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Therapeutic Effects of Time-limited Treatment with Brivaracetam on Posttraumatic Epilepsy after Fluid Percussion Injury in the Rat.

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Running Title: Effect of Brivaracetam on Posttraumatic Epileptogenesis

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Nonstandard Abbreviations: ASD, anti-seizure drug; BRV, brivaracetam; ECoG, electrocorticography; FPI fluid percussion injury; GAERS, genetic absence epilepsy rats from Strasbourg; LC-MS/MS, liquid chromatography tandem mass spectrometry; PTE, posttraumatic epilepsy; rpFPI, rostral parasagittal FPI; SV2A synaptic vesicle protein 2A.

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ABSTRACT

Mounting evidence suggests the synaptic vesicle glycoprotein 2A (SV2A) targeted by levetiracetam may contribute to epileptogenesis. Levetiracetam has shown anti-inflammatory, antioxidant, neuroprotective and possible antiepileptogenic effects in brain injury and seizure/epilepsy models, and a phase 2 study has signaled a possible clinical antiepileptogenic effect. Brivaracetam shows greater affinity and specificity for SV2A than levetiracetam and broader preclinical anti-seizure effects. Thus, we assessed the antiepileptogenic/disease-modifying potential of brivaracetam in an etiologically realistic rat posttraumatic epilepsy model optimized for efficient drug testing. Brivaracetam delivery protocols were designed to maintain clinical moderate-to-high plasma levels in young (5-week-old) male Sprague-Dawley rats for four weeks. Treatment protocols were rapidly screened in 4-week experiments using small groups of animals to ensure against rigorous testing of futile treatment protocols. The antiepileptogenic effects of brivaracetam treatment initiated 30min, 4hr and 8hr after rostral parasagittal fluid percussion injury (rpFPI) were then compared to vehicle-treated controls in a fully powered blind and randomized 16-week validation. Seizures were evaluated by video-electrocorticography using a 5-electrode epidural montage. Endpoint measures included incidence, frequency, duration and spread of seizures. Group sizes and recording durations were supported by published power analyses. Three months after treatment ended, rats treated with brivaracetam starting at 4 hr post-FPI (the best-performing protocol) experienced a 38% decrease in overall incidence of seizures, 59% decrease in seizure frequency, 67% decrease in time spent seizing, and a 45% decrease in the proportion of spreading seizures that was independent of duration-based seizure definition. Thus, brivaracetam shows both antiepileptogenic and disease-modifying properties after rpFPI.

Significance Statement: The rpFPI model, which likely incorporates epileptogenic mechanisms operating after human head injury, can be used to efficiently screen investigational treatment

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protocols and assess antiepileptogenic/disease-modifying effects. Our studies 1) support a role for SV2A in epileptogenesis, 2) suggest that brivaracetam and other drugs targeting SV2A should be considered for human clinical trials of prevention of post-traumatic epilepsy after head injury and 3) provide data to inform the design of treatment protocols for clinical trials.

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INTRODUCTION

Epilepsy is a disabling disorder affecting over 50 million people, world-wide (de Boer et al., 2008). Drug treatment is palliative and requires chronic treatment with anti-seizure medications (ASDs) with varied side effects (Gilliam et al., 2004; Loscher et al., 2013; Schmidt et al., 2014). One-third of epilepsy patients continue to suffer seizures despite the availability of about two dozen ASDs. Up to 40% of people with epilepsy have a history suggesting acquired causes (e.g. head injury, stroke; Schmidt and Sillanpaa, 2016). Such epilepsies are often difficult to treat, but are regarded to be preventable (Semah et al., 1998; French 2007). However, clinical trials have failed to identify any treatment to prevent epilepsy or modify its course after any brain insult (Klein and Tyrlikova, 2020). Thus, treatments to prevent or attenuate the development of acquired epilepsies remain an urgent unmet medical need (Garga and Lowenstein, 2006; Loscher, 2019; Klein and Tyrlikova, 2020).

Past antiepileptogenesis trials focused mainly on older ASDs administered after head injury. These trials were conducted in the absence of proof-of-principle demonstrations that the investigational treatments could prevent the development of seizures in animal models, and without benefit of suitable preclinical data to guide the specification of critical features of treatment protocols such as dose, duration and timing of treatment (Temkin, 2009; Mani et al., 2011; Schmidt et al., 2014). Thus, while the uniformly negative results of these trials do not support the antiepileptogenic efficacy of ASDs, factors unrelated to drug efficacy could have contributed to trial failures, and subsequent reviews have urged assessment of the antiepileptogenic potential of newer ASDs (Temkin, 2009; Schmidt, 2012). Racetam ASDs that target synaptic vesicle glycoprotein 2A (SV2A) are of particular interest (Loscher et al.,2016). SV2A gene knockout is associated with a severe epileptic phenotype and SV2A is reduced in both resected human epileptic brain tissue and in rodent epilepsy models (Crowder et al., 1999;

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Feng et al., 2009; van Vliet et al., 2009; Toering et al., 2009; Hanaya et al, 2012). The prototype antiseizure racetam, levetiracetam, has been reported to 1) inhibit inflammatory and oxidative processes thought to contribute to epilepsy and epileptogenesis (Oliviera et al., 2007, Kim et al., 2010, Itoh et al., 2016), 2) show neuroprotective effects in spontaneously epileptic rats (Yan et al., 2005; Sugata et al., 2011) and in rodent head injury and stroke models (Hanon and Klitgaard, 2001; Wang et al., 2006; Zou et al., 2013; Itoh et al., 2015), and 3) exhibit activity thought to be predictive of antiepileptogenesis in rodent kindling models (Loscher et al., 1998; Vinogradova and van Rijn, 2008; Russo et al., 2017). While levetiracetam has generally failed to prevent epilepsy in status epilepticus-based models, Sugaya et al (2010) reported that the mean duration of chronic seizures developing after intracerebral kainate administration was significantly reduced 2 months after cessation of levetiracetam treatment.

We have assessed the antiepileptogenic/disease-modifying potential of the recently approved racetam ASD, brivaracetam (BRV) in rats using the rostral parasagittal fluid percussion injury (rpFPI) model - an etiologically relevant posttraumatic epilepsy (PTE) model. BRV was rationally developed based on the discovery of SV2A as the principal target of levetiracetam (Rogawski, 2008; Kaminsky et al., 2012, Klitgaard et al., 2016). Compared to levetiracetam, BRV has an order of magnitude higher affinity for SV2A, a distinct mode of interaction with SV2A, greater potency in common seizure models, and efficacy in a wider range of models (Klitgaard et al., 2016; Klein et al., 2017; Wood and Gillard, 2017; Wood et al., 2018; Steinhoff and Staack, 2019). The rpFPI-PTE model is based on a widely accepted clinically relevant traumatic brain injury model (Thompson et al., 2005; Lyeth, 2016) and likely incorporates epileptogenic mechanisms that operate after human head injury. The model has been optimized for development of antiseizure and antiepileptogenic therapies, and permits adequately powered assessments of anti-epileptogenic treatments with manageable experimental group sizes (D'Ambrosio et al., 2013; Eastman et al., 2011; 2015; Curia et al.,

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2011; 2016). As in clinical trials, the detection of antiepileptogenic or disease-modifying effects requires that a sufficient dose of an effective agent be administered at an appropriate time for long enough to exert a therapeutic effect. BRV was assessed at plasma levels that would be in moderate-to-high therapeutic range in man, and at a duration of treatment that supported seizure prevention by focal cooling in the rpFPI model. Our study design incorporated pharmacokinetic experiments to establish a dosing protocol to maintain plasma levels in the target range for the duration of treatment, and screening experiments to guide choice of latencies to treatment.

Materials and Methods

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All experiments were approved by the University of Washington Institutional Animal Care and Use Committee (Animal Welfare Assurance #A3464-01). All surgery was performed under anesthesia, and all efforts were made to minimize suffering.

Animals: Outbred male Sprague-Dawley rats (Charles River, Hollister, CA) were 32-36 days of age at the start of experimentation (either rpFPI or the start of drug treatment). Animals were housed 2-3 per cage until use, and individually after start of experimentation. Animals were kept in a specific-pathogen-free facility with controlled light (14hr:10 hr light-dark cycle), temperature and humidity, and ad libitum access to food and water.

Surgical Procedures: rpFPI, epidural electrode implantation and subcutaneous osmotic pump implantation were performed as detailed previously (D'Ambrosio et al., 2013; Eastman et al., 2010; Curia et al., 2016). Animals were anesthetized (4% halothane), intubated and

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mechanically ventilated (1-1.5% halothane, 30% O_2 and air). Rectal temperature was monitored and maintained at 37°C with a heat pad. Osmotic pumps, when used, were implanted immediately prior to the rpFPI. For rpFPI, a 3 mm burr hole was drilled centered at 2 mm posterior to bregma and 3 mm from the midline over the right convexity. Animals were disconnected from the ventilator and a pressure pulse (8 ms, 3.5atm) was delivered with the rpFPI device (Scientific Instruments, University of Washington) and measured by a transducer (Entran EPN-D33-100P-IX, Measurement Specialties, Hampton, VA). Mechanical ventilation was resumed ten seconds after injury to standardize posttraumatic apnea and hypoxia, and terminated when spontaneous breathing resumed. Acute mortality rate due to rpFPI was ~3%.

Epidural electrodes were implanted 14-15 days after injury. Briefly, 1 mm diameter stainless-steel screw electrodes were implanted through 0.75 mm diameter guiding craniotomies. The full electrocorticography (ECoG) montage consisted of five epidural electrodes: a reference electrode placed midline in the frontal bone and two electrodes per parietal bone, placed at coordinates bregma 0 mm and -6.5 mm, 4.5-5 mm from the midline. Three anchoring screws (one frontal and two occipital) were implanted to help secure the headset. All electrodes were connected through insulated wire to gold-plated pins in a plastic pedestal (PlasticsOne inc., Roanoke, VA). Parts of the craniotomy not covered with thick connective tissue were covered with biocompatible silicone (Kwik-Cast., WPI, Sarasota, FL). The entire assembly was then cemented onto the skull with measured amount of dental acrylic (Jet, Lang Dental Manufacturing Co., Wheeling, IL) and further secured with VetBond (World Precision Instruments, Sarasota, FL) adhesive. Because unintended damage to the neocortex induces focal astroglial reactivity and is often associated with focally abnormal ECoG (D'Ambrosio et al., 2009), our surgical procedures incorporate routine precautions to minimize thermal and mechanical damage. During drilling, the skull and drill bit are cooled with room-temperature sterile saline to minimize frictional heating, and particular care is taken to avoid

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deforming or tearing the dura. In addition, the depth of anchoring screws and screw electrodes is carefully controlled under stereoscopic observation to avoid brain compression. During the exothermic phase of the curing process, the acrylic headset is cooled with compressed air to prevent excessive heating of underlying brain tissue.

BRV preparation and dosing: Brivaracetam (UCB, Brussels, Belgium) was administered intraperitoneally, orally, via subcutaneous osmotic pump or in drinking water (alone or in combination) according to the requirements of each experiment. BRV dissolves readily and is freely miscible in water. For bolus oral or intraperitoneal administration, BRV was dissolved in saline or water (target dose/cc) such that the target dose (mg/kg) could be delivered in a volume (ml) equal to the rat's weight in kg. Osmotic pumps (2ML1 or 2ML4, Durect Corp., Cupertino, CA) were loaded with up to 0.5 g/ml BRV in sterile ultrafiltered water, primed and implanted in a subcutaneous pocket immediately before rpFPI. While more dilute BRV solutions have been delivered via osmotic pumps without any reported problems (Nygaard et al., 2015), 10% of the BRV-filled pumps implanted for this study failed to deliver the expected quantity of BRV by the time of sacrifice. Pumps from which more than 1 g of fluid was recovered (thus < 83% of intended dose delivered) were deemed to have failed. Affected subjects were eliminated from the study. In order to delay treatment for 30 min 4 hr or 8 hr after rpFPI, pumps were equipped with calibrated lengths of empty tubing. For continuous oral administration in drinking water, a BRV solution was available ad libitum as the sole source of drinking water. Because the aversive flavor of BRV reduced fluid intake, BRV-containing drinking water was sweetened with up to 0.1% saccharine. Sweetened BRV solutions were formulated to deliver a designated dose given the mean weight and expected fluid intake of a cohort of rats. The designated dose was either the target dose or, when BRV was administered by more than one route, the balance of the target dose. BRV solutions were freshly prepared every 1-3 days to account for changes in the mean weights and fluid intakes of different cohorts of rats. Rats were weighed and fluid

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intakes determined prior to preparing fresh sweetened BRV solutions. We found that mean daily water consumption in animals administered BRV in sweetened drinking water was comparable to rats with ad lib access to unadulterated water (25-35 ml) throughout the experiment.

Video/ECoG Monitoring and seizure identification: ECoG was acquired continuously in 48 hr epochs. Animals were tethered to the amplifier headstage. Brain electrical activity was amplified (x 5000) and filtered (0.3 Hz high-pass, 300 Hz low-pass) using a Neurodata 12 or a M15 amplifier (Grass Instruments, Quincy, MA), acquired at 600 Hz per channel on computers equipped with SciWorks 4.1 or Experimenter V3 software (Datawave Technologies Inc., Longmont, CO), and DT3010 acquisition boards (DataTranslation Inc., Marlboro, MA). Videos were recorded in either VHS or in digital format using digital cameras. Each camera monitored a maximum of two cages (1 animal per cage). The seizures in this model have been extensively characterized (see D'Ambrosio et al., 2004,; Curia et al., 2011; and, especially D'Ambrosio et al., 2005; 2009).

Primary analyses of ECoG data were conducted blind to subject, treatment and any other treatment parameters. ECoG was visualized in Matlab (MathWorks inc., Natick, MA) and manually scrolled offline. This approach is laborious and requires expert raters, but it permits reliable analysis of all seizure activity -and its spread- generated by the epileptic focus and it is the approach used to evaluate human ECoG data. Seizure onset was characterized by 1) focal trains of spikes, each spike lasting about 150 ms, clearly distinct from baseline, 2) a sudden increase in spectral power in the theta band over the baseline (Butler et al., 2013; D'Ambrosio et al., 2004; Ikeda et al., 2009), and 3) simultaneous stereotyped ictal behavioral changes according to a behavioral scale previously described (D'Ambrosio et al., 2009) and according to the clinical practice of seeking evidence of abnormal neuronal activity paired to behavioral signs (D'Ambrosio et al., 2009; D'Ambrosio and Miller, 2010; Fisher et al., 2005). Identified seizures

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ranged from 1 s to over 5 minutes. Events occurring within 3s of each other were defined as a single seizure.

The following data were extracted for each seizure: 1) onset time, 2) duration, and 3) ECoG channel(s) at which the event started and spread to. The effects of BRV treatments were assessed on the basis of comparison of seizure frequency (events/hour), seizure incidence (proportion of rats that exhibited seizures), time spent seizing (seconds/hour), and proportion of spreading seizures, between treated groups and untreated controls.

Measurement of plasma BRV: Plasma BRV was determined in blood samples drawn from the tail vein. Blood (≥200 µl) was collected in Li-heparin coated Microtainer tubes (Beckton, Dickinson and Co., Franklin Lakes, NJ). Collection tubes were stored on crushed ice and centrifuged (10 min at 1500 x g) in a chilled rotor within 1 h of collection. Plasma samples (100 µl) were transferred to 0.5 ml microfuge tubes and stored at -20^{o}C prior to analysis. At the end of each pilot study, plasma samples were packed in dry ice and shipped to PRA Health Sciences (Assen, NL) for determination of plasma BRV levels by liquid chromatography tandem mass spectrometry (LC-MS/MS).

Study design: Success in detecting an antiepileptogenic effect of a drug requires it to be delivered and maintained at therapeutic levels for an adequate amount of time within a temporal window in which the disease process can be modified (Loscher, 2019). Because the mechanisms of posttraumatic epileptogenesis remain poorly understood, and few treatments have been reported to prevent epilepsy in any model (and none clinical trials), there is little data to guide the design of a treatment protocol and the wide range of reasonable treatment options cannot practically be rigorously investigated. BRV is well studied clinically and approved to treat

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epileptic seizures, and we elected to examine its antiepileptogenic potential in rats at plasma levels (3-5 mg/ml) that would be in a moderate to high therapeutic range for seizure control in humans and consistent with well tolerated acute iv administration in humans (Sargentini-Maier et al., 2007; Klein, 2016; Reimers et al., 2018). Based on our previous demonstration of potent and persistent prevention of rpFPI-induced epileptic seizures after four weeks of mild focal cooling of the perilesional neocortex (D'Ambrosio et al., 2013), we chose a four week duration of treatment. We investigated 3 delays to treatment (30 min, 4 hr and 8 hr) based on a hypothesis that early treatment would be most effective and the fact that very early treatment may not be clinically feasible. Our study protocol was designed to minimize the time and number of animals required to design an adequate treatment protocol.

A dosing protocol to maintain plasma BRV in the desired range was adaptively constructed based on a series of experiments of increasing duration (1, 2 and 3 weeks). Dosing parameters were adjusted after each experiment to more closely approximate the target exposure. In order to minimize the risk of prolonged rigorous testing of futile treatment protocols, delays to treatment were screened for potential antiepileptogenic activity on the last day of treatment (i.e. 4 weeks after injury). We reasoned that any antiepileptogenic or disease-modifying effect(s), which can only be confirmed upon prolonged follow-up after cessation of treatment, would be evident on the last day of treatment, even in the shorter 4-week protocols. Screening studies were conducted using small groups of animals (N = 6 – 8) and we selected $p<0.15$ in one-tailed Mann-Whitney or Fisher exact tests of pairwise comparisons of treatment groups with controls as a criterion for success (Fig. 1A). This criterion permitted screening with small groups of animals with comparable statistical power to detect treatment effects as more rigorous test conducted using groups of twenty animals, albeit with increased risk of false positive findings. This protocol was designed to provide objective screening of latencies to

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treatment in about 1 month, using small groups of animals (N=6-8), to inform the design of a more rigorous assessment of anti-epileptogenic or disease-modifying activity.

Three treatment protocols were rigorously validated in a fully powered study with 12 weeks follow-up after the four-week treatment to investigate the persistence of the antiepileptogenic effect (Fig 1B). Note that replication is built into the study design. Bootstrapped Monte-Carlo -based statistical power analyses for between groups assessments comparisons of seizure activity following rpFPI have been published previously (Eastman et al., 2015).

Screening and validation studies were conducted in a blind and randomized fashion. Injured animals were randomized to treatment groups using Matlab prior to start of treatment. Decisions to exclude animals from analysis (e.g. for poor health or pump failure) were made prior to examination of seizure data. Individual ECoG files were coded with numerical labels by an investigator (C.E.) who was not otherwise involved in the primary analysis of video/ECoG data, and the coded files were analyzed by personnel (J.F. and R.D.) who were kept blind to the identity and group assignment of the data records. Decisions to discard noisy ECoG files were made blind to file identity. Data files were decoded only when the primary analysis was deemed complete.

Statistics: Experimental data were analyzed with the aid of R (v. 3.1.3). Conventional statistical tests were performed using the using the base statistics (Fisher exact tests) and COIN (Mann-Whitney tests) packages. Bootstrapped confidence intervals were calculated using the boot package and are based on 10^5 replicates. Data from the more rigorous validation study were analyzed using randomization tests to assess the probability of observing differences as large

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as those obtained experimentally under the null hypothesis that all samples were drawn from the same population. which is detailed in Supplemental Methods. Briefly the experimental data were bootstrapped (10^6 replicates, unless indicated otherwise) to empirically estimate the distribution of the various outcome measures (the differences in the incidence or mean seizure frequency of seizures between the BRV treatment groups and the common control group, etc.) under the null hypothesis. For this purpose, control data and that from the three treatment groups were pooled, and sampled randomly with replacement to form four groups matched in size to the experimental data. Statistics (e.g. differences in mean frequency between the control group and the three other groups) were computed. This process was repeated 10^5 or 10^6 times as indicated. P-values for the experimentally observed outcome measures were then obtained by direct comparison with their estimated distributions under the null hypothesis. P-values were computed to account for the multiple comparisons to a common control (see Supplemental Methods for details).

For display of seizure frequency and time seizing data on a logarithmic scale, observations of zero seizures or zero s/hr spent seizing were conservatively assigned values of 1/30 seizures/hr or s/hr. These are just below the minimum frequencies or times seizing that can be detected in a 24 hr observation period.

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Results

Pharmacokinetic Pilot Studies: Because there were no pharmacokinetic data on chronic or subchronic administration of BRV to rats, a series of pilot studies were performed to develop dosing protocols capable of maintaining plasma levels that would be considered to be in the moderate to high therapeutic range (3-5 µg/ml) in humans for four weeks. In each pilot study 35 day old male Sprague Dawley rats were administered either a single dose or repeated doses of BRV and blood was drawn at specified intervals for determination of plasma BRV.

We first examined BRV levels in plasma from rats treated with BRV at doses in a range that had previously been shown to reduce stage 4-5 seizures and afterdischarge threshold and duration in hippocampal kindled rats and spike and wave discharges in GAERS rats (Matagne et al., 2008; Dupuis et al., 2015) Two groups of six rats each were administered 25 and 50 mg/kg BRV i.p., respectively, twice daily at 9 AM and 6 PM for 1 week. Blood samples were collected between 7 AM and 9AM (just prior to the next scheduled injection) after 1, 3, 5 and 7 days of treatment for determination of trough plasma BRV levels. Mean plasma levels were identically low (5-8 ng/ml) in both dosage groups for 3 days (Fig. 2A). Plasma BRV remained at this level in the 25 mg/kg group for the remainder of the treatment, while those in the 50 mg/kg group rose to 15-20 ng/ml on treatment days 5 and 7. Trough plasma BRV levels obtained with these dosing protocols were, at best, 2 orders of magnitude below target levels indicating that impractically frequent injections would be required to stably maintain plasma levels in the target ranges.

In a second pilot study, we examined both acute and subacute exposures. In the acute exposure (Fig. 2B), plasma levels were assessed at 1 hr (peak), 5 hr and 9 hr after administration of 5 mg/kg BRV by oral gavage. Consistent with a previous report (Iqbal et al., 2017) BRV was rapidly cleared after an oral dose with a plasma half-life of about 2.4 hours. In

the subacute exposure (Fig. 2C,D), plasma BRV was assessed after 1, 3, 5, 7, 9, 11 and 13 days of continuous exposure to BRV in sweetened drinking water at concentrations expected to deliver 120 mg/kg/day (5 mg/kg/hr) with normal ad lib water consumption. Blood was collected for plasma BRV determination between 7AM and 9AM. Plasma BRV levels in animals self-administering 120 mg/kg/day were stable at about 0.6 µg/ml for 5 days, and then rose to an apparent plateau at about 1.8 µg/ml by 9 days of treatment. The increase in plasma BRV levels, which was observed to some degree in all individuals (Fig. 2C) as well as in aggregate (Fig. 2D), cannot be explained by an increase in dose: the inset in figure 2D clearly shows that daily dosage remained stable near the target dose throughout the study. These data suggest a time or exposure (time x dose) dependent decrease in clearance. This could be related to a nephropathy that is induced specifically in male rats (and not female rats or humans) by exposure to a variety of chemicals (Read, 1991; Swenberg et al., 1989) including BRV (European Medicines Agency, 2015). Based on this evidence, we hypothesized that plasma levels in the male rat could be stabilized with a dosing protocol in which BRV doses were stepped down after a week to compensate for the time-dependent decrease in clearance.

We then targeted plasma levels of 3 µg/ml using a protocol in which animals self-administered (drinking water) 650 mg/kg/day for the first week, and 180 mg/kg/day for the following two weeks (Fig. 2E). Plasma BRV levels (4.2 ± µg/ml) were near target after 5 days of exposure, but increased steeply to a mean of 8 µg/ml by day 7, before the dosage step down to 180 mg/kg/day. This suggests that the exposure-induced decrease in BRV clearance was more intense or occurred earlier in animals receiving 650 mg/kg/day compared to those treated with the lower dose.

Based on these data, a protocol was developed to maintain about 4 µg/ml BRV in plasma for 4 weeks in a screening study designed to evaluate different latencies to the start of treatment after rpFPI. In outline, BRV would be administered at a high dose for the first 5 days

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after injury, ramped downward on days 6, 7 and 8 to compensate for the time dependent decrease in clearance, and administered at a lower dose expected to support 4 µg/ml plasma levels after the decrease in clearance through the last day of treatment.

Screening: Using the dosing protocol developed in the pharmacokinetic studies, we examined rpFPI-induced PTE in untreated rats and in rats treated with BRV for 4 weeks beginning 30 min, 4 hr and 8 hr after injury. Animals were prepared and treated in two batches (1 and 2), which were treated with similar, but not identical, dosing protocols (Fig. 3A). ECoG recordings (48 hr) were acquired from each rat during the last two days of BRV treatment.

Batch 1 rats were randomized to 4 groups after rpFPI: Untreated rpFPI (control; n=10), and 4-week BRV treatments starting at 30 min (n=7), 4 hr (n=7) and 8 hr (n=7) after injury. All rats survived injury, but one rat lost its headset prior to ECoG acquisition. BRV dosing was identical in the BRV-treated groups except for the delay to treatment. Based on the pilot studies, rats were administered 650 mg/kg/day on days 1-5, 500, 350 and 200 mg/kg/day on days 6, 7 and 8, and 200 mg/kg/day, thereafter. BRV was available in the drinking water throughout the treatment period. Because drinking is markedly suppressed for 1-3 days after injury, BRV was also orally administered (100 mg/kg, 3X daily at 8:00, 16:00 and 23:00) until rats consumed >50% of their target dose in drinking water. Each rat received an intraperitoneal loading dose of BRV at its designated treatment start time. Loading doses of 100 mg/kg/8hr were adjusted to account for the time remaining (TR) until the next scheduled oral dose: TR was rounded up to the nearest hour and the dose administered was TR/8 * 100 mg/kg. Thus, loading doses ranged from 12.5 mg/kg (administered less than 1 hr before scheduled oral dosing) to 100 mg/kg (administered more than 7 hrs before oral dosing). The resulting exposure is shown in figure 3A (left panel). At the end of 4 weeks of treatment all untreated rats had developed seizures, while seizure incidence was 43%, 57%, and 67% in the 30 min, 4 hr and 8hr BRV-treated groups,

respectively. Thus, incidence increased toward control levels with increasing delay to treatment (Fig. 3B, left panel), but reductions met our criterion for success in each treatment group. The median frequency of seizure was nominally lower than control in all treatment groups but met our criterion only in the 30 min and 8 hr groups (Fig. 3C, left panel). The differences in medians reflected a clustering of subsets of data points at zero and near-zero frequencies, rather than a uniform lowering of the frequency of seizure. In all experimental groups, there were some rats with very frequent seizures. Times spent seizing were distributed very similarly to seizure frequencies. Median times seizing were lower in all BRV-treated groups than controls, but met criterion only in the 30 min and 8hr groups (Fig. 3D, left panel)

In batch 2, we aimed to increase BRV exposure and ensure more stable plasma levels – especially in the first several days after injury when BRV was administered orally at intervals in excess of 2 plasma half-lives. Thus, target doses, loading doses and the oral doses administered in the first days after injury were increased relative to batch 1, and osmotic pumps were used to continuously supply a portion of the target dose that diminished from 200-250 mg/kg/day at the beginning of treatment to as little as 75 mg/kg/day as the animals gained weight. All BRV treatments started with a loading dose of 200 mg/kg i.p. injection of BRV at the designated time after rpFPI: 0.5, 4 or 8 hrs. Until animals started drinking at least half the expected volume, they were administered 150 mg/kg BRV thrice daily (8 AM, 4PM and 11 PM). The remainder of the target dose was administered in the drinking water. BRV-treated rats received a total of 750 mg/kg/day on days 1-5, 600, 400 and 300 mg/kg/day on days 6, 7 and 8, and 300 mg/kg/day, thereafter. The resulting exposure is shown in figure 3A (right panel). Thirty-four rats were randomized to treatment groups. One was lost to a technical failure, one was eliminated from the study after failing, for a week, to drink adequate quantities of the sweetened BRV solution and one was euthanized for health reasons. Thus, there were 10 untreated controls and 7 rats in each of the BRV-treated groups. Seizures were detected in 80%

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of control rats at the end of treatment, and in 86%, 43% and 71%, respectively, of rats treated with BRV starting 30 min, 4 hr and 8 hr after injury (Fig. 3B, right panel). Median frequencies of seizure were all lower than in control, but also reached criterion only in the 4 hr group (Fig. 3C, right panel). As in batch one, the effect of drug treatment was most evident in a clustering of data points at zero or near-zero frequencies in the BRV-treated groups. While median times spent seizing were nominally lower in all BRV-treated groups than controls, none of the differences reached the screening criterion.

The screening data consistently indicated that BRV treatment started 4 hr after injury was efficacious and that treatment was less effective when delayed by 8 hr. Treatment beginning just 30 min after injury was either comparably effective as treatment at 4hr after injury or ineffective, depending upon the dosing protocol. Because dosing protocol 1 produced nominally better outcomes, we chose its dosing targets for the validation study, but introduced the support of osmotic mini-pumps to achieve more stable and uniform dosing of BRV in the critical first days of the study. While our intent was to test just one protocol in the validation study, the diminished efficacy of treatments delayed 8 hr after injury, and the possibility that efficacy of treatment might follow an inverted U-shaped function of delay to treatment dictated that all three delays to treatment be reexamined in the fully-powered validation study.

Validation: The validation study employed larger experimental groups, vehicle-treated controls and twelve weeks of follow-up after cessation of BRV treatment for a fully powered assessment of antiepileptogenic potential (Fig 1). Animals (n=104) were randomized to 4 treatment groups: vehicle controls, and BRV treatments started at 30 min, 4 hr or 8 hr after injury. One animal died acutely after FPI, 12 were lost to technical failure (including 10 failed pumps), 5 were euthanized for health reasons (e.g. malocclusion, dehydration due to failure to consume BRV solution) and 8 animals lost their headsets during the course of the study. Final group sizes at week 16 of the

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study were: control (n=20), 30 min (n=18), 4 hr (n=18) and 8 hr (n=22) Immediately prior to rpFPI, rats were implanted with primed osmotic pumps. Vehicle control group pumps were filled with saline. Treatment group pumps were loaded with BRV at a concentration calculated to deliver 650 mg/kg/day to a group of rats at its projected mean weight during the 5 days after injury. Pumps were equipped with tubing calibrated to delay dosing by 0.5, 4 or 8 hours. A loading dose of 100 mg/kg was administered i.p. to each BRV treated rat at 0.5, 4 or 8 hours post-FPI, as required. Vehicle controls received a comparable volume of saline at 0.5, 4 or 8 hr after injury. Pumps were removed on day 5, and BRV treated rats were supplied with drinking water formulated to provide 500 mg/kg, 350 mg/kg and 200 mg/kg on days 6, 7 and 8, respectively. From day 8 to 28 postinjury, these rats were given drinking water formulated to provide 200 mg/kg/day BRV. Mean fluid intake and the resulting mean daily dose were comparable in all three BRV-treated groups (Fig. 4 B,C). This prolonged BRV treatment had no effect on weight gain. Growth during the treatment period was virtually identical in all treatment groups (Fig. 4A).

On the last day of treatment (week 4) and two weeks after cessation of treatment (week 6), the incidence of seizures was reduced in all BRV-treated groups compared to vehicle-treated controls (Fig 5A). By 12 weeks after cessation of treatment (week16), the decrease in the incidence of seizure had diminished in the 30 min and 8 hr groups, but not in the 4 hr group. While there was a trend toward diminished mean seizure frequency in all groups at all time points, the decrease was most reliable in 4 hr group (Fig. 5B). The time spent seizing was decreased, at all time points, only in the 4 hr group (Fig 5C). A more detailed view of these data (Fig. 6) shows that the predominant effect of BRV treatment, especially in the 4 hr treatment group, was to decrease the proportion of rats with no detected seizures. At all time points, the treatment groups all had a surfeit, compared to controls, of rats with no detected seizures (figure 6, top panels) and zero time spent seizing (Fig 6, bottom panels). At 4 and 6 weeks, this brought

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the median seizure frequency and time seizing to or near zero, and median frequencies and times seizing remained more than an order of magnitude below the control level at week 16. However, the seizure frequencies and times seizing in BRV-treated animals with detected seizures were distributed similarly to controls (Fig. 6) in most cases including frequencies and times seizing comparable to the highest observed in controls. Representative seizures recorded from BRV-treated non-responders at 16 weeks post-FPI are shown in figure 7. Among animals with detected seizures (Fig 8), there was a week trend toward reduced seizure frequency and time seizing in the 4 hr group, but no suggestion of an effect of BRV treatment, otherwise. Thus, the decreases in seizure frequency and time seizing were largely, if not wholly, attributable to prevention of the precipitation of seizures at a frequency sufficient to permit detection.

To further characterize the long-term effects of BRV treatment, we specifically examined seizures that spread beyond the perilesional focus (Fig. 9) at 16 weeks post-injury. The overall incidence of spreading seizures in BRV treated groups ranged from 39% (standard deviation [SD]=10%) in the 4 hr group to 50% (SD=11%) in the 8hr group versus 80% (SD=9%) in vehicle-treated controls. Among rats with detected seizures at 16 weeks post-injury, the incidences of spreading seizures were from 67% (SD=14%) in the 30 min group, 78% (SD=14%) in the 4hr group and 79% (SD=11%) in the 8 hr group versus 100% (SD=0%) in controls. Confidence intervals for the difference in incidence between BRV treatment groups and controls indicate a trend toward lower incidence of spreading seizures compared to controls. (Fig 9A). Except for the animals with no detected seizures, which were found only in the BRV-treated groups, frequencies (Fig.9B) and times spent in spreading seizures (Fig. 9C) in the 30 min and 8 hr groups were distributed similarly to controls, and there was no indication of an independent reduction of either measure among animals with spreading seizures Fig 9D,E). Among the 8 rats with spreading seizures the 4hr group, there appeared to be a weak trend toward reduced frequency of spreading seizures (Fig. 9D), but larger numbers will have to be

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examined to confirm that trend. Thus, while the main long-term effect of BRV treatment was to reduce the proportion of animals with detected seizures, our exploratory analyses suggests that it also decreased the incidence of spreading seizures and, in the 4hr group, may have independently reduced frequency of seizures.

We have previously demonstrated that, all other things being equal, counting all seizures sensitively detected by an electrode montage that includes an electrode placed near an epileptic focus, increases statistical power to detect changes in the frequency and incidence of seizures, and in time spent seizing (Eastman et al., 2015). Accordingly, we routinely count all identifiable seizures down to as little as 1 s in duration. In order to rule out the possibility that the BRV-induced reduction in the proportion of animals with detected seizures observed in this study might be due to a selective effect on the more numerous brief seizures that are seldom monitored in other laboratories, we re-analyzed this data set using common duration-based seizure definitions ranging from 1-15 s minimum duration (Fig. 10). Regardless of seizure definition, the incidence of seizures (Fig. 10, top panels) was lower in BRV treated groups than controls. The apparent incidence of seizures tended to decrease with the stringency of seizure definition in all experimental groups, but this decrease was most pronounced in the vehicle group at 4 and 6 weeks post-FPI. The ratios of seizure incidences in the BRV-treated groups to that in controls did not increase appreciably with the stringency of seizure definition, as would be expected if BRV treatment selectively affected short seizures. In fact, this ratio tended to decrease in the 4 hr group that showed the best response to BRV-treatment. P-values for comparisons of BRV-treated groups and vehicle controls (Fig. 10, bottom panels) were stable across seizure definitions on week 4, varied non-monotonically on week 6. On week 16, when longer seizures accounted for a larger proportion of the seizure burden, p-values declined as shorter seizures were increasingly defined away. These results are incompatible with a selective

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effect of treatment on short seizures, and suggest that longer seizures may be preferentially affected by BRV treatment initiated 4 hr after head injury.

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DISCUSSION

We assessed the antiepileptogenic/disease-modifying potential of BRV using an optimized, etiologically realistic PTE model (Eastman et al., 2015; Curia et al., 2016) based on a well-accepted, clinically relevant experimental head injury (Thompson et al., 2005; Lyeth, 2016). BRV was tested using a complex dosing protocol designed to maintain plasma BRV in the human moderate-to-high therapeutic range for 4 weeks based on a series of pharmacokinetic experiments. The four-week duration was chosen based on the antiepileptogenic efficacy of mild focal cooling when applied for four weeks in the rpFPI model (D'Ambrosio et al., 2013). We examined injury-treatment latencies of 30 min, 4 hr and 8 hr after FPI . to optimize translatability of the findings to human studies. Animals were formally randomized to treatment groups, and ECoG data were analyzed blind to the identity of the data files. The principal finding of these studies is that BRV, dosed to maintain plasma levels in the human moderate-to-high therapeutic range for 4 weeks after experimental TBI persistently prevents the emergence of posttraumatic epileptic seizures in a subset of rats and may reduce incidence of spreading seizures in rats that develop seizures. The results constitute the most direct evidence to date that SV2A can be targeted to prevent or modify epilepsy after brain injury.

While this study was not powered to distinguish variably effective treatment protocols, the data indicate that there may be a relatively narrow post-injury time window in which treatment must be started to attain maximal effect. Treatment started 4 hrs after rpFPI had comparable or larger effects on all outcome measures than treatments started 30 minutes or 8 hours after injury (Figs. 3, 5, 6). More work will be required to verify this therapeutic "sweet spot", and determine its physiological substrate(s). The most striking effect of BRV treatment was to prevent the development of seizures in a subset of responsive rats. A surfeit of animals with no detected seizures in BRV treated groups compared to controls is evident both in the screening data (Fig. 3) and at all time points in the validation study (Fig. 6), and evidence for a

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treatment-induced reduction of seizure frequency or time seizing is weak, at best – even in the 4 hr group, which displayed the best response to treatment (Fig 8).

The data support the existence of distinct populations of responders and non-responders, although BRV treatment may provide some long-term benefit to “non-responders”, as well. In each BRV-treated group, non-responders – animals with detected seizures - included animals with times spent seizing and seizure frequencies comparable to the highest observed in the control group, and seizure frequencies and times seizing appear comparably distributed among animals with detected seizures in all experimental groups (Fig. 6) – particularly at the four and six week time points. This could either reflect genetic variability among subjects or individual differences in the kinetics of whatever processes underlie the apparent transience of BRV’s therapeutic window. By 16 weeks post-FPI, however, there was a trend toward lower seizure frequencies and times seizing in the BRV-treated groups compared to controls (Fig. 6), which was principally due to progression in frequency and time seizing in the control group. While the median seizure frequencies and times seizing in BRV-treated groups were nominally higher at 16 weeks post-FPI than at 4 and six weeks, the distributions of both appeared comparable. Thus, BRV treatment appears to have diminished or retarded the well-documented progression of seizure frequency and time seizing after rpFPI (D’Ambrosio et al., 2005; 2013; Eastman et al., 2011; Curia et al., 2011). The effect of BRV on spreading seizures further supports the hypothesis of diminished PTE progression after BRV treatment. Spreading seizures, which are typically rare in the early weeks after FPI, progressively increase to become the predominant seizure type by 3-4 months post-injury (D’Ambrosio et al., 2005, Curia et al., 2011). At 16 weeks post-injury, all rats with detected seizures in the vehicle group exhibited spreading seizures, which accounted for 87% (95% CI=72%-98%) of their seizure burden (i.e. time spent seizing). The incidence of spreading seizures in BRV treated rats with detected seizures ranged from 67% in the 30 min group to 79% in the 8 hr group.

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Strategy for discovery of anti-epileptogenic and disease-modifying treatments: Antiepilepto-genesis is regarded as the holy grail of epilepsy research (Loscher, 2020) and the quest faces daunting challenges. Since no treatment has ever been shown to prevent acquired epilepsy in man, epileptogenesis models cannot yet be rigorously validated for therapy development. The mechanisms of posttraumatic and other acquired epilepsies are poorly understood, placing reliable mechanism-based high-throughput screening assays beyond our reach. In addition, clinical trials for prevention of acquired epilepsies are necessarily large, lengthy, logistically challenging and very expensive (Herman, 2006, Mani et al., 2011; Schmidt and Sillanpaa, 2016). Within any at-risk patient population, only a fraction of patients will be diagnosed with epilepsy within any set time period, and the latency to first detected epileptic seizure may vary from weeks to decades after the precipitating insult. The sample size required to detect a specified effect depends upon the magnitude of the effect to be detected and the proportion of untreated patients expected to develop epilepsy in the study period. Estimates of group sizes required for 80% power to detect ~50% effect on epileptic outcome, given the 10%-20% risk of epilepsy (10%-20%) in feasible patient populations, range from 200 to 1000 (Herman, 2006; van Tuijl et al., 2011; Klein and Tyrlikova, 2017). For head injury patients, a follow-up of at least two years is recommended (Mani et al., 2011). The expense of clinical anti-epileptogenesis trials and hurdles to their success demand that investigational treatments should be selected based on the most reliable preclinical proof-of-principle studies and on preclinical data addressing therapeutic windows (duration and latency to treatment) and plasma levels required for therapeutic effect.

Until we attain a detailed mechanistic understanding of epileptogenesis, epilepsy models that feature the development of spontaneous seizures will be required to assess the antiepileptogenic potential of experimental treatments, and the use of etiologically relevant

models has been advocated based on the likelihood that different etiologies may recruit different epileptogenic mechanisms (Curia et al., 2016; Loscher, 2016; 2017). A variety of etiologically relevant syndrome-specific acquired epilepsy models have been developed over the past two decades (Kelly et al., 2001; D'Ambrosio et al., 2004; 2005; Dube et al., 2006; Stewart et al. 2010; Rakhade et al., 2011; Reid et al., 2016; Ping and Jin, 2016). The rpFPI model is based on a well-accepted clinically relevant TBI model (Thompson et al., 2005; Lyeth, 2016). Its brief, temporally discrete epileptogenic stimulus guarantees that injury modification cannot be mistaken for disease modification (Sloviter, 2011). The rpFPI model has been optimized for drug discovery (Curia et al., 2011; Curia et al., 2015; Eastman et al., 2015) and has demonstrated both negative and positive prediction. Studies using the rpFPI model stood alone in predicting the weak efficacy of carisbamate against drug resistant partial seizures in clinical trials (Eastman et al., 2011; Halford et al., 2011). The subsequent discovery that four weeks of mild focal cooling of an incipient epileptic focus persistently and potently prevented posttraumatic epileptic seizures after FPI (D'Ambrosio et al., 2013) was recently validated by reports that brain cooling reduces the risk of epilepsy after neonatal encephalopathy (Liu et al., 2017; Lugli et al., 2018).

Detecting the effect of an effective antiepileptogenic agent requires that it be delivered at an adequate dose for sufficient duration at an appropriate time, and exhaustive exploration of these variables is expensive. We screened a range of latencies to treatment using a streamlined procedure that allowed rapid (1 month versus 4 months) assessment of antiepileptogenic potential using small numbers of animals (n=7-8) for each drug treatment group. For novel, or less well characterized compounds, this strategy could be extended to survey doses and durations of treatment, as well. Maintenance of clinically relevant doses is problematic when drugs are administered chronically or subchronically to rodents (Loscher, 2007). Many drugs (e.g. BRV) are rapidly metabolized and cleared by rodents and/or may autoinduce their own

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metabolism, complicating efforts to maintain plasma levels in a therapeutic range. In this study, an empirically designed dosing regimen was likely instrumental in detecting the effects of BRV.

Limitations: This study has some limitations that are important to note. First, and most obvious, is the absence of female subjects, which may limit the scope of the findings. The rpFPI-PTE model was developed using young male rats, which are arguably representative of the most frequent victims of TBI, and the model has been optimized and characterized using young male rats. Since the development of epileptic seizures after rpFPI has not yet been documented in female rats, their inclusion in these studies could potentially diminish the power to detect antiepileptogenic effects. Second, based on a previously published power analysis (Eastman et al., 2011), this study was powered to reliably detect a 60% decrease in seizure incidence or a much larger “pure” decrease in seizure frequency. Thus, it was barely powered to detect the decrease in incidence in the 4hr group, and inadequately powered to confirm smaller changes such as decreased incidence of spreading seizures.

Conclusions: Time-limited treatment with BRV after head injury reduced the incidence of detected seizures in a subset of responsive rats, may have diminished or retarded the progression of PTE in “non-responders”. These results were obtained in a blind and randomized study using a clinically relevant exposure in an etiologically relevant PTE model. They suggest that brivaracetam has antiepileptogenic potential after TBI that should be evaluated in human studies. More broadly, it is possible that this potential may extend to other drugs targeting SV2A.

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Authorship Contributions.

Participated in research design: D'Ambrosio, Eastman, Fender and Klein.

Conducted experiments: Eastman and Fender.

Performed or contributed to data analysis and interpretation: D'Ambrosio, Eastman and Klein

Wrote or contributed to the writing of the manuscript: D'Ambrosio and Eastman.

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Footnotes:

a) Funding for this study was provided by UCB Pharma, which consulted in the design of the study and reporting of the findings, but did not contribute to the study execution, data analysis, or interpretation.

b) Financial Disclosure: PK has received consulting and speaker honoraria from UCB Pharma who makes brivaracetam.

c) Reprint requests should be e-mailed to Clifford L. Eastman: cliff@uw.edu

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Figure Legends

Figure 1. Experimental Design. A) Treatment protocols were screened in four-week experiments using experimental groups of 6-8 rats each and a relaxed statistical criterion ($p<0.15$) for success. ECoG data were acquired in two 24 hr epochs on the last days of treatment. B) Screened treatment protocols were validated in a longer experiment using experimental groups of 18-22 rats each and a conventional statistical criterion ($p<0.05$). ECoG was acquired on the last days of treatment (week 4), two weeks after cessation of treatment (week 6) and 12 weeks after cessation of treatment.

Figure 2. Development of a dosing protocol to maintain plasma BRV levels in rat plasma in a moderate to high (human) therapeutic range for four weeks. A) Mean trough plasma BRV levels during twice daily oral gavage administration of 25 and 50 mg/kg BRV. Mean BRV levels are more than 2 orders of magnitude below target, throughout. Plasma BRV rose abruptly after 3 days of dosing in rats administered 50, but not 25, mg/kg. B) Mean plasma BRV levels 1, 5 and 9 hours after oral administration of 5 mg/kg BRV. Data indicate plasma half-life of about 2.4 hours. C-D) Plasma BRV levels during administration of 120 mg/kg/day BRV for two weeks in drinking water. Individual and aggregate data are shown in C and D, respectively. Note that mean $[BRV]_{plasma}$ rose from a 5-day plateau at about 600 ng/ml to about 2000 ng/ml by the end of the second week of exposure, despite a mean daily dose of BRV that closely approximated the 120 mg/kg/day target (D, inset above), throughout. E) Mean plasma BRV levels measured during a stepped dosing protocol (inset, above) designed to compensate for the rise in plasma BRV during the second week of exposure. This resulted in plasma BRV levels that mostly ranged from 3-5 µg/ml, as desired, for the three weeks of exposure. Error bars in A, D and E indicate standard deviation.

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Figure 3. Screening Latencies to Treatment. Row A) Doses delivered in two independent screening tests. Doses under protocol 1 (left) overshot target for the first 5 days, but adhered closely to target thereafter. Protocol 2 targeted higher doses. The smaller variance during the first 5 days of exposure was due to use of osmotic pumps. Row B) Incidence of seizures in independent tests of dosing protocols shown in A. Under protocol 1 (left) seizure incidence increased with delay to treatment. Under protocol 2 (right), treatment started 4 hr after FPI appeared uniquely effective. P-values were computed with a one-tailed Fisher exact test comparing BRV-treated groups with controls. Rows C-D) Plots show binned (width=0.04) raw frequency and time seizing data overlaid on boxplots. Note that zero-valued frequencies and times seizing were assigned a value of 1/30 for presentation on a logarithmic scale and the lowest points in each plot correspond to rats with no detected seizures. Displayed p-values serve only as objective decision boundaries. Row C) Median (bold horizontal line) seizure frequencies are zero or near zero in the 30 min and 4 hr groups under both protocols, and median seizure frequencies in all BRV treated groups are below those of corresponding controls. Row D) Under protocol 1 (left) the median times spent seizing increase and the number of rats without seizures decrease with delay to treatment. Under protocol 2 (right) the median times spent seizing in BRV-treated groups are below those of controls.

Figure 4. Growth, fluid consumption and BRV dosing in validation study. BRV was administered via subcutaneous osmotic pumps for 5 days after injury and self-administered via sweetened BRV-containing drinking water thereafter. [BRV] in drinking water was adjusted, based on rats' mean body weight and anticipated fluid intake, to deliver target doses. A) Mean ± SD body weight during treatment. Weight gain was nearly identical in all experimental groups, and there was no difference between BRV- and saline-treated groups. A small inflection at day

6 reflects surgical removal of osmotic pumps. B) Mean ± SD consumption of BRV-containing drinking water. Fluid intake was measured in BRV-treated animals after removal of osmotic pumps. The low intake recorded on day 6 and the inflection at day 15 reflect post-surgical suppression of drinking after pump removal and headset implantation, respectively. C) Mean ± SD dose delivered to BRV-treated rats. Exposure was comparable in all treatment groups.

Figure 5. Confidence Intervals for Effect of BRV Treatment on Primary Endpoints. A) Bootstrapped 95% confidence intervals for the differences in incidences between BRV-treated groups and vehicle controls. Seizure incidence is clearly reduced in all treatment groups at 4 and 6 weeks postinjury and remains decreased in the 4 hr group at 16 weeks. B) Bootstrapped 95% confidence intervals for the differences in mean frequency of seizures between BRV-treated groups and vehicle controls. The mean frequency of seizure is reduced in the 4 hr group at all time points. C) Bootstrapped 95% confidence intervals for the differences in mean time seizing between BRV-treated groups and vehicle controls. The mean time spent seizuring is reduced in the 4 hr group at all time points

Figure 6. Seizure Incidence, frequency and time spent seizing in validation study. Top) Incidence of seizures 4, 6, 14 and 16 weeks post-FPI. The incidence of seizure was lower in the BRV-treated groups than in controls at all time points. Note that seizure incidence in the 4 hr group remained within the 40% to 50% range for 12 weeks after cessation of treatment. Middle and bottom) Plots show binned (width=0.04) seizure frequency (middle) and time seizing (bottom) data overlaid on boxplots. The lowest data points in all plots represent rats with no detected seizures. At the end of treatment (week 4) and two weeks after cessation of treatment (week 6), the median frequency of seizure and time spent seizing is zero or near-zero in all

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three BRV-treated groups. Twelve weeks after the end of treatment (week 16), median seizure frequencies and times spent seizing in the BRV-treated groups remain more than an order of magnitude lower in than controls. Note that the BRV-treated groups include individuals with frequencies and times seizing comparable to the highest in vehicle controls, and that the lower median values in the BRV-treated groups are mainly attributable to a surfeit of rats with no detected seizures.

Figure 7. Representative seizures from BRV-treated non-responders at 16 weeks post-FPI. Multi-channel EEG was recorded using the montage shown in the left inset. Numbered black dots indicate electrode locations. The perilesional electrode (e4) is located just rostral to the FPI craniotomy, which is indicated by a circle. The top traces show a focal non-spreading seizure that is detected exclusively by the perilesional electrode. The top inset shows the indicated segment of the signal from the perilesional electrode on an expanded time scale to show the details of the waveform. The bottom traces show a spreading seizure that is first detected by the perilesional electrode and then spreads to the contralateral cortex. The seizures recorded in BRV-treated non-responders were indistinguishable from those recorded in vehicle-treated controls.

Figure 8. Seizure frequency and time spent seizing of rats with seizures. Plots show 95% confidence intervals for vehicle - treatment group differences and p-values for each comparison. A) Seizure frequency. Eight out of nine confidence intervals overlap zero difference, and no apparent trend toward reduced frequency was improbable under the null hypothesis. The data do not support an effect of BRV on seizure frequency independent of a decrease in seizure incidence. B) Time spent seizing. Eight out of nine confidence intervals overlap zero difference, and no apparent trend toward reduced time seizing was improbable under the null hypothesis. While there may be a weak trend toward lower seizure frequency and time seizing in the 4 hr

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group, the data do not support an effect of BRV on time seizing independent of a decrease in seizure incidence.

Figure 9. Effect of BRV treatments on spreading seizures at 16 weeks post-FPI. A) Incidence of spreading seizures in animals with detected seizures (N= 16, 12, 9 and 14 in the vehicle, 30 min, 4 hr and 8 hr groups, respectively). Bootstrapped 95% confidence intervals for the difference (treatment-vehicle) indicate that the incidence of spreading seizures was diminished in BRV-treated groups. B-C) Plots show spreading seizure frequency and time spent in spreading seizures in rats with detected seizures. The lowest data points in all plots represent rats with no spreading seizures. Epileptic animals with exclusively focal seizures were observed only in BRV-treated groups, and fully accounted for the trend toward lower frequencies of spreading seizures (B) and times spent in spreading seizures (C) in the 30 min and 8 hr groups. D-E) Frequency (D) and time spent in spreading seizures (E) in animals with spreading seizures (N=16, 8, 7 and 11 in the vehicle, 30 min, 4 hr and 8 hr groups, respectively). Bootstrapped 95% confidence intervals do not indicate an independent effect of BRV treatment on the frequency or time spent in spreading seizures.

Figure 10. Effects of BRV treatment are independent of seizure definition. Top Row) Seizure incidence in the 4 experimental groups as a function of duration-based seizure definition. Apparent incidence decreases with the stringency of the seizure definition. This is most dramatic in the vehicle controls, which have a higher incidence of seizure, overall. Middle Row) The ratio of incidences in the BRV treatment groups to incidence in vehicle controls is plotted as a function of duration-based seizure definition. This ratio is relatively stable across seizure definitions, but appears to decline in the 4 hr group that displays the best response to

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BRV. Bottom Row) p-values obtained for comparisons of the incidence of seizures in the 3 BRV treatment groups with vehicle controls are plotted as a function of seizure definition. P-values do not increase with the stringency of the seizure definition. The decline in both the treatment/vehicle ratio and pvalues at the most stringent definitions suggests that longer seizures could be more sensitive to BRV than briefer seizures.

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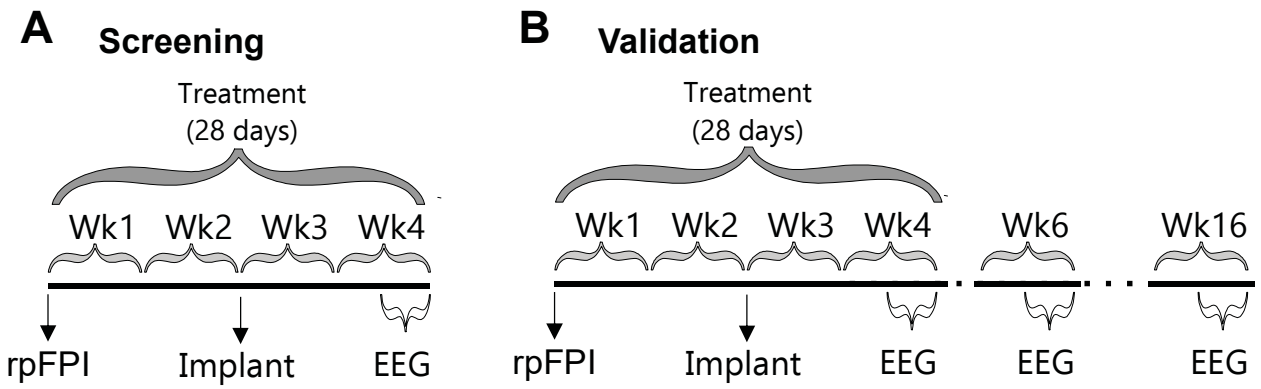

Fig. 1

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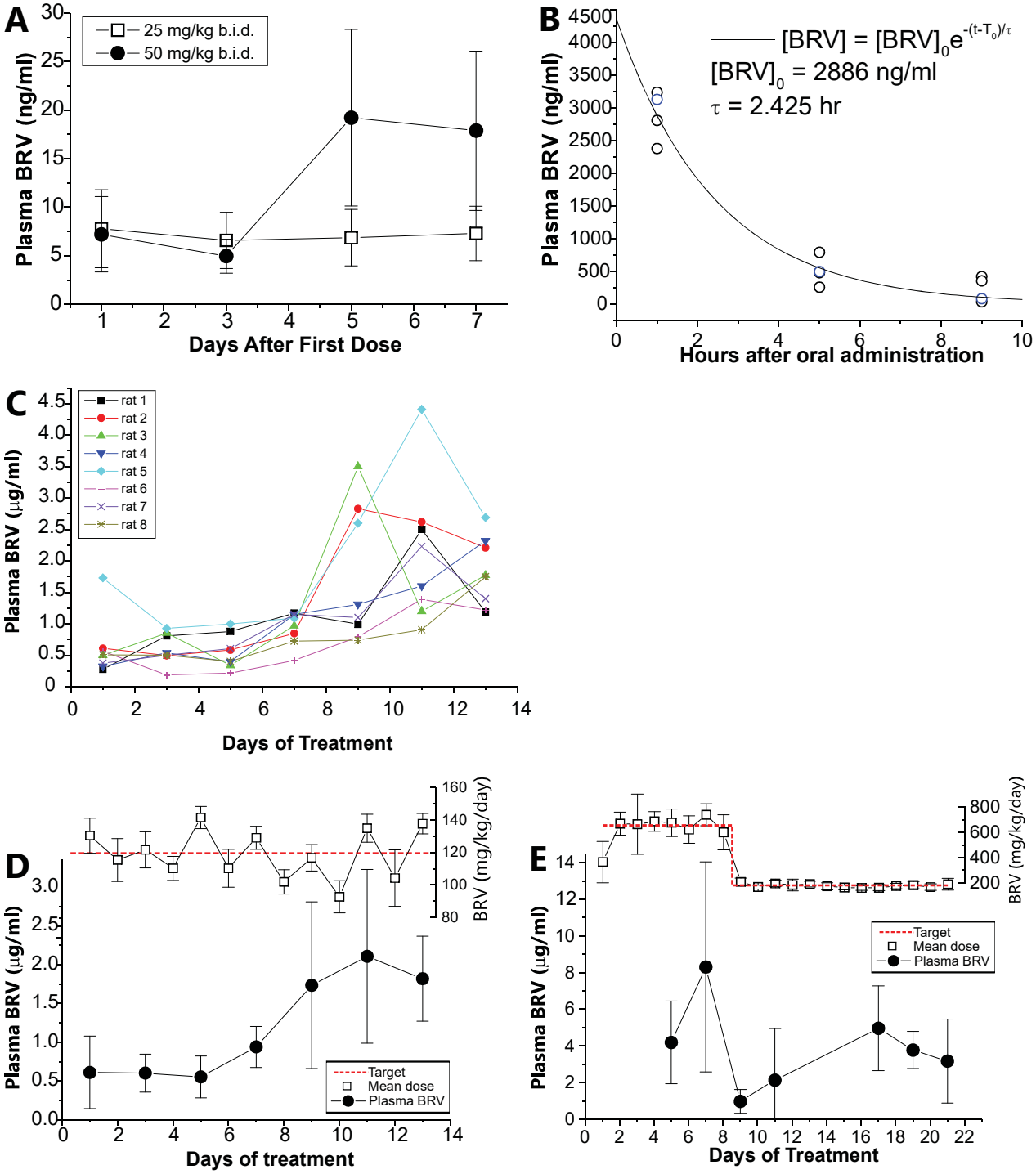

Fig. 2

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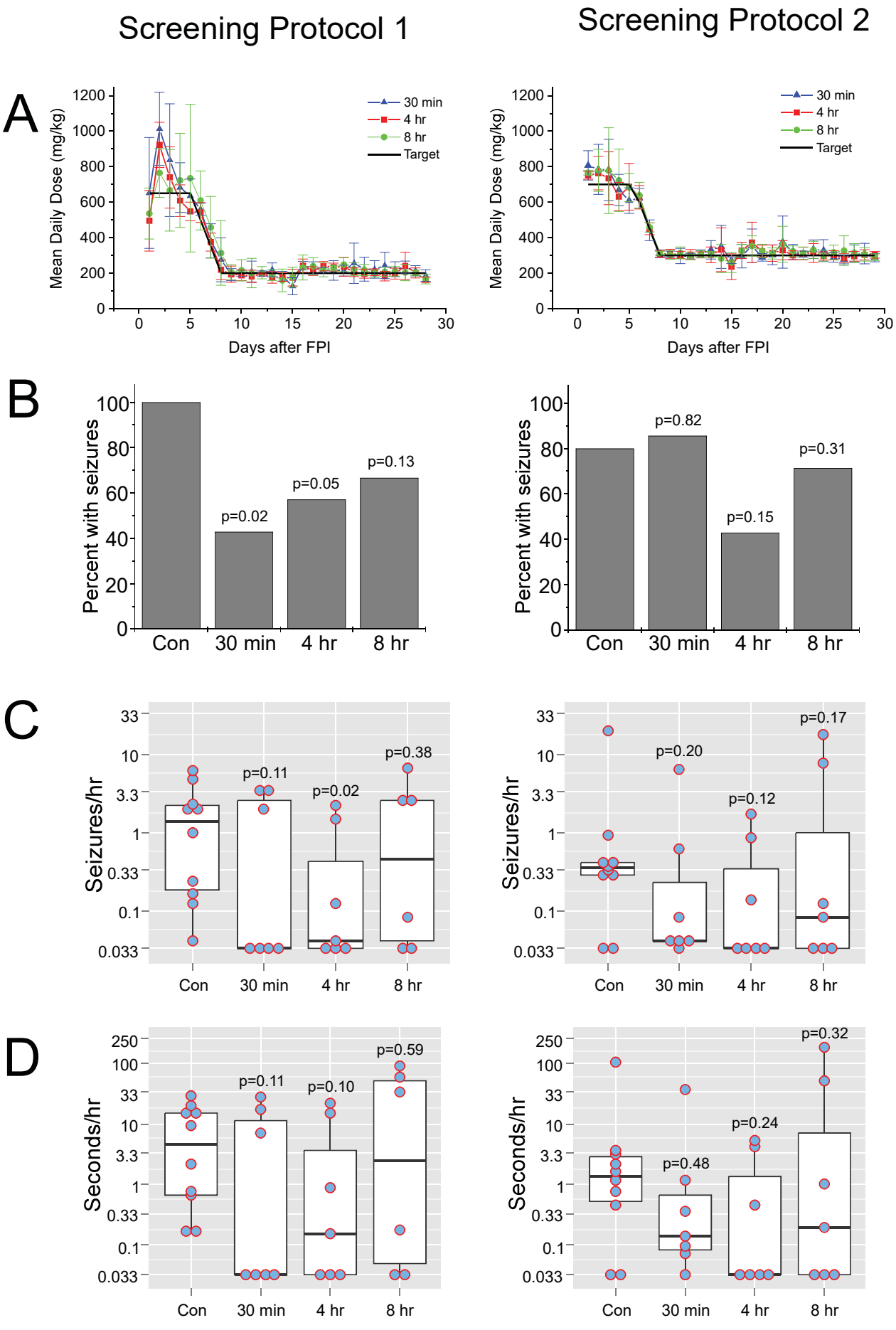

Fig. 3

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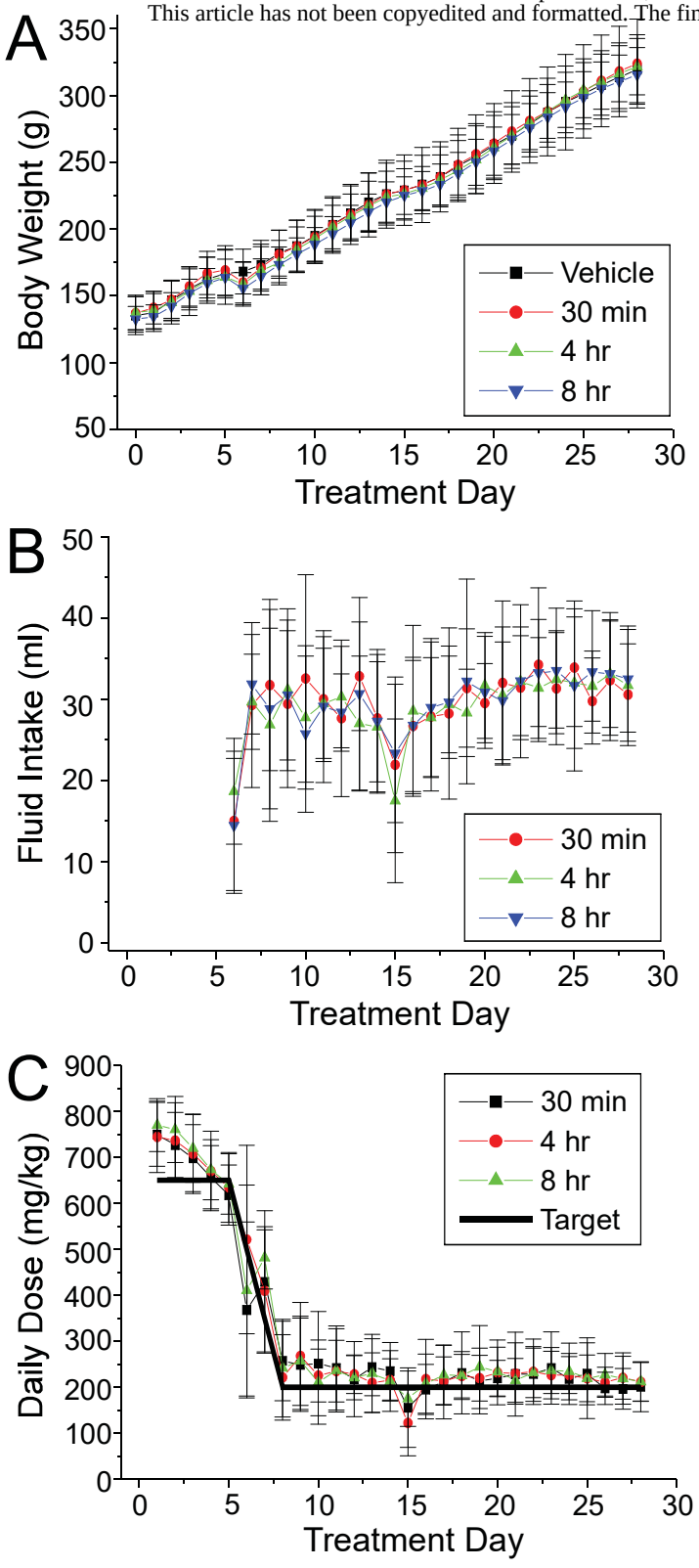

Fig. 4

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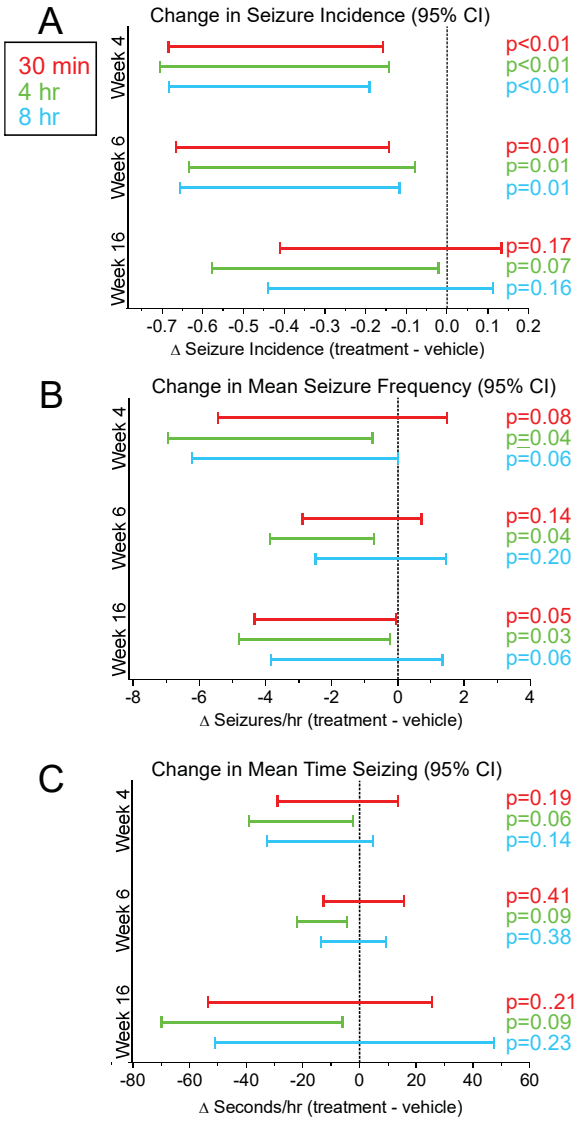

Fig. 5

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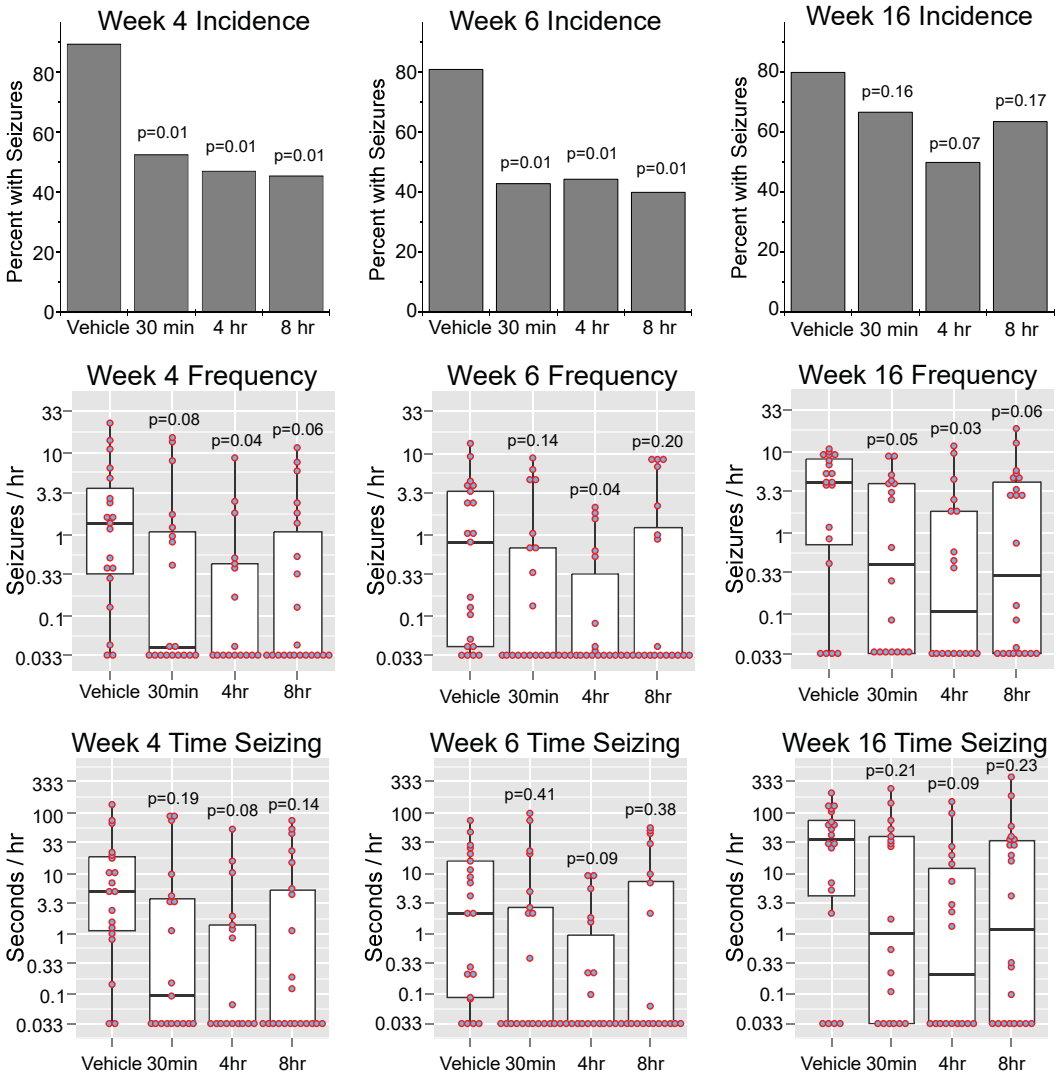

Fig. 6

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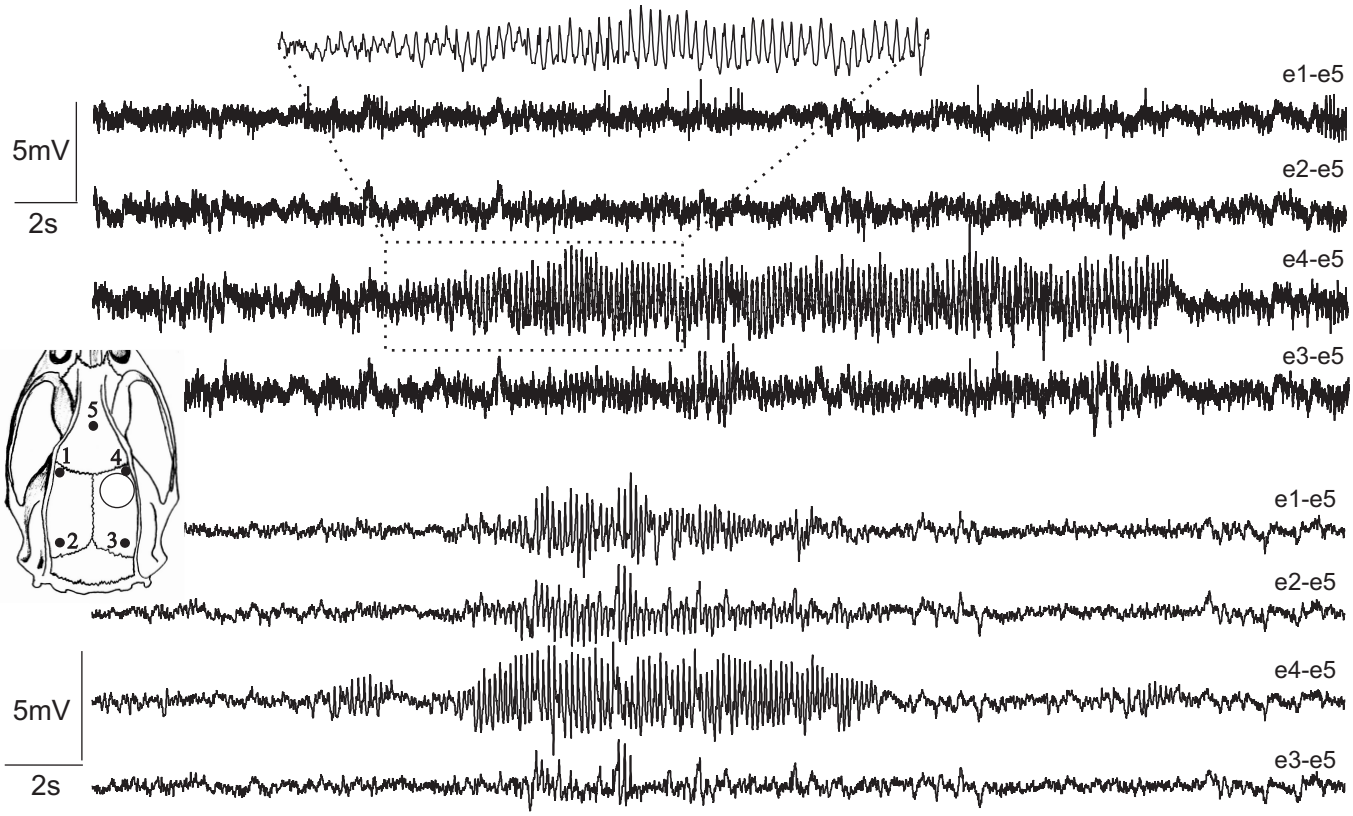

Fig. 7

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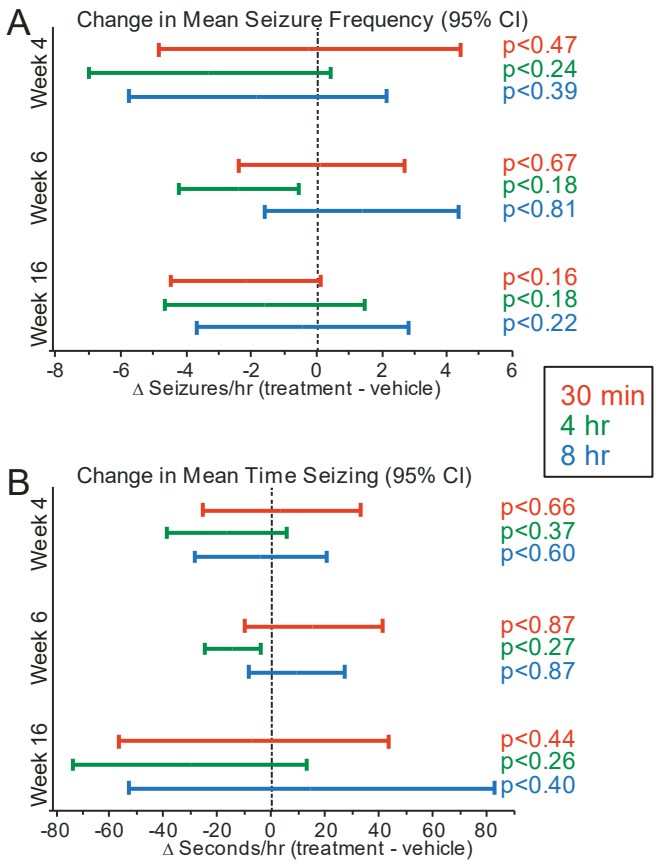

Fig. 8

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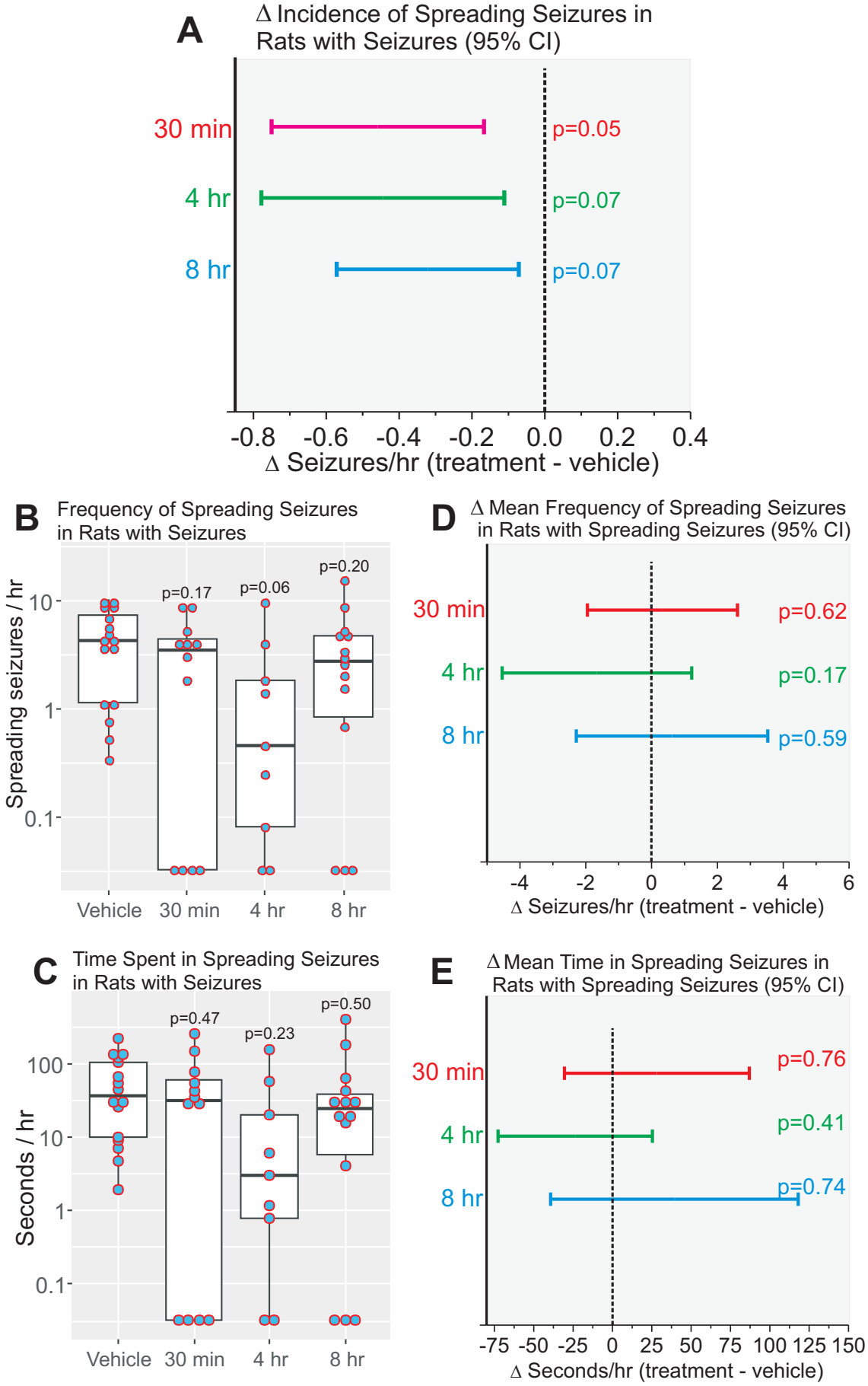

Fig. 9

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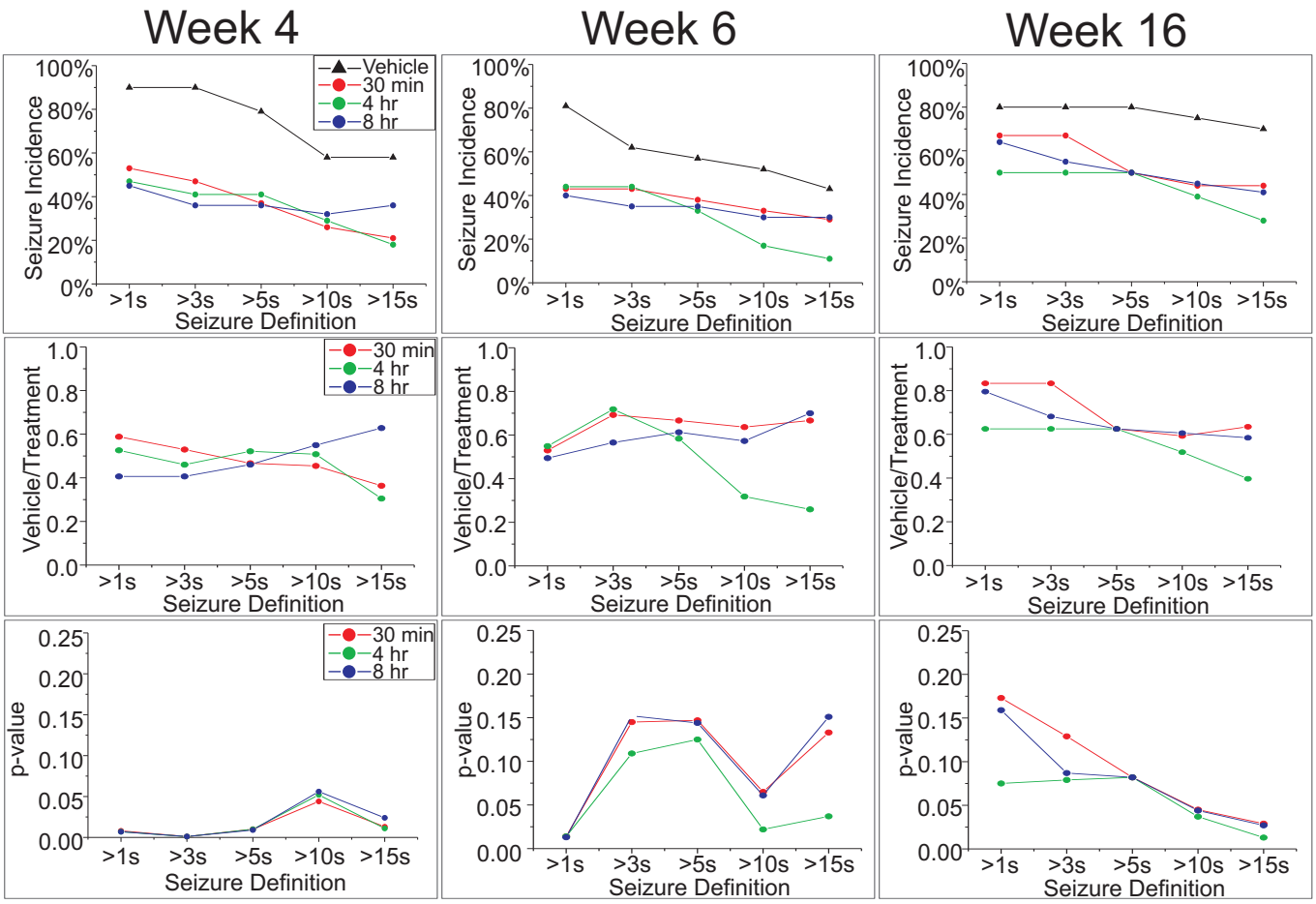

Fig. 10