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Post-exposure administration of diazepam combined with soluble epoxide hydrolase inhibition stops seizures and modulates neuroinflammation in a murine model of acute TETS intoxication



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ABSTRACT

Tetramethylenedisulfotetramine (TETS) is a potent convulsant poison for which there is currently no approved antidote. The convulsant action of TETS is thought to be mediated by inhibition of type A gamma-aminobutyric acid receptor (GABA_AR) function. We, therefore, investigated the effects of post-exposure administration of diazepam, a GABA_AR positive allosteric modulator, on seizure activity, death and neuroinflammation in adult male Swiss mice injected with a lethal dose of TETS (0.15 mg/kg, ip). Administration of a high dose of diazepam (5 mg/kg, ip) immediately following the second clonic seizure (approximately 20 min post-TETS injection) effectively prevented progression to tonic seizures and death. However, this treatment did not prevent persistent reactive astrogliosis and microglial activation, as determined by GFAP and Iba-1 immunoreactivity and microglial cell morphology. Inhibition of soluble epoxide hydrolase (sEH) has been shown to exert potent anti-inflammatory effects and to increase survival in mice intoxicated with other GABA_AR antagonists. The sEH inhibitor TUPS (1 mg/kg, ip) administered immediately after the second clonic seizure did not protect TETS-intoxicated animals from tonic seizures or death. Combined administration of diazepam (5 mg/kg, ip) and TUPS (1 mg/kg, ip, starting 1 h after diazepam and repeated every 24 h) prevented TETS-induced lethality and influenced signs of neuroinflammation in some brain regions. Significantly decreased microglial activation and enhanced reactive astrogliosis were observed in the hippocampus, with no changes in the cortex. Combining an agent that targets specific anti-inflammatory mechanisms with a traditional antiseizure drug may enhance treatment outcome in TETS intoxication.

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Abbreviations: ANOVA, analysis of variance; CI, confidence interval; DZP, diazepam; EEG, electroencephalogram; GABA_AR, type A gamma-aminobutyric acid receptor; GFAP, glial fibrillary acidic protein; GMR, geometric mean ratio; H&E, hematoxylin and eosin; Iba-1, ionized calcium binding adaptor molecule-1; ip, intraperitoneal; PTZ, pentylenetetrazole; sEH, soluble epoxide hydrolase; sEHI, soluble epoxide hydrolase inhibitor; SE, status epilepticus; TETS, tetramethylenedisulfotetramine; TUPS, (1-(1-methanesulfonyl-piperidin-4-yl)-3-(4-trifluoromethoxy-phenyl)-urea.

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Introduction

Tetramethylenedisulfotetramine (TETS; tetramine; TMDT) is a heteroadamantane compound originally synthesized as a condensation product of sulfamide and formaldehyde (Wood and Battye, 1933). It was subsequently found to be a highly toxic convulsant poison and was widely used as a rodenticide until banned in China in 1984 (Whitlow et al., 2005). TETS is, however, still produced illicitly and has been implicated in the accidental or intentional poisoning of as many as 14,000 individuals in China between 1991 and 2010, as well as more than 50 human poisonings in Western countries since 2002 (Li et al., 2012; Zhang et al., 2011). TETS meets the criteria for inclusion in the World Health Organization's list of "extremely hazardous pesticides" (Whitlow et al., 2005) and is considered a high priority chemical threat agent by the National Institutes of Health (Jett and Yeung, 2010).

Based on clinical reports, exposure to low doses of TETS causes dizziness and headaches while high dose exposures trigger acute seizures

and status epilepticus (SE) and can lead to death. Exposure to sublethal doses of TETS can cause persistent neurologic sequelae in survivors, including epileptogenesis that lasts for years following the initial exposure to TETS (Bai et al., 2005; Barrueto et al., 2003; Li et al., 2012; Whitlow et al., 2005). Recently, our group characterized rodent models of acute TETS intoxication, which confirmed the high potency of TETS as a convulsant in mice and rats (Zolkowska et al., 2012). Our findings were confirmed in a study by Shakarjian et al. (2012), which demonstrated that treatment with high dose diazepam (5 mg/kg, ip), a GABA_AR positive allosteric modulator, protected adult male C57BL/6 mice from further motor seizures and increased survival at 1 h following TETS exposure. This is consistent with experimental evidence indicating that TETS is a noncompetitive antagonist of the GABA_AR [reviewed by Zhao et al., 2014]. However, this high dose of diazepam did not stop electrographic seizures, and the animals died several hours after treatment (Shakarjian et al., 2012). These observations are consistent with case reports indicating that benzodiazepine therapy is not always efficacious in treating humans poisoned with TETS (Lu et al., 2008; Poon et al., 2005). Collectively, these data suggest that benzodiazepines alone are insufficient to protect against acute TETS poisoning.

Emerging evidence implicates neuroinflammation, specifically reactive astrogliosis and microglial activation, in the pathogenesis of spontaneous recurrent seizures following SE (Clasadonte et al., 2013; Devinsky et al., 2013; Hunt et al., 2013; Pineda et al., 2013; Rossi et al., 2013). Interestingly, we found in our earlier study that exposure to sublethal convulsant doses of TETS significantly increased reactive astrogliosis and microglial activation in the cortex and hippocampus of Swiss mice several days after TETS exposure (Zolkowska et al., 2012). This suggests the possibility that combinatorial therapy with anticonvulsant and anti-inflammatory agents may better protect the brain following acute TETS intoxication. Epoxygenated fatty acids are produced in the brain and have been shown to be potent anti-inflammatory compounds (Iliiff and Alkayed, 2009; Inceoglu et al., 2007; Wang et al., 2013). The beneficial effect of these naturally occurring epoxy fatty acids is severely limited by their rapid metabolism via soluble epoxide hydrolase (sEH) (Imig and Hammock, 2009; Newman et al., 2005). Pharmacologic inhibition of sEH stabilizes these bioactive molecules (Spector and Norris, 2007) and small molecule sEH inhibitors (sEHI) have been shown to have significant anti-inflammatory and neuroprotective activity in animal models of pain, inflammation and ischemic stroke (Iliiff and Alkayed, 2009; Inceoglu et al., 2008; Wang et al., 2013). In addition, we recently demonstrated that sEHI pretreatment delays the onset of clonic seizures and prevents lethality in NIH Swiss mice exposed to a lethal dose of other GABA_A receptor antagonists, including picrotoxin and pentylentetrazole (PTZ) (Inceoglu et al., 2013).

The goal of this study was to characterize the effects of high dose diazepam administered alone or in combination with the sEHI TUPS on TETS-induced seizures, death and neuroinflammation. Neuroinflammation was assessed by quantifying GFAP and Iba-1 immunoreactivity as biomarkers of reactive astrogliosis and microglial activation, respectively, in the cortex and hippocampus, which are key brain regions implicated in the generation of seizures (Hunt et al., 2013). Our findings support the argument that a combinatorial approach targeting different pathogenic mechanisms of acute TETS intoxication may be more efficacious than using benzodiazepines alone to protect the brain following acute TETS intoxication.

Materials and methods

Chemicals. Paraformaldehyde, sulfamide, hydrochloric acid, acetone, and hexane were obtained from Thermo Fisher Scientific (Waltham, MA). All chemicals were of the highest purity available. TETS was synthesized as previously described (Zolkowska et al., 2012). A final recrystallization step was performed to ensure no water remained in the crystals and characterization of the final product by gas chromatography–mass spectrometry supported a purity of >98%. USP grade

diazepam manufactured by Hospira (in 40% propylene glycol, 10% alcohol, 5% sodium benzoate and 1.5% benzyl alcohol) was purchased from Western Medical Supply (Arcata, CA). The sEHI TUPS (1-(1-methanesulfonyl-piperidin-4-yl)-3-(4-trifluoromethoxy-phenyl)-urea) was synthesized as previously described (Tsai et al., 2010). TETS was dissolved in 100% DMSO and stored as stock solutions at 10 mg/ml. TUPS was formulated in pure polyethylene glycol 400 (PEG400). Chemical structures of TETS and TUPS are illustrated in Fig. 1.

Animals. Animals were maintained in facilities fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, and all studies were performed under protocols approved by the UC Davis Institutional Animal Care and Use Committee. Male NIH Swiss mice (22–25 g) were obtained from the Animal Resource Program, Center for Cancer Research, National Cancer Institute (Bethesda, MD) and were housed four per cage. All animals were kept in a vivarium under controlled environmental conditions (22–26 °C; 40–50% humidity) with a 12 h normal light/dark cycle and free access to food and water. Animals were allowed to acclimate to the vivarium for at least 5 days prior to experimentation. Experiments were performed during the light phase of the light/dark cycle after at least 30 min of acclimation to the laboratory setting.

Dosing paradigm. Mice were randomly divided into the following 4 treatment groups: (1) TETS + diazepam, (2) TETS vehicle (10% DMSO in sterile saline) + diazepam, (3) TETS + diazepam + TUPS, and (4) TETS + diazepam + TUPS vehicle (PEG400). On the day of each experiment, stock solutions of TETS were sequentially diluted in saline with 10% DMSO at 40–60 °C to a final concentration of 0.015 mg/ml. TETS solutions were kept at 35–36 °C and administered ip in a volume of 10 ml/kg. Immediately after dosing, animals were observed for up to 60 min for seizure behavior, and time to seizure onset and duration of each seizure were recorded for each animal. Mice in treatment groups 3 and 4 also received either TUPS (1 mg/kg, ip) or an equal volume (1 ml/kg) of vehicle (PEG400) 1 h following treatment with diazepam, and every 24 h thereafter until euthanized.

Electroencephalogram (EEG) recording. EEG recordings were obtained in a subset of animals to validate behavioral seizure scoring. To place electrodes, mice were anesthetized using a mixture of ketamine

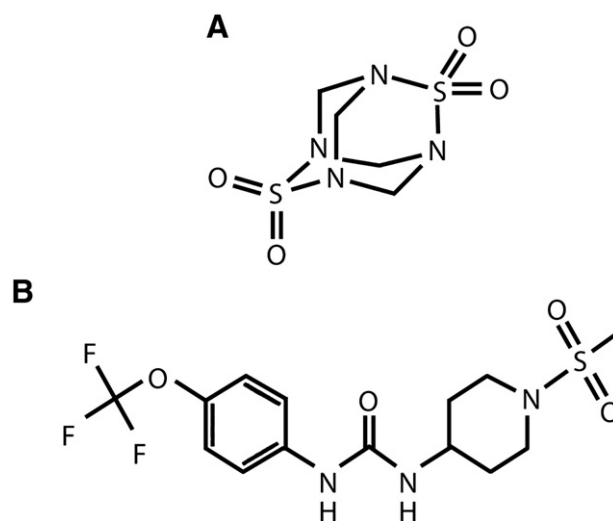


Fig. 1. Chemical structures of (A) the cage convulsant 2,6-dithia-1,3,5,7-tetraazacyclo[3.3.1.1.3.7]decane-2,2,6,6-tetraoxide (TETS) and (B) the soluble epoxide hydrolase inhibitor 1-(1-methylsulfonyl-piperidin-4-yl)-3-(4-trifluoromethoxy-phenyl)-urea (TUPS).

(100 mg/kg, ip) and dexmedetomidine (1 mg/kg, ip) and then surgically implanted with a 2 EEG/1 EMG mouse head mount (8201 mouse head mount, Pinnacle Technology, Lawrence, KS). The head mount was affixed to the skull with stainless steel screws that also acted as EEG electrodes. The front edge of the implant was placed 3.0–3.5 mm anterior of bregma. In this configuration, all four screws rest on the cerebral cortex. The right frontal (EEG2) and parietal (EEG1) screws were used for EEG recording. The lowest noise EEG signal was selected for display in Fig. 2; both EEG signals are shown in Supplementary Fig. 2C. EMG electrodes were implanted in the neck. EEG recordings were conducted with respect to the left parietal cortex. The left frontal screw electrode was ground. Mice were allowed to recover for 1 week before initiating experimentation. On the day of each experiment, mice were acclimated to the recording cage and a 30 min baseline recording was obtained prior to administration of TETS (0.15 mg/kg, ip). Within 1–2 min after the second clonic seizure, diazepam (5 mg/kg, ip) was administered to half the animals. Following diazepam administration, EEG activity was continuously recorded for at least 1 h in each mouse. EEG signals were acquired and analyzed using Sirenia software (Pinnacle Technology).

Histology. At 1, 2, 3 and 7 days post-TETS exposure, anesthetized mice were serially perfused with ice-cold saline and 4% paraformaldehyde. Fixed brains were flash frozen in O.C.T. compound (Sakura Finetek, Torrance, CA) and cut with a cryostat into 10 μ m sagittal sections. Hematoxylin and eosin (H&E) staining was used to assess tissue integrity while Fluoro-Jade B (FJB) staining was used to identify neuronal cell damage as previously described (Zolkowska et al., 2012). To assess reactive astrogliosis and microglial cell activation, brain sections adjacent to those used for histological assessment were immunostained for glial fibrillary acidic protein (GFAP) or ionized calcium binding adaptor molecule-1 (Iba-1), respectively, as previously described (Zolkowska et al., 2012). GFAP and Iba-1 immunoreactivity were visualized by confocal microscopy and quantified using image analysis software (Metamorph, Molecular Devices, Sunnyvale, CA) as previously described (Zolkowska et al., 2012). Individuals blinded to the treatment condition determined the number of activated versus resting Iba-1 immunopositive microglia according to published morphologic criteria (Matthews and Kruger, 1973). Activated versus resting microglia were quantified across the entire hippocampus and along the entire dorsal

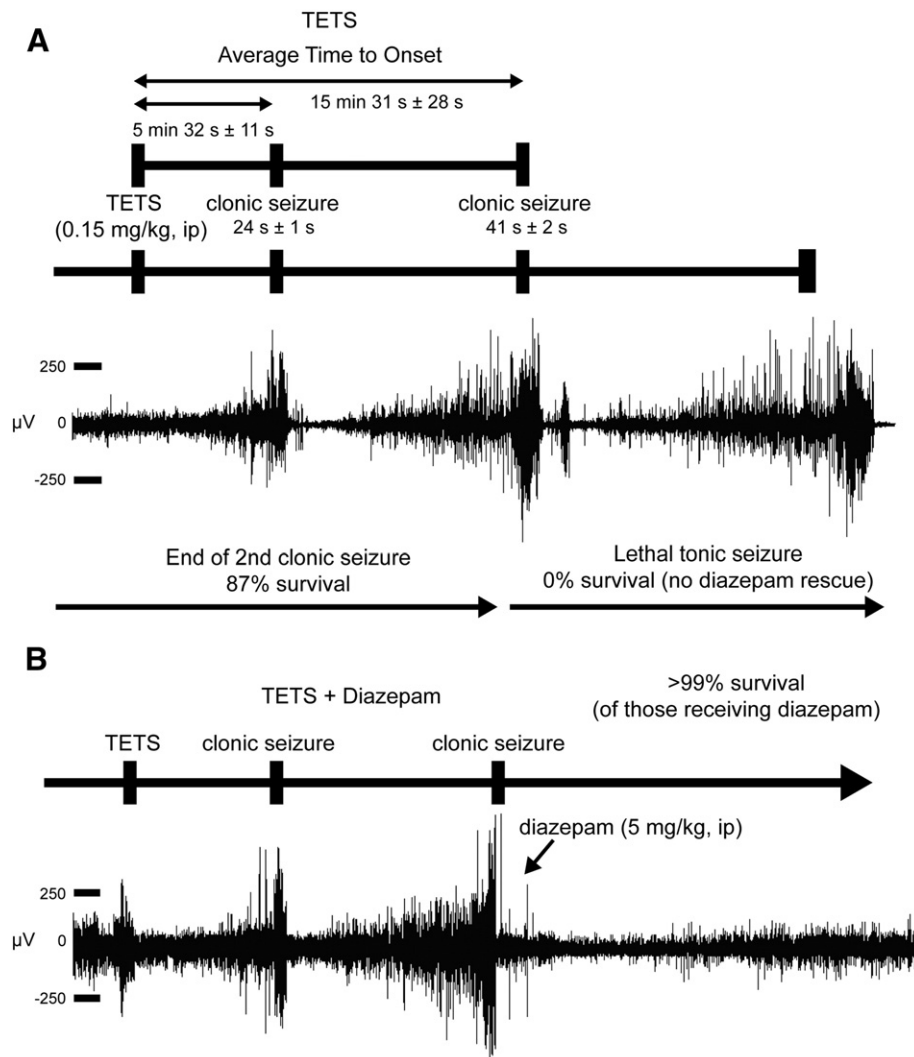


Fig. 2. Diazepam blocks TETS-induced tonic seizures and prevents mortality when administered after the second clonic seizure. (A) Animals administered TETS at 0.15 mg/kg (ip) typically experienced two clonic seizures followed by a tonic seizure. All animals died within minutes of exhibiting tonic-clonic seizures ($n = 68$ animals). (B) Administration of diazepam (5 mg/kg, ip) immediately following the second clonic seizure prevented subsequent seizures and significantly reduced mortality as evidenced by a survival rate of > 99% ($n = 142$ animals). Time-to-onset and duration of seizures are expressed as the mean \pm SE. Representative EEG recordings are shown from mice administered TETS (0.15 mg/kg, ip) with post-exposure administration of vehicle (top trace) or diazepam (bottom trace). EEG recordings for 1 h post-TETS exposure in animals rescued by diazepam confirmed no additional seizure activity. The supplementary data file includes additional EEG traces from other animals (Figs. S1A and B), and a trace with a finer temporal resolution (Fig. S1C).

boundary of the cortex to an inward depth of 500 μm from the dorsal edge. All endpoints were examined in at least 3 serial sections per animal and data were collected from the same level of the brain across all animals.

Statistical analyses. Histological data were analyzed using Stata 13 software (StataCorp, College Station, TX). Graphics were produced using the R 3.0.1 software suite (Vienna, Austria; package ggplot2 version 0.9.3.1 by Hadley Wickham). Because fluorescent staining and glial counts were performed on multiple sections from different brain regions of each animal, mixed-effects regression models employing repeated measures taken from the same animal were used to account for within-animal correlations and to improve the efficiency of estimates. Repeated measures analysis of variance (ANOVA) methods were inappropriate due to missing observations and to their inability to deal with count outcomes, as in the microglial data.

GFAP and Iba-1 immunostaining were analyzed with respect to the area of immunoreactivity. Exploratory analysis indicating that a logarithmic transformation of the data was required to stabilize the variance and normalize the distribution to accord with the linear model specification. Linear mixed effects models with animal random effects were applied unless residual scatterplots indicated that the error variance was approximately proportional to the mean. In the latter case, Poisson mixed effects models (log link) were used. In every model, candidate fixed effects were as follows: treatment condition, brain region (cortex or hippocampus), number of days after treatment administration, and their interactions. Variable selection was performed using the Akaike information criterion and Wald tests for pairwise comparisons of interaction effects; when interaction terms were not significant the model was simplified by removing them. Glial activation data were expressed as counts and Poisson mixed effects models were applied with variable selection proceeding as described above.

Statistical analysis results are presented as geometric mean ratios between two treatment conditions. These ratios are directly interpretable as fold changes. That is, a ratio of 1.5 corresponds to a 50% increase, and a ratio of 0.5 corresponds to a 50% decrease. In the figures, point estimates of the ratio are presented as dots with the associated 95% confidence interval extending above and below. The confidence interval provides an indication of the strength of the effect. When the confidence interval includes 1, there is no statistical evidence of any difference between the treatment and its comparison group. When the interval does not include 1, the estimate of the effect is considered significant at the 5% level. However, any value lying within the confidence interval is considered a plausible value for the true treatment effect. Using a fully interacted model (that is, all interaction terms are included), there is a confidence interval for every brain region and day number. When the variable selection process indicated that the model could be simplified, the appropriate interaction terms were removed. In this case, the confidence intervals act as a summary measure across brain region, day number, or both.

Results

Post-exposure administration of diazepam prevents lethality in TETS-intoxicated mice

Consistent with previous observations (Zolkowska et al., 2012), mice dosed with 0.15 mg/kg TETS displayed a brief period of hyperactivity followed by a brief period of quiescence, Straub tail, twitches, imbalance, clonic seizures, and subsequently, tonic seizures and death. The majority of animals (>80%) exhibited a period of wild running immediately prior to the onset of tonus. Mice intoxicated with this lethal dose of TETS exhibited a stereotypic pattern of seizure behavior consisting of two clonic seizures of short duration (<1 min) beginning within 5 to 6 min after TETS injection, followed by a lethal tonic seizure within 20–30 min after TETS injection (Fig. 2A). The time to onset and duration

of seizures (Fig. 2A) were as observed previously (Zolkowska et al., 2012). In the absence of therapeutic intervention, 87% of mice exposed to TETS survived the two clonic seizures, but none (0%) survived the tonic seizure. The 13% of animals who died during this period characterized by clonic seizures transitioned to tonus after a single clonic seizure or immediately following the second seizure. To determine whether animals could be protected from death by a treatment delivered before the lethal tonic seizure, diazepam at 5 mg/kg (ip) was administered within 1–2 min after the second clonic seizure (approximately 20 min after TETS injection). This dose of diazepam was chosen based on previous studies (Shakarjian et al., 2012). When administered shortly following the end of the second clonic seizure, diazepam stopped seizure behavior and prevented mortality in >99% of TETS-intoxicated animals (Fig. 2B). Administering diazepam at later times (e.g., at the start of the third seizure) also decreased mortality relative to animals that received no therapeutic intervention, but the results were inconsistent and fewer animals survived (data not shown).

To assess whether diazepam also terminated electrographic seizure activity, EEG recordings were performed in TETS-intoxicated mice, a

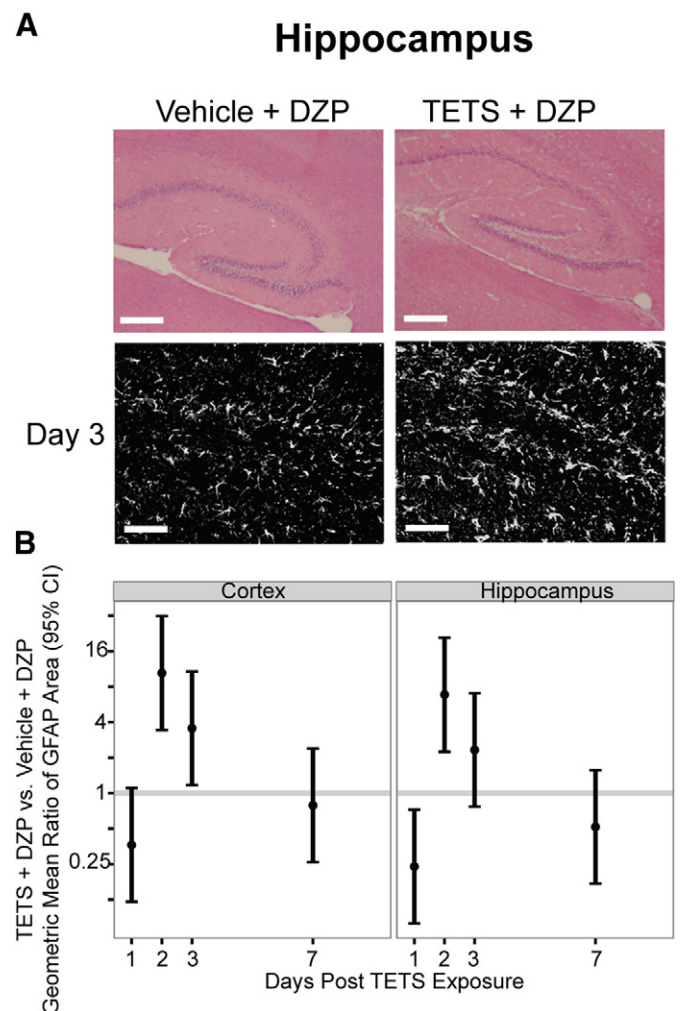


Fig. 3. Post-exposure administration of diazepam (DZP) does not prevent reactive astrogliosis in mice acutely intoxicated with TETS. (A) Representative photomicrographs of H&E staining (top panels) and GFAP immunoreactivity (bottom panels) in the hippocampus 3 days post-injection of mice exposed to either vehicle or TETS (0.15 mg/kg, ip) followed by administration of diazepam (5 mg/kg, ip) ~20 min later. Bar = 50 μm . (B) The area of GFAP immunoreactivity per field was quantified in the cortex (left) and hippocampus (right) as a measure of the number of GFAP-immunopositive cells. Dots represent the geometric mean ratio of TETS + diazepam versus vehicle + diazepam-treated animals; bars represent 95% confidence intervals ($n = 3\text{--}6$ animals per treatment). Data from individual animals within each treatment group are provided in the Supplementary Data file (Fig. S2).

subset of which were treated with diazepam following the second clonic seizure ($n = 4$ animals per group). Behavioral seizure activity was also evaluated throughout the recording period. As shown in representative EEG recordings of TETS-intoxicated mice (Figs. 2A and B), there was a good correlation between observations of behavioral seizure activity and electrographic seizure activity. Electrographic seizures were characterized by >5 s of noticeably increased spike amplitude and frequency relative to the baseline signal. In each case, an increased EMG signal coincident with EEG peaks was recorded (see Supplementary data, Fig. S1). Treatment with diazepam following the second clonic seizure stopped subsequent electrographic seizures for at least 1 h post-diazepam treatment (Fig. 2B). Diazepam also caused an initial slight and transient decrease in the amplitude of EEG peaks from baseline levels. Recordings from the other 3 animals in each treatment group are provided in the Supplementary data file (Fig. S1).

Post-exposure administration of diazepam does not prevent TETS-induced neuroinflammation

Our earlier study (Zolkowska et al., 2012) demonstrated that sublethal convulsive doses of TETS triggered time- and region-dependent neuroinflammatory responses as determined by GFAP and Iba-1 immunohistochemistry. To determine whether treatment with diazepam mitigated the neuroinflammation associated with TETS-induced seizures, brains were collected at 1, 2, 3, and 7 days post-TETS exposure from mice injected with TETS (0.15 mg/kg, ip) then rescued with diazepam (5 mg/kg, ip). Mice treated with the vehicle for TETS (10% DMSO in saline) followed 20 min later by injection with diazepam (5 mg/kg, ip) were used as controls for these studies. Similar to our earlier report

of sublethal exposures to TETS (Zolkowska et al., 2012), there were no gross pathological lesions or neurodegeneration in the cortex or hippocampus of mice rescued from TETS lethality by diazepam, as indicated by H&E staining (Fig. 3A) and Fluoro-Jade B staining (data not shown). However, the cortex and hippocampus of mice rescued from TETS-induced tonus and death by diazepam (TETS rescue mice) displayed a time-dependent increase in reactive astrogliosis relative to these same brain regions of mice treated only with diazepam (Fig. 3B). Calculation of the geometric mean ratio (GMR) of the area of GFAP immunoreactivity in the cortices and hippocampi of TETS rescue mice relative to that of control mice revealed a significant decrease in reactive astrogliosis at 1 day post-treatment but a significant increase in reactive astrogliosis at 2 and 3 days post-treatment (Fig. 3B). At 2 days post-treatment, a 10-fold increase in the GMR of the area of GFAP immunoreactivity was observed in the cortex, whereas a 7-fold increase was observed in the hippocampus. By 7 days post-exposure, there was no significant difference in the area of GFAP immunoreactivity between TETS rescue mice and control animals (Fig. 3B).

The area of Iba-1 immunoreactivity was assessed as a biomarker of microglial activation (Fig. 4). Calculation of the GMR of the area of Iba-1 immunoreactivity in the cortices and hippocampi of TETS rescue mice relative to that of control mice indicated that rescue by diazepam did not block TETS-induced microglial activation in these brain regions (Fig. 4C). Mice treated with TETS and diazepam exhibited a statistically significant increase (20%) in the area of Iba-1 immunoreactivity in the cortex and hippocampus at 1, 2, 3 and 7 days post-treatment, suggesting that microglia were recruited to these brain regions in TETS-intoxicated animals. Iba-1 immunopositive cells were classified as resting or activated microglial cells using morphological criteria (Matthews and Kruger,

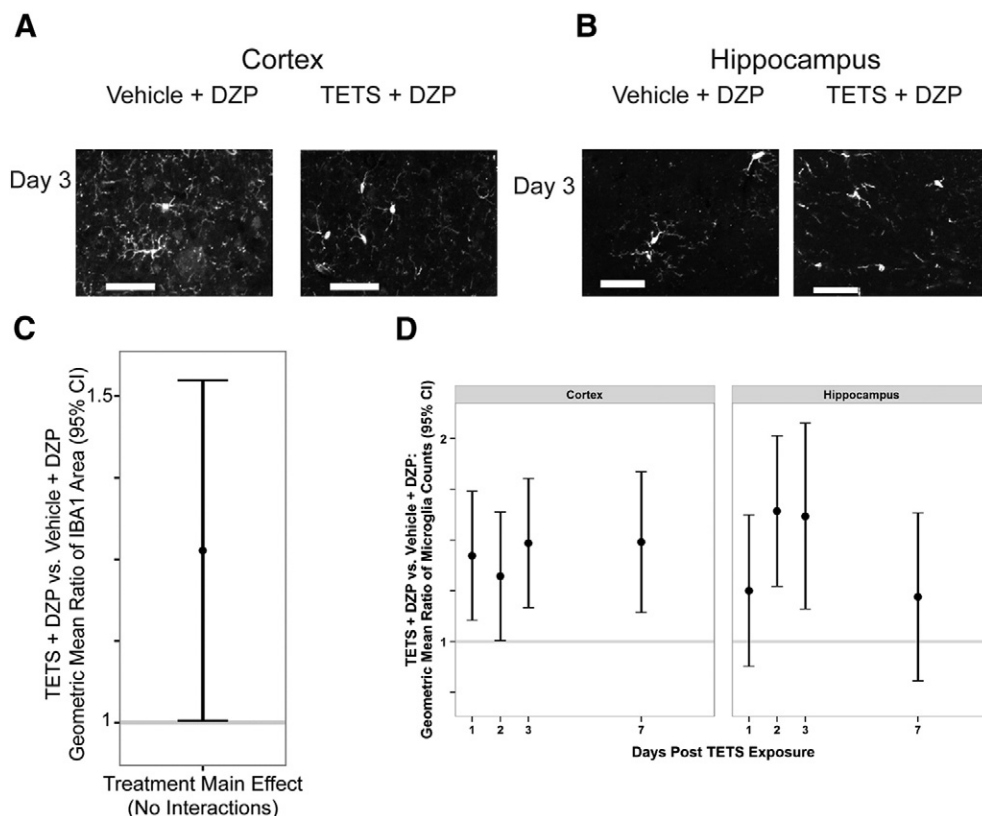


Fig. 4. Post-exposure administration of diazepam (DZP) does not prevent microglial activation in mice acutely intoxicated with TETS. Representative fluorescence photomicrographs of Iba-1 immunoreactivity 3 days post-TETS injection in the cortex (A) and hippocampus (B) of mice exposed to either vehicle or TETS (0.15 mg/kg, ip) followed by administration of diazepam (5 mg/kg, ip) ~20 min later. Bars = 50 μ m. Microglial activation was assessed at 1, 2, 3 and 7 days post-TETS injection with respect to: (C) the area of Iba-1 immunoreactivity per field and (D) the number of activated microglia per field as determined using morphological criteria. Dots represent the geometric mean ratio of TETS + diazepam versus vehicle + diazepam-treated animals; bars represent 95% confidence intervals ($n = 3$ –6 animals per treatment). Analyses of the area of Iba-1 immunoreactivity revealed no significant day- or region-specific interactions so the data were compressed to a single geometric mean that included all days and regions sampled. Data from individual animals within each treatment group are provided in the Supplementary Data file (Fig. S3).

1973). Consistent with results from the quantitative analyses of the area of Iba-1 immunoreactivity, the morphometric assessment of the number of activated microglial cells per unit area indicated significantly increased microglial activation in both the cortex and hippocampus of TETS intoxicated mice compared to controls (Fig. 4D). The temporal profile of microglial activation differed between brain regions, with significant increases observed in the cortex at 1, 2, 3 and 7 days post-TETS exposure and in the hippocampus at 2 and 3 days post-TETS exposure.

Pharmacologic inhibition of soluble epoxide hydrolase (sEH) differentially modulates TETS-induced reactive astrogliosis and microglial activation

Since inhibition of sEH has been shown to exert potent anti-inflammatory action (Iliff and Alkayed, 2009; Inceoglu et al., 2008; Wang et al., 2013), we quantified the effects of administering TUPS, a small molecule inhibitor of sEH, on TETS-induced seizures and neuroinflammation. Initial experiments focused on determining whether pharmacologic inhibition of sEH was sufficient to protect against TETS-induced tonus and death in the absence of diazepam. Pretreatment with TUPS (1 mg/kg, ip) 1 h prior to injection of a lethal dose of TETS (0.15 mg/kg, ip) had no effect on clonic seizures but decreased the number of mice that experienced tonic seizures and died (Figs. 5A and B), although the survival rate was not as high as observed in TETS-intoxicated mice treated with high dose diazepam following the second clonic seizure. Administration of TUPS to TETS-intoxicated mice immediately following the second clonic seizure did not prevent tonus and death in the absence of diazepam (Fig. 5C). When administered to TETS intoxicated mice 1 h after treatment with diazepam (5 mg/kg, ip), TUPS did not diminish the protective efficacy of diazepam against TETS-induced tonus and mortality (Fig. 5D).

To determine whether sEH inhibition would prevent TETS-induced neuroinflammation, GFAP and Iba-1 immunoreactivity were quantified

in the cortex and hippocampus of TETS-intoxicated mice rescued with diazepam and then treated with TUPS (1 mg/kg, ip) beginning 1 h after diazepam treatment, with repeated administration every 24 h until animals were euthanized. Control mice were similarly treated with TETS and diazepam but then administered the TUPS vehicle (PEG400) instead of the sEH. The GMR of the area of GFAP immunoreactivity in TETS intoxicated mice treated with both diazepam and TUPS relative to controls indicated no significant treatment effect in the cortex at 1, 2, 3 or 7 days post-TETS exposure (Fig. 6). In contrast, the GMR of the area of GFAP immunoreactivity was significantly increased in the hippocampus at 1, 2, 3 and 7 days post-TETS exposure (Fig. 6), indicating that treatment with the sEH significantly enhanced astrogliosis in this brain region.

The effects of TUPS on TETS-induced microglial activation also varied between the cortex and hippocampus. The GMR of the area of Iba-1 immunoreactivity in the cortex of TETS intoxicated mice treated with diazepam and TUPS relative to cortical Iba-1 immunoreactivity in control mice did not differ significantly from a ratio of 1 at any of the times examined post-TETS exposure (Fig. 7A), indicating no significant effect of TUPS on TETS-induced microglial cell recruitment in the cortex. However, the GMR of the area of Iba-1 immunoreactivity was significantly decreased in the hippocampus at 1, 3 and 7 days post-TETS exposure (Fig. 7A), indicating that inhibition of sEH significantly inhibited microglial recruitment in the hippocampus. Quantification of the number of activated microglia as identified using morphometric criteria further indicated that administration of TUPS significantly decreased microglial cell activation in the hippocampus, but not the cortex, at 1, 2, 3 and 7 days post-TETS exposure, as indicated by a GMR significantly <1 in the hippocampus at all post-exposure times analyzed (Fig. 7B).

Discussion

The present study supports and extends our previous characterization of TETS-induced seizures in adult male NIH Swiss mice (Zolkowska et al.,

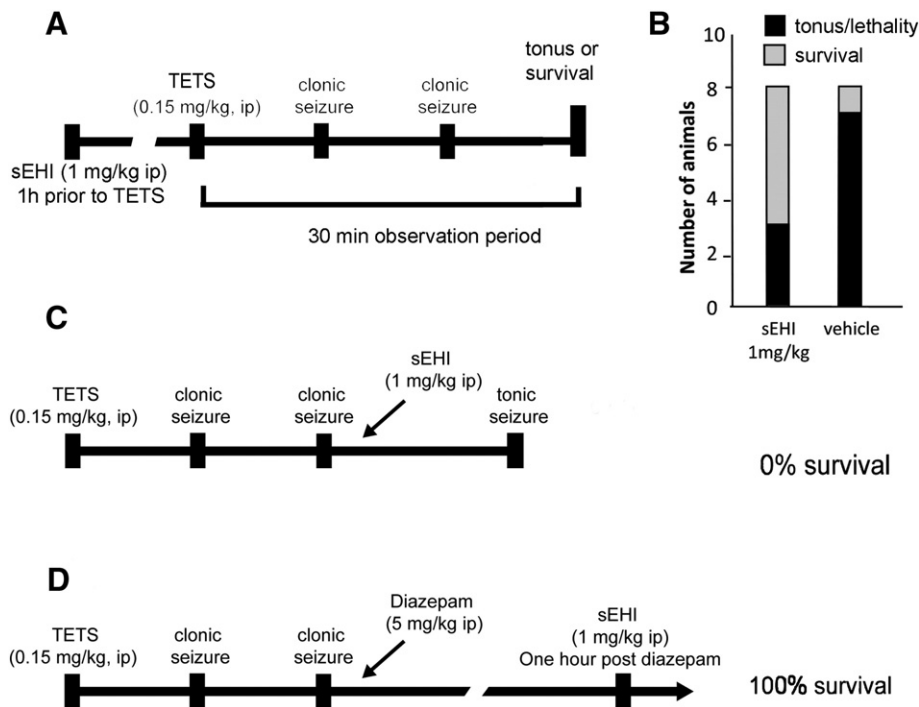


Fig. 5. Pretreatment but not post-exposure administration of sEH reduces TETS lethality. (A) Schematic representation of the experimental paradigm used to evaluate the prophylactic efficacy of the sEH TUPS. (B) Administration of TUPS (1 mg/kg, ip) 1 h prior to TETS protects against TETS-induced tonic seizures and mortality. (C) Schematic representation of the therapeutic experimental paradigm used to evaluate the efficacy of TUPS when administered post-TETS exposure. Inhibition of sEH following the second clonic seizure was ineffective in protecting mice against the TETS-induced tonic seizures and mortality. (D) Schematic representation of the experimental paradigm used to evaluate polytherapy with diazepam and TUPS. Administration of TUPS 1 h after injection of diazepam did not alter the rescue of TETS-intoxicated mice by diazepam (5 mg/kg ip) administered immediately following the second clonic seizure.

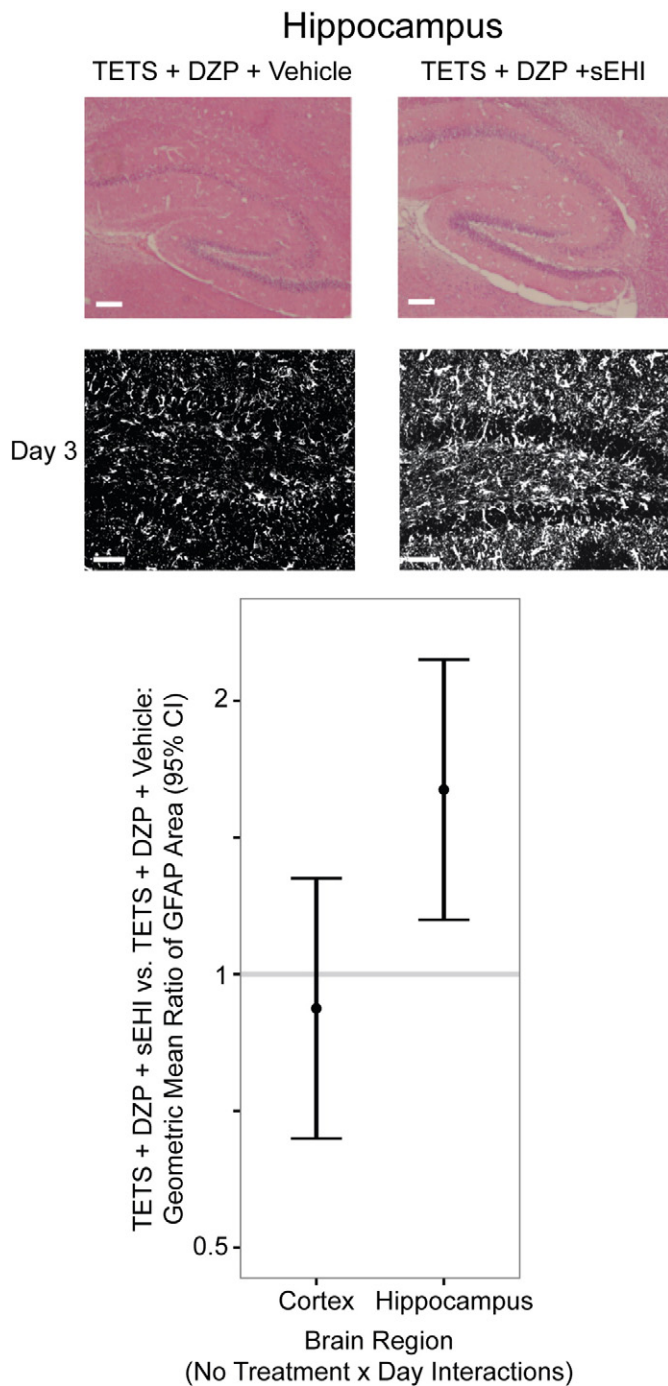


Fig. 6. Combinatorial post-exposure treatment with diazepam and the soluble epoxide hydrolase inhibitor (sEHI) TUPS modulates reactive astrogliosis in a region-dependent manner. To determine whether inhibition of sEH alters TETS-induced reactive astrogliosis, TETS-intoxicated mice were administered TUPS (1 mg/kg, ip) or an equal volume of vehicle (PEG400) 1 h following rescue with diazepam (5 mg/kg, ip) with additional sEHI injection every 24 h until animals were euthanized. (A) Representative photomicrographs of H&E staining (top) and GFAP immunoreactivity (bottom) in the hippocampus 3 days post-TETS injection of mice treated with diazepam followed by either vehicle or sEHI. Bars = 50 μ m. (B) The area of GFAP immunoreactivity per field was quantified as a measure of the number of GFAP-immunopositive cells. Combinatorial therapy had no significant effect on reactive astrogliosis in the cortex, but caused a significant, albeit modest, increase in the hippocampus. Dots represent the geometric mean ratio of TETS + diazepam + sEHI versus TETS + diazepam + vehicle-treated animals; bars represent 95% confidence intervals ($n = 3$ –6 animals per treatment). Data from individual animals within each treatment group are provided in the Supplementary Data file (Fig. S4).

2012). We now show that the behavioral seizures are accompanied by electrographic seizure activity, and that “rescue therapy” with a high dose of diazepam (5 mg/kg, ip) administered upon cessation of the

second clonic seizure (approximately 20 min post-TETS) effectively stops both behavioral and electrographic seizures for at least 1 h. This extends the therapeutic window of 3–5 min post-TETS for high dose diazepam rescue previously reported for adult male C57BL/6 mice intoxicated with a lethal dose of TETS (Shakarjian et al., 2012). We also observed that rescue therapy with diazepam prevented death in TETS-intoxicated mice, as evidenced by >99% survival at 24 h post-TETS exposure. These rescued animals remained healthy and did not exhibit behavioral seizures or weight loss during the period prior to euthanasia, which ranged from 1 to 7 days after TETS injection. Our observations are in contrast to an earlier report (Shakarjian et al., 2012) in which administration of high dose diazepam (5 mg/kg, ip) 3–5 min after TETS injection in C57BL/6 mice temporarily terminated behavioral seizure activity and prevented immediate lethality for about 2 h, but did not stop ongoing electrographic seizures and death at 4–8 h. In our studies thus far [(Zolkowska et al., 2012) and the present study], we have not observed continuous behavioral or electrographic SE. Possible reason(s) for the discrepancies between our studies of NIH Swiss mice and the earlier studies of C57BL/6 mice (Shakarjian et al., 2012) include: (1) strain differences in seizure susceptibility (Esplin et al., 1994; McKhann et al., 2003; McLin and Steward, 2006; McLin et al., 2006; Schauwecker, 2012, 2013); (2) strain differences in TETS or diazepam pharmacokinetics; and (3) differences in the total injected dose of TETS (0.15 mg/kg, ip in NIH Swiss mice versus 0.4 mg/kg, ip in C57BL/6 mice). If the latter is a contributing factor, it suggests that the therapeutic efficacy of benzodiazepines for treating humans poisoned with TETS may depend on the total body burden of TETS; high TETS exposures may be more difficult to treat.

A key finding of the present study is that even though high dose diazepam effectively stopped behavioral and electrographic seizures and prevented mortality in TETS-intoxicated mice, it did not mitigate the neuroinflammatory response to sublethal and lethal doses of TETS (Zolkowska et al., 2012), as evidenced by increased GFAP and Iba-1 immunoreactivities in both the hippocampus and cortex. The spatiotemporal profile of GFAP immunoreactivity was similar, but the magnitude of the response greater than, and the microglial response generally occurred earlier and persisted longer than those previously reported in TETS-intoxicated mice not treated with diazepam (Zolkowska et al., 2012). Seizures induced by GABA_AR antagonists are believed to involve generalized thalamo-cortical mechanisms and are distinguished from limbic-like seizures involving the hippocampus and other temporal lobe structures (Velisek, 2005). However, intense GABA_A receptor antagonist-induced seizures strongly recruit hippocampal structures, including the dentate gyrus and to a lesser extent CA3 and CA1 (Willoughby et al., 1995). Therefore, it is not surprising that astrocytic changes were observed in the hippocampus and cortex.

Neuroinflammation has been observed in a number of seizure models. Electroconvulsive seizures (Steward, 1994) and kindled seizures (Steward et al., 1992) as well as organophosphate nerve agent-induced seizures (Baille-Le Crom et al., 1995; Baille et al., 2005; Liu et al., 2012; Zimmer et al., 1997) and kainic acid-induced SE (Drexel et al., 2012; Ravizza et al., 2005; Rizzi et al., 2003) are associated with reactive astrogliosis. Prolonged seizures have also been shown to rapidly activate microglia (Baille et al., 2005; Du et al., 1993; Rizzi et al., 2003; Zimmer et al., 1997). Based on data collected in these seizure models, it is postulated that the neuroinflammatory response is a consequence of neurodegeneration, and that the extent or severity of neurodegeneration and neuroinflammation is directly correlated to the duration and/or intensity of seizure activity (Drexel et al., 2012; McDonough et al., 1995, 1998; Shih et al., 2003). However, in our previous study of sublethal exposures to TETS in the absence of diazepam (Zolkowska et al., 2012) and in this study of TETS rescue mice, reactive astrogliosis and microglial activation were observed in the absence of overt neuronal injury and cell death. Moreover, the persistence and/or magnitude of the neuroinflammatory response were greater in the TETS rescue mice relative to that observed in the TETS intoxicated mice not treated with diazepam (Zolkowska et al., 2012) even though the behavioral seizures in the TETS rescue

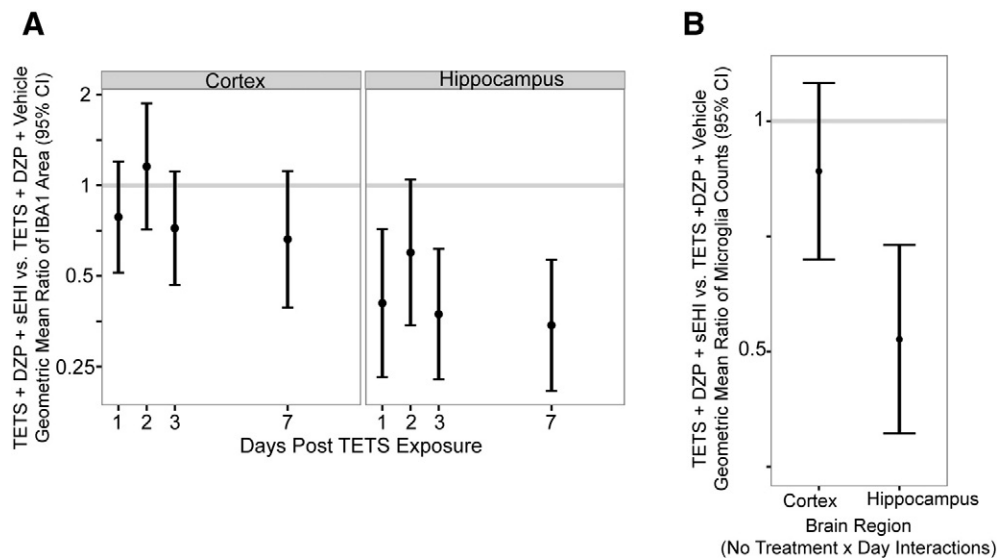


Fig. 7. Post-exposure combinatorial therapy with diazepam (DZP) and the sEHI TUPS decreases microglia activation. Mice were exposed to TETS (0.15 mg/kg, ip) followed ~20 min later with diazepam (5 mg/kg, ip) and 1 h later with either vehicle or TUPS (1 mg/kg, ip). Microglia activation was assessed in these 2 brain regions at 1, 2, 3 and 7 days post-TETS intoxication by quantifying: (A) the area of Iba-1 immunoreactivity per field and (B) the number of activated microglia per field as determined using morphological criteria. Dots represent the geometric mean ratio of TETS + diazepam + TUPS versus TETS + diazepam + vehicle; bars represent 95% confidence intervals ($n = 3$ –6 animals per treatment). Data from individual animals within each treatment group are provided in the Supplementary Data file (Fig. S5).

mice were similar to or less severe than those observed in the TETS intoxicated mice not treated with diazepam (Zolkowska et al., 2012). Collectively, these findings suggest that, contrary to current dogma, neuroinflammation is not a consequence of neurodegeneration, and it is not correlated with the duration of seizure activity. These observations also suggest that TETS-induced neuroinflammation is mediated by pathogenic mechanisms other than those that underlie TETS-induced seizures, a possibility reinforced by the observation that diazepam, a GABA_AR positive allosteric modulator, stops TETS-induced seizures, but not TETS-induced neuroinflammation.

The observation that diazepam prevented death but did not mitigate the neuroinflammatory response in TETS intoxicated mice suggests that the neuroinflammation triggered by TETS likely does not contribute to lethality. However, it does raise the possibility that once triggered, neuroinflammation develops independently and perhaps contributes to chemical-induced epilepsy or spontaneous recurrent seizures (Clasadonte et al., 2013; Devinsky et al., 2013; Hunt et al., 2013; Pineda et al., 2013; Rossi et al., 2013). While we did not see any behavioral evidence of spontaneous recurrent seizures in mice that survived a lethal dose of TETS, this possibility cannot be ruled out in the absence of continuous EEG monitoring over longer periods of time post-exposure. If true, this possibility reinforces the rationale that combining an antiseizure agent and a specific anti-inflammatory treatment may be a more effective therapeutic approach than using an antiseizure agent alone. To test this possibility, we employed a small molecule inhibitor of the enzyme sEH. This cytosolic enzyme regulates the levels of the epoxy fatty acids, which are bioactive lipid metabolites with anti-inflammatory properties (Iliff and Alkayed, 2009; Inceoglu et al., 2007). The anti-inflammatory effects of epoxy fatty acids and sEHIs have been demonstrated in other models of neuroinflammation, including a mouse model of scrapie (Poli et al., 2013), a mouse model of neurodegeneration following cardiac arrest (Wang et al., 2013), and an in vitro model of amyloid peptide-induced neurotoxicity (Sarkar et al., 2011). We have previously shown that epoxy fatty acids and sEHIs protect against seizures induced by PTZ, and this anticonvulsant activity seems to be mediated, in part, by augmentation of GABAergic signaling (Inceoglu et al., 2013). These observations suggested that sEHIs may protect against both the convulsant and neuroinflammatory actions of TETS. We found that pharmacologic inhibition of sEH with TUPS increased survival when administered prior to TETS; however, in contrast to observations of PTZ-induced

seizures (Inceoglu et al., 2013), TUPS did not delay the onset of TETS-induced seizures (Inceoglu, unpublished observations). Moreover, TUPS did not stop TETS-induced seizures when administered following the second clonic seizure. These findings are consistent with our earlier observations that the toxic profile of TETS varies from that of picrotoxin and PTZ (Zolkowska et al., 2012), and reinforces the hypothesis that TETS may have mechanisms of toxicity in addition to GABA_AR antagonism.

Pharmacologic inhibition of sEH did, however, modulate the neuroinflammatory response in TETS-intoxicated mice rescued by diazepam, increasing reactive astrogliosis and decreasing microglial activation. Interestingly, these sEH-related changes in neuroinflammation were observed in the hippocampus but not in the cortex. The reason for the region-dependent effects is not known, nor is it known why sEH exerts differential effects on astrocytes versus microglial cells. The sEH enzyme is localized to astrocytes (Marowsky et al., 2009; Rawal et al., 2009; Sura et al., 2008) where it is thought to regulate levels of epoxy fatty acids in the brain (Marowsky et al., 2009; Terashvili et al., 2012), but there is very little information regarding the expression or function of sEH in microglial cells. Similarly, there is little known about the effect of sEH inhibition on the activation status of either astrocytes or microglial cells with the exception of a recent report that pharmacologic inhibition of sEH alters the transcriptional profile of activated microglia to selectively induce anti-inflammatory and neuroprotective cytokine expression (Wang et al., 2013).

Perhaps the more important question raised by these data is the functional significance of increased reactive astrogliosis and decreased microglial activation with sEH treatment. The impact of these changes is difficult to predict since there is evidence that microglial activation can be detrimental in some circumstances and beneficial in others (Harry, 2013). Similarly, there is evidence that activated astrocytes can cause or exacerbate neuronal injury (Davalos et al., 2005; Liu et al., 2011) or play a neuroprotective role following brain and spinal cord injury via secretion of anti-inflammatory factors that suppress the activation of microglia (Faulkner et al., 2004; Tichauer et al., 2007; Zhao et al., 2003). In a model of organophosphate nerve agent-induced seizures, neuronal cell death is reduced when there is increased astrocyte activation and is increased when the number of activated astrocytes decreases (Collombet et al., 2005, 2007). The protective role of activated astrocytes in this model is thought to be mediated by the release of neuroprotective cytokines (Collombet, 2011). Functional studies are

needed to determine whether the modulation of neuroinflammation by sEH inhibition is of therapeutic value, but based on observations in the organophosphate nerve agent-induced seizure model (Collombet, 2011), it seems plausible that the enhanced reactive astrogliosis and decreased microglial activation observed following sEH treatment is a beneficial outcome.

In summary, our findings suggest that TETS may have mechanisms of toxicity in addition to GABA_AR antagonism that lead to neuroinflammation. Diazepam, a GABA_AR positive allosteric modulator, effectively protected against seizures and lethality when administered up to 20 min after TETS injection; however, diazepam was ineffective in mitigating TETS-induced neuroinflammation. In the treatment of TETS intoxication, administering an agent that targets relevant anti-inflammatory mechanisms as an adjunct to a traditional antiseizure agent may lead to better therapeutic outcomes.

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Conflict of interest statement

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.taap.2014.10.001>.

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