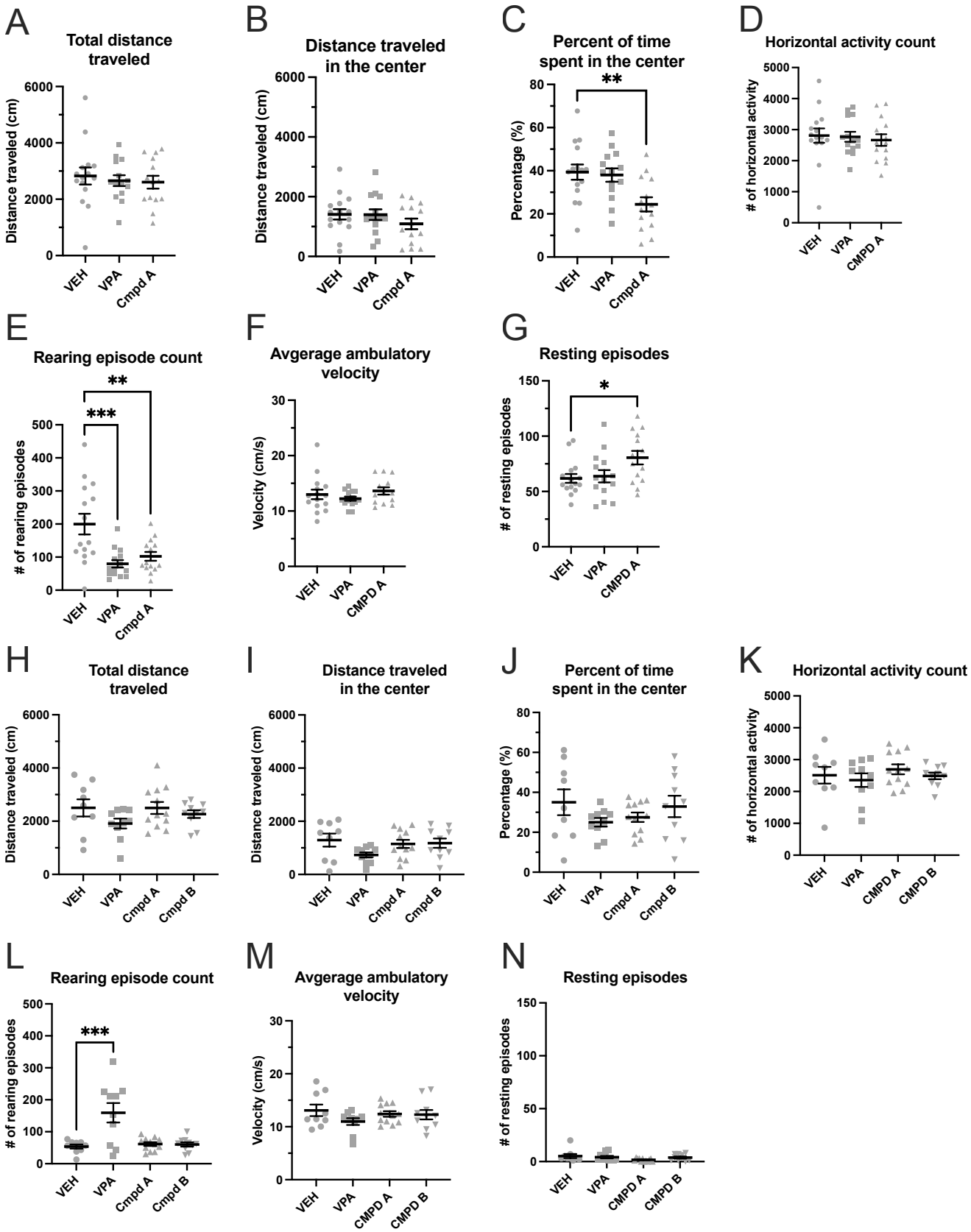

Figure 5. Day 1 post-kindling open-field test of anxiety in **(A-G)** males (VEH n= 15, VPA n= 15, CMPDA n = 14) and **(H-N)** females (VEH n= 10, VPA n= 10, CMPD A n= 12, CMPD B n= 10). Data include **(A&H)** total distance a mice traveled, **(B&I)** distance traveled when mice is in the center zone, **(C&J)** percent of time spent in the center zone, **(D&K)** horizontal activity count (measured by count of horizontal sensor beam breaks), **(E&L)** rearing episode count (the animal must go below the level of the vertical sensor for at least 1 second before the next rearing count can be registered), **(F&M)** average ambulatory velocity (the mean of the velocities for each ambulatory episode), and **(G&N)** resting episodes (a resting period is defined as a period of inactivity greater than or equal to 1 sec). **(E)** ***P < 0.001 (VEH vs. VPA) **p = 0.004 (VEH vs. CMPD A). **(G)** *P = 0.028 (VEH vs. CMPD A) **(L)** ***P < 0.001 (VEH vs. VPA). Data presented as a mean ± SEM and evaluated by one-way ANOVA, with Dunnett's post-hoc test.

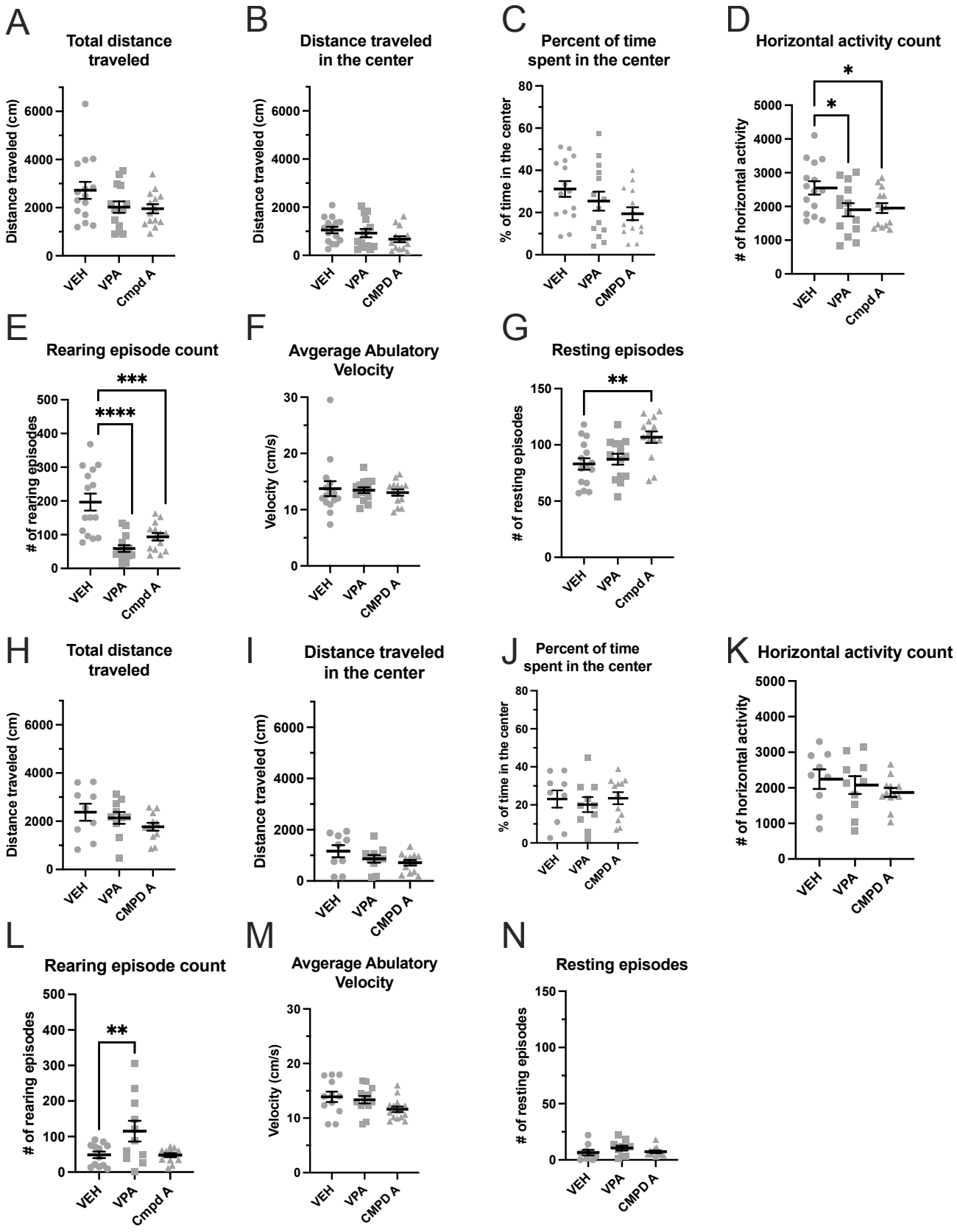

Figure 6. Day 2 post-kindling open-field test of habituated locomotor activity test in **(A-G)** males (VEH n= 15, VPA n= 15, CMPD A n= 14) and **(H-N)** females (VEH n= 10, VPA n= 10, CMPD A n= 12, CMPD B n= 10). Data include **(A&H)** total distance a mice traveled, **(B&I)** distance traveled when mice is in the center zone, **(C&J)** percent of time spent in the center zone, **(D&K)** horizontal activity count (measured by count of horizontal sensor beam breaks), **(E&L)** rearing episode count (the animal must go below the level of the vertical sensor for at least 1 second before the next rearing count can be registered), **(F&M)** average ambulatory velocity (the mean of the velocities for each ambulatory episode), and **(G&N)** resting episodes (a resting period is defined as a period of inactivity greater than or equal to 1 sec). **(E)** ****P < 0.001 (VEH vs. VPA) ***p < 0.001 (VEH vs. CMPD A). **(G)** **P = 0.003 (VEH vs. CMPD A) **(L)** **P = 0.008 (VEH vs. VPA). Data presented as a mean ± SEM and evaluated by one-way ANOVA, with Dunnett's post-hoc test.

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Supplementary Materials

Supplemental Table 1. 3 vehicle formulations were tested to improve solubility and anticonvulsant activity of CMPD A and B. Formulation #1 consisted of 5% ethanol/5% Kolliphor EL in 90% sterile saline and was made into a 20 mg/mL solution. Formulation #2 consisted of 2.5% DMSO/5% Kolliphor EL/2.5% PEG-400 in PBS and was made into a 20 mg/mL solution. Formulation #3 consisted of 1:3 PEG 200 / 20% Tocopherol Polyethylene Glycol Succinate (TPGS) in H2O and was made into a 15 mg/mL solution. Each solution underwent a 3-minute ultrasonification at around 20 kHz. Table includes pictured suspensions in each formulation and descriptions.

Formulation	**CMPD A**	**CMPD B**
<u>Formulation #1:</u> 5% ethanol/5% Kolliphor EL in 90% sterile saline; 20 mg/mL	 white to off-white, opaque suspension	 clear solution that is slightly yellow
<u>Formulation #2:</u> 2.5% DMSO/5% Kolliphor EL/2.5% PEG-400 in PBS; 20 mg/mL	 white to off-white, opaque suspensions	 clear, colorless solution
<u>Formulation #3:</u> 1:3 PEG 200 / 20% Tocopherol Polyethylene Glycol Succinate (TPGS) in H2O; 15 mg/mL	 colored solution	 clear solution that is slightly yellow

Supplemental Table 2. Formulation testing summary. 6 Hz 32 mA seizure test in CF-1 male mice following i.p. administration to determine optimal formulation (refer to Supplemental Table 1) with best anticonvulsant activity for CMPD A and B at 10 mg/kg. 1 Hr TPE based off old formulation determination. Formulation 3 was determined to exhibit the most anticonvulsant activity therefore, it was used in continued study. Data represented by the number of mice protected or impaired / total number of tested mice. n= 2-3 mice per formulation

CMPD A, 10 mg/kg, 1 hr TPE	**Protected**	**Motor impairment**
Formulation #1	0/2	0/2
Formulation #2	0/2	0/2
Formulation #3	1/2	1/2

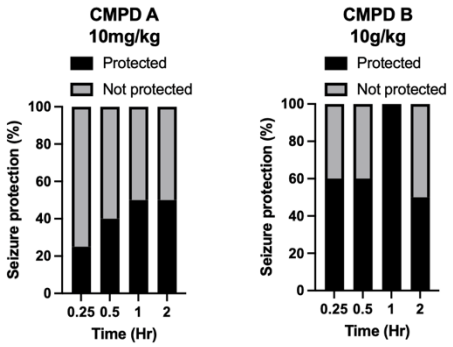
CMPD B, 10 mg/kg, 0.25 hr TPE	**Protected**	**Motor impairment**
Formulation #1	0/3	0/3
Formulation #2	0/3	0/3
Formulation #3	2/3	0/3

Supplemental Table 3. Formulation #3 6 Hz 32 mA time course testing in CF-1 male mice. New vehicle formulation may change drug delivery and pharmacokinetics in the body therefore a pilot study was performed at varying timepoints (0.25, 0.5, 1, and 2 hr) to further confirm anticonvulsant activity in CMPD A and B. Data represented by the number of mice protected or impaired / total number of tested mice. n= 4-5 mice per formulation

CMPD A, 10 mg/kg	**Protected**	**Motor impairment**
0.25 hr	1/4	0/4
0.5 hr	2/5	0/5
1 hr	2/4	0/4
2 hr	2/4	1/4

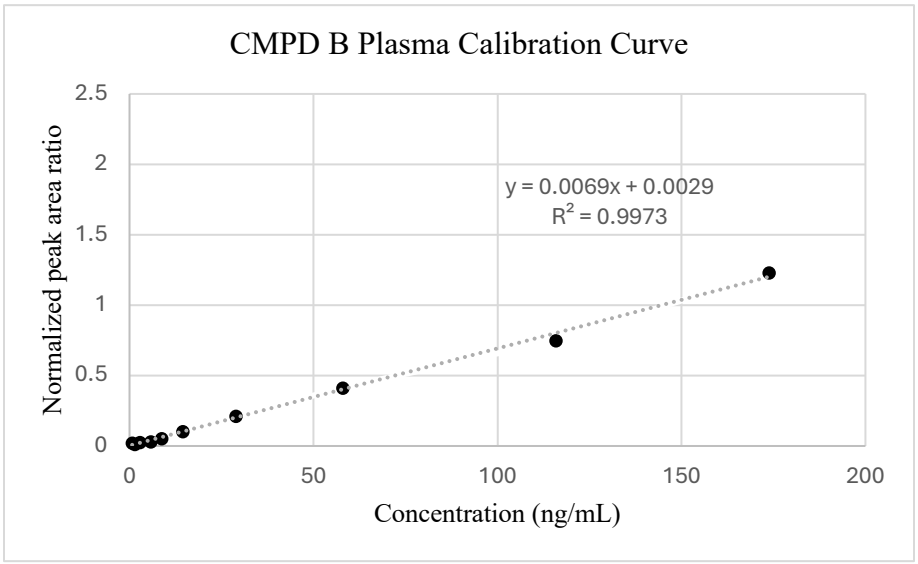
CMPD B, 10 mg/kg	**Protected**	**Motor impairment**
0.25 hr	3/5	1/5
0.5 hr	3/5	1/5
1 hr	4/4	1/4
2 hr	2/4	1/4

Supplemental Figure 1. Formulation #3 6 Hz 32 mA time course testing in CF-1 male mice (graphical representation of Supplemental Table 3). Pilot study with formulation #3 was performed at varying timepoints (0.25, 0.5, 1, and 2 hr) to further confirm anticonvulsant activity in CMPD A and B. Data represented by the number of mice protected or not protected. n= 4-5 mice per formulation.



Supplemental Figure 2. Standard calibration curve for quantifying CMPD B. Calibration standards were prepared by spiking blank matrix (plasma or brain homogenate) with known concentrations of CMPD B to yield final concentrations of 0.72 ng/mL to 173.88 ng/mL. Calibration curves for **(A)** plasma and **(B)** brain was generated by plotting the analyte-to-internal standard peak area ratio versus the nominal concentration. The curve was fitted using a least-squares linear regression model and was considered acceptable if the correlation coefficient (R^2) was ≥ 0.995.

A



B