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Characterization of Seizures Induced by Acute and Repeated Exposure to Tetramethylenedisulfotetramine

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Abbreviations: GFAP, glial fibrillary acidic protein; Iba1, ionized calcium binding adaptor molecule; TETS, tetramethylenedisulfotetramine; PTZ, pentylenetetrazol

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ABSTRACT

Tetramethylenedisulfotetramine (tetramine; TETS) is a potent convulsant poison that is considered to be a chemical threat agent. To provide a basis for the investigation of antidotes for TETS-induced seizures, we characterized the convulsant activity of TETS in mice and rats when administered by the intraperitoneal, intravenous, oral and intraventricular routes as a single acute dose and with repeated sublethal doses. In mice, parenteral and oral TETS caused immobility, myoclonic body jerks, clonic seizures of the forelimbs and/or hindlimbs, tonic seizures and death. The CD_{50} values for clonic and tonic seizures following oral administration were 0.11 and 0.22 mg/kg, respectively. Intraventricular administration of TETS (5–100 μg) in rats also caused clonic-tonic seizures and death. In mice, repeated sublethal doses of TETS at intervals of 2, 24, and 48 h failed to result in the development of persistent enhanced seizure responsivity ("kindling") as was observed with repeated pentylenetetrazol treatment. In mice, sublethal doses of TETS that produced clonic seizures did not cause observable structural brain damage as assessed with routine histology and Fluoro-Jade B staining 7 days after treatment. However, 1 to 3 days following a single convulsant dose of TETS the expression of glial fibrillary acidic protein, an astrocyte marker, and ionized calcium binding adaptor molecule 1, a microglia marker, were markedly increased in cortex and hippocampus. Although TETS doses that are compatible with survival are not associated with overt evidence of cellular injury or neurodegeneration, there is transient reactive astrocytosis and microglial activation, indicating that brain inflammatory responses are provoked.

Introduction

Tetramethylenedisulfotetramine (tetramine; TETS; Fig. 1), a heteroatom-substituted tricyclodecane, was originally synthesized in 1933 as a condensation product of sulfamide and formaldehyde (Wood and Battye, 1933). It was subsequently found to be a highly toxic convulsant poison (Hecht and Henecka, 1949), with an LD_{50} of 0.1–0.2 mg/kg when administered parenterally to mice or rats (Haskell and Voss, 1957; Voss et al., 1961; Casida et al., 1976). TETS has been used as a rodenticide, although it is now banned in most parts of the world (Whitlow et al., 2005). Nevertheless, TETS continues to be used illicitly in some regions and human poisonings still occur (Wu and Sun, 2004). Moreover, TETS in considered a chemical threat agent and there is concern that it could result in mass casualties if released accidentally or as an act of terrorism (Jett and Yeung, 2010). Within 30 min to a few hours after exposure, humans poisoned with TETS exhibit generalized clonic-tonic convulsions that may be followed by coma and death (Barrueto et al., 2003; Croddy, 2004). The lethal dose in humans is believed to be similar to that in rodents (0.1 mg/kg; Guan et al., 1993). TETS has no major effects on peripheral neuromuscular or autonomic transmission, and its toxicity appears to be due exclusively to actions on the central nervous system (Haskell and Voss, 1957). Despite its extreme central nervous system toxicity, even prolonged exposure of rats to sublethal doses of TETS is not associated with histopathological changes in organs (Haskell and Voss, 1957).

The convulsant action of TETS has been attributed to blockade of $GABA_A$ receptor-mediated neurotransmission. In the superior cervical ganglion, TETS at concentrations greater than approximately 13 μM produced a reversible block of GABA-evoked currents in a manner suggesting noncompetitive inhibition with respect to GABA (Bowery et al., 1975). Similar results were obtained in studies of GABA responses at the crab neuromuscular junction (Large, 1975) and of *Limulus* neurons (Roberts et al., 1981). In vivo recordings in rats demonstrated that intravenous TETS inhibits

responses to iontophoretically applied GABA and produces prolonged epileptiform spiking in the cortical electroencephalogram (Dray, 1975).

In accordance with an effect on $GABA_A$ receptors, TETS was found to displace specific [^{35}S]*t*-butylbicyclophosphorothionate ([^{35}S]TBPS) binding to rat brain membranes with an IC_{50} in the range of 1 μM (Squires et al., 1983; Esser et al., 1991; Ratra et al., 2001). This is markedly more potent than the convulsant pentylenetetrazol, but not substantially more potent than picrotoxin or its active component picrotoxinin (Squires et al., 1983; Cole and Casida, 1986; Ratra et al., 2001). Moreover, TETS and picrotoxin have similar potencies for inhibition of $GABA_A$ receptor mediated GABA-activated $^{36}Cl^-$ uptake by membrane vesicles from rat cerebral cortex (IC_{50} for TETS is 1.75 μM; Obata et al., 1988). Although TETS and picrotoxin have similar potencies on $GABA_A$ receptors, TETS is 30–100-fold more potent as a convulsant and lethal toxin in the mouse than picrotoxin (Duka et al., 1979; Lamanna and Hart, 1968; Shandra et al., 1996; Vogel, 2008). The basis for the high in vivo convulsant potency of TETS is not well understood. Since TETS may represent a novel type of convulsant for which there is no specific antidote, in the present study we sought to characterize its proconvulsant actions in mice and rats with single and repeated exposures, and we also determined the propensity of sublethal convulsant doses of TETS to cause brain inflammation and injury. Our results define various animal models for the evaluation of therapeutic strategies and provide information on the time window available for the administration of postexposure treatments.

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Materials and Methods

Chemical Synthesis

Chemicals. Paraformaldehyde, sulfamide, hydrochloric acid, acetone, and hexane were obtained from Fisher Scientific (Hanover Park, IL). All chemicals were the highest purity available unless otherwise specified.

Analytical Procedures. ^{1}H and ^{13}C NMR spectra were obtained with a mercury-300 spectrometer for compounds dissolved in acetone-d6. ^{1}H NMR data are reported as chemical shift, multiplicity, coupling constants (Hz), and number of protons. ^{13}C NMR has chemical shifts in ppm.

The purity and mass spectra of compounds were determined by gas chromatography-mass spectrometry (GC-MS) with a Hewlett Packard Agilent 6890 gas chromatograph, controlled by ChemStation, fitted with a 100% methylsilicone Durabond capillary column DB1 (30 m, 0.25 mm i.d., 0.25-μm film thickness) with a Hewlett Packard 5873 mass spectrometer at 70 eV as detector. Helium was the carrier gas. The injector port was 250 °C. The temperature program (100 ºC, 1 min; 15 ºC/min to 350 ºC; 350 ºC for 2 min) gave retention times (min) for TETS of 8.7 and for hexamethylenetrisulfohexamine (HEXS) of 15.8. The analyte was dissolved in 50% acetonitrile/water (20 μg/ml). Detection was by ion monitoring for ions at m/z 212 and 240, after a 3 min solvent delay at an acquisition rate of 20 Hz.

Synthetic procedures. TETS was prepared by a modification of the procedure of Esser et al. (1991). In brief, 126 mg of paraformaldehyde was dissolved in 5 ml of concentrated HCl and 80 mg of sulfamide was added. The reaction was capped and allowed to stir overnight at room temperature. The solution was then filtered and the fine crystals were washed with ethanol before dissolving in acetone. TETS was recrystallized by the slow addition of hexane until crystals began to form. The crystals were recovered by filtration and redissolved in a minimal amount of acetone, which was then removed by vacuum. The final product consisted of clear cubic crystals. The final yield was 56%; ^{1}H

NMR δ5.63 ppm (s, 8H); ^{13}C NMR δ 71.42; MS m/z 240 (56, M+), 212 (l00), 149 (5), 132 (12), 121 (10), 92 (13), 76 (6). Two batches of TETS were used. In the first case, the final recrystallization was not performed. For this batch, referred to as the "hydrated" batch, the total ion chromatogram from GC-MS analysis indicated that 12% of the crystalline material is HEXS (Fig. 1). This compound is weak or inactive as a $GABA_A$ receptor antagonist as assessed by displacement of [^{35}S]TBPS binding to mouse brain membranes (Esser et al., 1991). Additionally, this material contained water so that the overall purity was 70%. The second batch, referred to as the "anhydrous" batch, contained 2% HEXS. Doses are reported in terms of the mass of the mixture and are not corrected for the HEXS impurity or for hydration.

Animal Seizure Assessment

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 University of California, Davis Institutional Animal Care and Use Committee in strict compliance with the Guide for the Care and Use of Laboratory Animals of the National Research Council (National Academy Press, Washington, DC; http://www.nap.edu/readingroom/books/labrats/). Male NIH Swiss mice (22–30g) were obtained from the Animal Resource Program, Center for Cancer Research, National Cancer Institute (Bethesda, MD) and were housed four per cage. Male Sprague-Dawley rats (300 to 400 g) were obtained from Taconic Farms (Germantown, NY) and were housed individually. All animals were kept in a vivarium under controlled laboratory conditions (temperature, 22–26 ºC; humidity, 40–50%) with an artificial 12-h light/dark cycle and free access to food and water. Animals were allowed to acclimate to the vivarium for ≥5 days. The experiments were performed during the light phase of the light/dark cycle after a ≥30-min period of acclimation to the experimental room.

TETS and Picrotoxin Solutions. Solutions of TETS were made in 50% dimethyl sulfoxide (DMSO) in sterile saline (hydrated batch) or in 100% DMSO (anhydrous batch) at a concentration of 10 mg/ml. Further dilutions of the hydrated batch were made with 10% DMSO in 0.9% sterile saline. The anhydrous batch material did not remain in solution with further dilution in 10% DMSO so dilutions to 1 mg/ml were made in 50% DMSO in saline; 10% DMSO in saline was used for more dilute solutions. Picrotoxin (Sigma-Aldrich, St. Louis, MO) was prepared in distilled water at a concentration of 0.4 mg/ml for intraperitoneal administration and 1 mg/ml for intravenous administration. TETS (anhydrous batch) and picrotoxin were prepared in 100% DMSO for intraventricular administration.

Intraperitoneal Administration. TETS was administered intraperitoneally to mice at doses of 0.1 to 0.6 mg/kg and rats at a dose of 0.2 mg/kg to determine the incidence and time of onset of various seizure signs. Picrotoxin was administered at a dose of 4 mg/kg. The volumes used for intraperitoneal injection were 10 ml/kg in the case of mice and 1 ml/kg in the case of rats. Animals were observed for a period of 1 h following injection. During this period, the occurrence and time of onset of myoclonic jerks, clonus, tonic extension and death was recorded. In experiments with mice, dose-response curves were constructed allowing a determination of CD_{50} values, defined as the dose in mg/kg estimated to produce the specific seizure endpoint or mortality in 50% of animals.

Intravenous Administration. The threshold for induction of various seizure signs was determined by intravenous infusion of TETS (0.02 mg/ml solution) into the tail vein of unrestricted freely moving mice at a rate of 0.1 ml/min with an infusion pump (Harvard Apparatus, South Natick, MA). The time to onset of the following signs, which occurred in sequence during infusion, were recorded: (1) first myoclonic twitch (sudden jerk of the whole body); (2) clonus (generalized clonus entire body and repeated clonic jerks of the forelimbs and hindlimbs with loss of the righting reflex); and (3) tonic hindlimb extension (hindlimbs extended in line with the body). The threshold dose (in

mg/kg) for each endpoint was calculated using the formula (infusion duration [s] × infusion rate [ml/min] × drug concentration [mg/ml] × 1000)/(60 s × weight of mouse [g]). In an experiment to compare the rate of onset of seizure signs induced by TETS and picrotoxin (1 mg/ml solution), a faster infusion rate (0.05 ml/min) was used.

Oral Administration. TETS was administered orally to mice through a syringe fitted with a feeding needle (20 G) with a rounded tip in a volume equaling 10 ml/kg. The occurrence of seizure signs was monitored as in the case of intraperitoneal administration.

Intraventricular Administration. Rats for intraventricular infusion studies had a 26-gauge guide cannula (Plastics One, Roanoke, VA) assembly implanted under ketamine (60–75 mg/kg, i.p.)/dexmedetomidine HCl (0.25–0.5 mg/kg) (Dexdomitor; Pfizer Animal Health, New York, NY) anesthesia at stereotaxic coordinates with respect to bregma of anteroposterior, –0.90 mm; mediolateral, 1.4 mm; and dorsoventral, –3.0 mm. The anesthesia was reversed with atipamezole HCl (1 mg/kg, i.p.; Antisedan, Pfizer). Animals were allowed 7 days to recover. At the time of infusion, a 33-gauge infusion cannula (Plastics One) was inserted through the guide cannula so that the tip extended 1 mm beyond the end of the guide cannula to a dorsoventral depth of –4.0 mm within the right lateral ventricle. Toxins solutions (TETS and picrotoxin) (10 mg/ml) in 100% DMSO were infused at a rate of 20 μl/min and animals were observed for a period of 1 h. The incidence and time of onset of myoclonic jerks, clonus and tonic extension, and lethality were recorded. Control animals were infused with 100% DMSO. Correct placement of the intraventricular cannula was verified in each animal by injection of a green food color solution. Animals that had not expired were deeply anesthetized and decapitated. The brains were visually examined. The results presented only include animals in which the correct catheter placement was verified.

Repeated Sublethal TETS Dosing and Comparison with Repeated Pentylenetetrazol (PTZ). To assess the response to repeated sublethal dosing with TETS, a low dose of TETS (0.02

and 0.05 mg/kg) was administered to mice that caused only a minimal behavioral response in naïve animals. We sought to determine if repeated dosing caused an augmentation of the seizure response. The interval between doses was 2 h, 24 h (daily) and 48 h (alternate day). For comparison, we treated mice with a minimally convulsant dose of pentylenetetrazol (PTZ; Sigma-Aldrich; 35 mg/kg, i.p. in sterile water) on a 48 h dose schedule, which led to permanent augmentation of the seizure response ("kindling") (Corda et al., 1991). Immediately after dosing, the animals were observed for 60 min in the case of TETS or 30 min in the case of PTZ for the occurrence of seizure signs, which were rated on the basis of a modified Racine scoring system (Racine, 1972), in which points were assessed as follows: 0 = no response; 1 = immobility; 2 = brief myoclonic body jerks; 3 = clonic seizures (repetitive clonic jerking of forelimbs or forelimbs and hindlimbs bilaterally that persist for at least 5 s). A composite score was calculated as the sum of the individual point values with a maximum score of 6. Animals exhibiting full-blown clonus invariably exhibited immobility and myoclonus and therefore achieved the maximal score. In one experiment, we administered a "challenge" dose of PTZ (30 or 35 mg/kg, i.p.) to animals that had received repeated vehicle (sterile water or 10% DMSO in 0.9% sterile saline), PTZ or TETS to assess whether there was augmented seizure susceptibility.

Analysis of Data in Animal Seizure Tests. In dose-response experiments, the response to TETS is expressed as the percentage of animals exhibiting specific seizure signs (clonic seizures, tonic seizures) or mortality. CD_{50} values representing the estimated doses causing the seizure sign or mortality in 50% of animals with their 95% confidence limits (95% CL; given in brackets) were determined by log-probit analysis using the Litchfield and Wilcoxon method (PHARM/PCS Version 4.2; Microcomputer Specialists, Philadelphia, PA). Logistic curves to the dose-response data were fitted by eye with slopes of 6.2–7.0. Latencies and the threshold doses for the induction of seizures by intravenous infusion of TETS were expressed as the mean ± S.E.M.

Histology

TETS Treatment. Mice were treated with single intraperitoneal doses of TETS (0.05–0.20; hydrated batch). The animals were allowed to survive for 24 h or 2, 3 or 7 days. In some preliminary experiments, brains were collected 4 h and 24 h after TETS (0.13, 0.15 and 0.20 mg/kg; hydrated batch).

Tissue Harvesting and Preparation. Animals were deeply anesthetized with 4% isoflurane in oxygen and then perfused transcardially with cold PBS followed by cold 4% paraformaldehyde solution in phosphate-buffered saline (PBS; pH 7.2). The fixed brains were removed and post-fixed with 4% paraformaldehyde at 4 °C for 24 h, and then placed in 30% sucrose in PBS and stored at 4 °C. Fixed brains were frozen in O.C.T. Compound (Sakura Finetek, Torrance, CA) on dry ice and then sagitally sectioned on a cryostat, generating sections of 10-µm thickness. Sections were stored at –80 ºC until further use.

Histological Assessment of Tissue Integrity. Sections were stained with the nuclear stain 4',6-diamidino-2-phenylindole (DAPI) by applying Vectashield mounting medium supplemented with DAPI (1.5 µg/ml; Vector Laboratories, Burlingame, CA) directly to tissue slices; stained sections were imaged by fluorescence microscopy. Adjacent sections were stained with hematoxylin and eosin (H&E), by sequential processing in 95% ethanol, Accustain hematoxylin (Sigma-Aldrich), water, 0.1% sodium bicarbonate, 95% ethanol, and Accustain eosin Y (Sigma-Aldrich). Sections were then dehydrated in 95% ethanol, 100% ethanol, and xylene before mounting with Permount adhesive (Fisher Scientific).

Analysis of Neurodegeneration. Sections adjacent to those stained with DAPI and H&E were stained with Fluoro-Jade B as previously described (Li et al., 2011). As a positive control, paraformaldehyde-fixed sections from the brain of a rat treated with diisopropyl fluorophosphate (9 mg/kg, i.p.) were processed concurrently with sections from TETS-treated mouse brains.

Immunohistochemical Analysis of Glial Activation. Glial fibrillary acidic protein (GFAP) immunoreactivity in astrocytes and ionized calcium binding adaptor molecule 1 (Iba1) immunoreactivity in microglia were examined in brain sections adjacent to those used for histological assessment. Nonspecific binding was blocked by incubating brain sections in blocking buffer (PBS supplemented with 10% normal goat serum, 1% bovine serum albumin, and 0.03% Triton X-100) for 1 h. Sections were then incubated overnight at 4 ºC in rabbit anti-GFAP (1:500 dilution; DakoCytomation, Glostrup, Denmark) or rabbit anti-Iba1 (1:1000 dilution; Wako Chemicals, Richmond, VA) diluted in blocking buffer. Sections were washed 3 times with PBS and subsequently incubated for 1 h at room temperature in goat anti-rabbit Alexa Fluor 555 (1:2000; Molecular Probes, Eugene, OR) or goat anti-rabbit Alexa Fluor 488 (1:1000; Molecular Probes), diluted in PBS with 0.03% Triton. Sections were washed three times in PBS and mounted with Vectashield Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA). Negative control sections were treated identically except that the primary antibodies were omitted.

Images were captured using an Olympus spinning disk confocal microscope, and optical sections were collected at 1-µm z-steps. To quantify GFAP and Iba1 average fluorescence intensity and area, fluorescence falling within a predetermined threshold was binarized and quantified with MetaMorph imaging software (Molecular Devices, Sunnyvale, CA). In each section, images were captured of the hippocampus, cortex, and olfactory bulb and quantified; 2–3 sections were analyzed per animal.

To assess microglial activation, an individual unaware of the treatment examined slides under a fluorescent microscope and counted activated microglia in the cortex and hippocampus of three successive sections. Activated microglia were distinguished from resting microglia by enlarged (and sometimes elongated) cell bodies with fewer ramifications and short hypertrophic processes (Matthews and Kruger, 1973). Phagocytic microglia were rarely seen, and were not counted.

Analysis of Histological Data. In each animal, multiple coded sections were analyzed by an operator who was blind to the treatment and the numerical values were averaged; mean ± S.E.M. values presented in the bar graphs were calculated from these averages. Statistical significance of paired comparisons were made using Student's *t*-test whereas multiple comparisons were made using one-way ANOVA with Newman-Keuls post hoc analysis.

Results

Characterization of Acute TETS-induced Seizures and Comparison with Picrotoxin

Intraperitoneal Administration. In mice, intraperitoneal TETS caused a sequence of immobility, myoclonic body jerks, clonic seizures of the forelimbs and/or hindlimbs, tonic seizures (falling on the side followed by forelimb tonic contraction and hindlimb tonic extension), and death. At low doses, only the initial signs (immobility and myclonic body jerks) were observed. Higher doses produced clonic and tonic seizures, which was nearly always associated with lethality. Animals that failed to exhibit tonic seizures survived without apparent long-term impairment. As the dose of TETS (hydrated batch) was increased within the range of 0.02 to 0.6 mg/kg, there was a dose-dependent increase in the incidence of the seizure signs (Fig. 2). In animals that exhibited clonic seizures without progressing to tonic seizures, clonic seizure clusters occurred repetitively (1–5 clusters each lasting between 9 s and 58 s) during the 1 h observation period. The CD_{50} value for induction of clonic seizures was 0.14 [95% CL: 0.13–0.15], for tonic seizures was 0.25 [95% CL: 0.19–0.33], and for mortality was 0.28 [95% CL: 0.21–0.37]. For doses that resulted in clonic seizure but were not lethal during the 1 h observation, the total time spent in clonic seizures was largely independent of dose (78 ± 20 s for 0.2 mg/kg and 79 ± 14 s for 0.4 mg/kg). The time to onset of clonic seizures was dose-dependent (Fig. 3): at low intraperitoneal doses, the onset of clonic seizures was delayed (mean value, 54 min) whereas with higher doses, the onset to clonic seizures occurred more rapidly (~2 min). A similar experiment with intraperitoneal administration of anhydrous batch TETS provided a CD_{50} for tonic seizures and mortality of 0.12 [95% CL: 0.10–0.15], indicating that the anhydrous batch is approximately twice as potent as the hydrated batch.

In 6 rats, intraperitoneal injection of TETS at a dose of 0.2 mg/kg resulted in immobility, followed by myclonic jerks and then forelimb clonus followed by forelimb tonus; 1 animal exhibited

generalized clonus and no tonus. The time to onset of clonus was 106 ± 16 s. Four of 6 animals died during the 1 h observation period.

The convulsant mechanism of TETS is thought to be similar to that of picrotoxin. To provide a comparison, 4 mice were treated with intraperitoneal picrotoxin at a dose of 4 mg/kg that caused clonic seizures in all animals. The progression of seizure signs was similar to that obtained with TETS. The mean times to onset of immobility, myoclonic jerks and clonic seizures were 206 ± 6 s, 543 ± 63 s, and 860 ± 60 s, respectively. None of the animals exhibited tonic extension at the dose used. For doses of TETS within the range of 0.15–0.20 mg/kg (hydrated batch) (Fig. 3) and 0.10 mg/kg (anhydrous batch) (data not shown) that caused clonic seizures in most animals and few or no tonic seizures and mortality, the onset time was comparable to that obtained with picrotoxin. This indicates that there is a similar latency to the onset of clonic seizures for equieffective doses of picrotoxin and TETS.

Intravenous Infusion. Intravenous infusion of TETS in mice resulted in a similar sequence of seizure signs as for intraperitoneal administration except that an immobility phase was not readily apparent. Initial intravenous delivery experiments assessed the speed of onset of seizure signs with TETS solution (hydrated batch, 0.02 mg/ml) using an infusion rate of 0.5 ml/min. For comparison, a group of mice were infused with picrotoxin (1 mg/ml) at the same rate. In 6 animals receiving TETS (mean body weight, 24.7 ± 0.7 g), the time of onset of seizure signs were as follows: myclonic jerks, 39.3 ± 0.9 s; clonic seizures, 41.7 ± 0.8 s; and tonic extension, 71.8 ± 7.4 s. In 6 animals receiving picrotoxin (mean body weight 25.3 ± 0.7 g), the corresponding values were as follow: myoclonic jerks, 44.7 ± 1.1 s; clonic seizures, 47.0 ± 1.3 s; tonic extension, 74.5 ± 1.5 s. Given the slow onset of seizure signs with these agents, to determine the threshold value a separate experiment was conducted with a slower infusion rate (0.1 ml/min). Mean threshold values for myoclonic jerks, clonic seizures and tonic extension are presented in Fig. 4.

Oral Administration. After oral administration, TETS (anyhydrous batch) produced a similar sequence of seizure signs as with intraperitoneal administration but was less potent (accounting for the greater purity of the test material). Dose response curves are shown in Fig. 5. The CD_{50} values for induction of clonic seizures, tonic seizures and mortality were 0.11 mg/kg [95% CL: 0.10–0.13], 0.22 mg/kg [95% CL: 0.19–0.26], and 0.22 mg/kg [95% CL: 0.19–0.26], respectively. As for intraperitoneal administration, the time to the onset of seizures diminished as the oral dose increased (Fig. 6).

Intraventricular Administration. Intraventricular administration of TETS (anhydrous batch) into the right lateral ventricle of rats resulted in the dose-dependent occurrence of agitation (with increased respiratory rate and increased locomotor activity) followed by myoclonic jerks, wild running, clonic seizures, tonic seizures and lethality (Table 1). With a 5 μg dose, only 1 of 5 animals exhibited clonic seizures and all animals survived; with 50 and 100 μg doses, all animals exhibited clonic seizures; and with a 100 μg dose all animals died (within 13 min after the onset of clonus). The mean time to onset of clonic seizures diminished with increasing dose as shown in Fig. 7.

For comparison, we examined picrotoxin in 2 animals each at doses of 5, 10, 25, and 50 μg. As shown in Table 1 and Fig. 7, picrotoxin caused a comparable dose-dependent induction of seizure signs and lethality. Since picrotoxin is an equimolar mixture of the active convulsant picrotoxinin and its ~50-fold less potent saturated analog picrotin (Jarboe et al., 1968), picrotoxinin, when administered intraventricularly, is at least as potent as TETS if not modestly more potent.

Repeated Sublethal Dosing. To determine whether repeated administration of sublethal doses of TETS leads to a reduction in seizure threshold (a phenomenon often referred to as "chemical kindling"; Mason and Cooper, 1972) or other untoward effects, mice received multiple low doses of TETS (0.02, 0.03, 0.04 or 0.05 mg/kg; hydrated batch). In preliminary experiments, dosing of 0.02 mg/kg at 24 h intervals failed to produce a consistent response other than an

occasional body jerk. In additional preliminary experiments with 0.04 and 0.05 mg/kg administered at 24 h intervals, there was no consistent kindling-like augmentation of the seizure score. We then turned to 48 h dosing. Fig. 8 provides the results of such an experiment. For comparison, a group of mice were treated with a nonlethal dose of the convulsant PTZ (35 mg/kg). As shown in Fig. 8A, repeated dosing with PTZ resulted in an increase in mean seizure score so that following the 7th treatment, all 8 mice exhibited the maximum seizure score. None of the 8 control animals treated in parallel exhibited seizure signs following injection. After a 7 day waiting period, both groups received a PTZ challenge dose (35 mg/kg, i.p.). All animals in the PTZ-treated group exhibited the maximum seizure score whereas the vehicle-treated group animals exhibited minimal seizure scores. These results indicate that the increase in seizure susceptibility that occurred during the repeated treatment regimen is persistent. In contrast, as shown in Fig. 8B, repeated sublethal TETS treatment (0.05 mg/kg) was not associated with a monotonic increase in seizure score: there was a transient increase on the 2nd and 3rd treatments that was not sustained. Moreover, the magnitude of the response to a PTZ challenge dose administered 7 day after the last treatment was similar in the TETS and vehicle groups. Therefore, in contrast to repeated PTZ, repeated sublethal test was not associated with a persistent increase in seizure susceptibility.

In an additional experiment, a sublethal dose of TETS was administered repetitively on a 2 h interval schedule for 4 treatments, with a 5th challenge dose the following day. As shown in Fig. 9, there was a monotonic rise in the mean seizure score with each 2 hourly treatments. However, only a minimal response was obtained with the challenge dose, again demonstrating a lack of persistent increase in seizure susceptibility. The increase in seizure score with each of the 2 hourly treatments may be due to a short-term increase in seizure susceptibility or more likely to the accumulation of TETS with repeated dosing.

Histology

Groups of 8–9 mice received a single intraperitoneal 0.05, 0.07, 0.11, 0.15 or 0.20 mg/kg dose of TETS (hydrated batch). None of the animals receiving the 0.05 mg/kg dose exhibited an observable behavioral response. All mice receiving the 0.07 mg/kg dose were tremulous and agitated and exhibited Straub tail and whole body jerks. With the 0.11 mg/kg dose, all animals exhibited clonic seizures and 1 had tonic seizures. All animals receiving the 0.15 mg/kg dose also exhibited clonic seizures. All animals receiving the 0.20 mg/kg dose experienced clonic seizures and ~58% of animals experienced tonic seizures followed by the death of the animal. The animals were euthanized and the brains removed for histology 24 h or 2, 3 or 7 day after dosing. Doses that produced clonic seizures but were generally not lethal (0.11 and 0.15 mg/kg) were grouped together and are considered "sublethal" doses. The higher 0.20 mg/kg dose, which was generally not compatible with long-term survival, was considered "lethal;" only the small number of animals surviving for 24 h were used for histological analysis.

General Tissue Integrity. As illustrated in Fig. 10, brains from mice exposed 7 days previously to a sublethal dose of TETS (0.11 mg/kg) failed to exhibit degenerating neurons as assessed with Fluoro-Jade B staining. Furthermore, DAPI and H&E stains of adjacent sections revealed no observable loss of cells or changes to general brain architecture. In additional experiments, Fluoro-Jade B staining was not observed in any brain sections from animals receiving TETS, regardless of dose (0.05–0.20 mg/kg) or time after exposure (4 h – 7 d) (not shown). However, as shown in Fig. 10, there were many Fluoro-Jade B positive cells in brain sections from DFP-treated rats processed in parallel.

GFAP Immunohistochemistry. Fig. 11 shows GFAP immunoreactivity in the cortex and hippocampus 24 h and 2,3 and 7 days following treatment with sublethal and lethal doses of TETS. As illustrated by the control images, the hippocampus exhibits substantially greater baseline GFAP immunoreactivity than the cortex (or any of the other brain regions that were examined, including the cerebellum). Mean total area of GFAP immunoreactivity was quantified to estimate changes in the

number of astrocytes expressing GFAP, whereas mean fluorescence intensity was quantified to assess changes in the levels of GFAP expression. In both cortex and hippocampus, there were no changes in the mean area of GFAP immunoreactivity at 24 h following either lethal or sublethal TETS doses, but sublethal dosing was associated with marked increases in GFAP area at 2 and 3 days. At 7 days, GFAP area was not statistically different from control levels. Mean GFAP fluorescence intensity was generally unchanged by the treatments except that there was a small increase with respect to control at 2 days following sublethal dosing in both brain regions.

Iba1 Immunohistochemistry. Iba1 immunoreactivity, a marker of microglia, was present throughout the brain and was observed at high levels in the cortex and hippocampus (Fig. 12). There was a significant increase in the area of Iba1 immunostaining at 24 h in the cortex of animals exposed to the lethal dose of TETS but not to the sublethal dose. A similar trend was observed in the hippocampus although the TETS-related increase did not reach statistical significance (except in the comparison with the value from the sublethal group). Sublethal TETS caused a significant increase in the area of Iba1 immunostaining in the cortex and hippocampus at 2 days. There were no changes in Iba1 fluorescence intensity except for a slight increase at 2 days in the hippocampus. These results suggest that TETS-induced seizures cause a transient increase in the number of cells expressing Iba1.

Microglial activation may represent a more relevant measure of the microglial response to brain injury than simple quantification of Iba1 immunostaining since Iba1 is expressed in resting (ramified) microglia (Ahmed et al., 2007). Accordingly, the morphology of Iba1 immunoreactive cells was examined, and the number of activated microglia cells was determined in the hippocampus and cortex (Fig.13). In both brain regions, the number of activated microglia increased following exposure to a lethal dose of TETS, although only in the cortex did the treatment-related increase reach statistical significance. Sublethal dosing was associated with an increase in activated microglia

at 2 days; again the increase only reached significance in the cortex. Overall, the counts of activated microglia are consistent with the area measurements presented in Fig. 12.

Discussion

In the present study we confirmed the high potency of TETS as a convulsant in mice and rats, and for the first time demonstrate its efficacy and potency when administered by the intravenous, oral and intraventricular routes. TETS is among the most potent known chemical convulsants, with a potency in the same range as organophosphate nerve agents (Clement et al., 1981; Clement, 1983) and more potent than the most potent cage convulsants, including TBPS (Holland et al., 1992). The seizure activity and lethality of TETS has previously been attributed exclusively to its action on the brain (Haskell and Voss, 1957). Our demonstration that intraventricular TETS causes a similar behavioral response and lethality as with systemic administration supports this conclusion. The seizures induced by TETS appear similar to those produced by GABA antagonists such as PTZ and picrotoxin (Dhir et al., 2011). The rate of onset of seizures induced by low sublethal doses of TETS was similar to that of picrotoxin but slower than for PTZ. Therefore, in order to obtain accurate estimates of threshold values in the intravenous infusion model, slower infusions were required than is the case with PTZ (Dhir et al., 2011). With higher (lethal) doses the latency was reduced and seizures occurred rapidly.

It is generally believed that the convulsant action of TETS relates to its picrotoxin-like noncompetitive, reversible block of $GABA_A$ receptors. Indeed, the sequence of behavioral seizure types induced by TETS is similar to that produced by picrotoxin and PTZ, both of which are believed to induce seizures by blockade of $GABA_A$ receptors (Squires et al., 1984). When injected intraventricularly, TETS and picrotoxin have comparable potencies as expected from their similar potencies as antagonists of $GABA_A$ receptors (Squires et al., 1983; Cole and Casida, 1986; Ratra et al., 2001). However, our results indicate that TETS is approximately 40-times more potent as a convulsant when administered systemically. In addition, whereas TBPS is an order of magnitude more potent as a functional antagonist of $GABA_A$ receptor Cl^- flux than TETS (Obata et al., 1988;

Van Renterghem et al., 1987), TBPS is less potent than TETS as a convulsant in mice (Holland et al., 1992). Therefore, TETS is a far more potent convulsant in vivo than expected from its known activity as a $GABA_A$ receptor antagonist. TETS has excellent parenteral and oral bioavailability as its potency when administered intraperitoneally and orally are similar to that obtained with intravenous delivery. The enhanced potency of TETS compared with other $GABA_A$ receptor blocking convulsants could be due to greater blood-brain barrier diffusion or transport. Greater diffusion seems unlikely because TETS is a hydrophilic molecule with a predicted octanol:water partition coefficient of 0.07 (QikProp, Schrödinger). This compares to the calculated octanol:water partition coefficient for picrotoxinin of 0.5. The estimated blood:brain concentration ratios for TETS and picrotoxinin are 0.20 and 0.35, respectively. Therefore, if TETS is concentrated in brain, this must occur through a transport process rather than by diffusion. An alternative explanation for the extreme systemic potency of TETS is that it is metabolized in the periphery to a more potent convulsant compound; at present, there is no evidence that this occurs.

Repeated administration of low sublethal doses of TETS in mice failed to elicit a clear progressive development of increased seizure response as was observed in the present study with PTZ (Fig. 8; see also, Dhir et al., 2005) and is also obtained with other $GABA_A$ receptor antagonists including picrotoxin (Shanda et al., 1996). Moreover, there was no persistent enhanced seizure susceptibility when animals treated with repeated doses of TETS were exposed to a subthreshold challenge dose of PTZ 7 days after the last dose of TETS. It has previously been demonstrated that chemical kindling with one $GABA_A$ receptor antagonist results in a persistent reduced threshold for other $GABA_A$ receptor antagonists such as PTZ (Grecksch et al., 1990; Corda et al., 1991; De Sarro et al., 1999). The failure of TETS to cause chemical kindling represents a difference from other $GABA_A$ receptor antagonists.

Lower levels of exposure to TETS caused a variety of behavioral effects including clonic seizures that animals survived without apparent long-term behavioral or neurological sequelae. These

seizures were not associated with evidence of overt neuronal injury and cell death. Certain seizure types including electroconvulsive (Steward, 1994) and kindled seizures (Steward et al., 1992) as well as nerve agent induced seizures (Baille-Le Crom et al., 1995; Zimmer et al., 1997; Baille et al., 2005) and kainic acid induced status epilepticus (Rizzi et al., 2003; Ravizza et al., 2005) are associated with the conversion of astrocytes to a reactive state that can be monitored by GFAP expression. There is often a rapid increase in GFAP that may be present at 4 h and persist for up to 4 days (Steward, 1994; Zimmer et al., 1997); with prolonged seizures, the changes can persist for 7 days or more (Baille et al., 2005). Even the seizures associated with sublethal doses of TETS evoked marked increases in GFAP immunoreactivity in cortex and hippocampus 2 and 3 days following exposure. In contrast to reports in the literature with other seizure types, no increase was measurable at 24 h. The increase was manifest as an increase in the area of immunoreactivity, suggesting an increase in the number of reactive astrocytes with no or only minimal increases in the expression of GFAP per astrocyte. Reactive astrocytosis occurs after many types of central nervous system injury. In some situations, there may be harmful long-term consequences although recent evidence supports the view that reactive astrocytes limit inflammation and have other beneficial effects (Sofroniew, 2005). Therefore, the implications of the transient reactive astrocytosis that occurs following TETS-induced seizures remain to be determined.

Microglia are also rapidly activated by prolonged seizures (status epilepticus) (Rizzi et al., 2003; Du et al., 1993; Baille et al., 2005; Zimmer et al., 1997). In the present study, we observed evidence of microglial activation as assessed by increased Iba1 immunoreactivity in mice that survived for 24 h following a dose of TETS that often caused lethality and at 2 days following non-lethal doses. The results from Iba1 immunostaining were confirmed with increased counts of activated microglia in the cortex but not the hippocampus. Microglial activation is generally believed to be a marker of central nervous system inflammation and may be a concomitant of neurodegeneration (Dirnagl et al., 1999; Turrin and Rivest, 2004). Therefore, we cannot exclude the

possibility that TETS-induced seizures may cause some neurodegeneration despite the failure to observe evidence of cellular injury and death as assessed by routine histology and Fluoro-Jade B staining.

In conclusion, TETS exposure causes intense seizures in mice and rats. Doses that induce tonic seizures are generally lethal, although the time to onset of tonic seizures varies inversely with dose providing a variable window in which postexposure treatments can be administered. Lower doses associated with clonic seizures are survivable and there are no detectable neuropathological consequences, although there is evidence of transient reactive astrocytosis and inflammation in some brain regions. The models we have described provide opportunities to evaluate therapies for TETS-induced seizures and lethality and for the further assessment of the long-term consequences of the seizures produced by this extremely potent convulsant agent.

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Authorship Contributions

Designed animal experiments: Zolkowska, Dhir, and Rogawski

Designed histology experiments: Banks and Lein

Conducted animal experiments: Zolkowski, Dhir, and Inceoglu

Conducted histology experiments: Banks and Bruun

Contributed new reagent: Sanborn, McCoy, and Hammock

Performed data analysis: Zolkowska, Banks, Dhir, Lein, and Rogawski

Wrote or contributed to the writing of the manuscript: Rogawski, Zolkowska, Banks, Lein, McCoy, and Hammock

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Footnote

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LEGENDS FOR FIGURES

Fig 1. Structures of tetramethylenedisulfotetramine (2,6-dithia-1,3,5,7-tetraazatricyclo[3.3.1.1^{3,7}]decane-2,2,6,6-tetraoxide; TETS) and hexamethylenetrisulfohexamine (HEXS). **2.** Dose-response relationships for induction of clonic seizures, tonic seizures and mortality following intraperitoneal injection of TETS (hydrated batch) in mice. Each point represents 8 animals.

Fig. 3. Time to onset of clonic seizures in mice following intraperitoneal TETS (hydrated batch). The data are from the experiment shown in Fig. 2. Each point represents the mean ± S.E.M.

Fig. 4. Threshold values for myoclonic jerks, clonic seizures, and tonic seizures during intravenous infusion of TETS (hydrated batch) in mice. TETS solution (0.02 mg/ml) was infused at a rate of 0.1 ml/min via the tail vein in 6 mice. The times to onset of the seizure signs were recorded and converted to threshold values as described in Materials and methods. Each bar represents the mean ± S.E.M. of the threshold values.

Fig. 5. Dose-response relationships for induction of clonic seizures, tonic seizures and mortality following oral gavage of TETS (anhydrous batch) in mice. Each point represents 8 animals.

Fig. 6. Time to onset of clonic seizures following administration of TETS (anhydrous batch) by oral gavage. The data are from the experiment shown in Fig. 5. Each point represents the mean ± S.E.M.

Fig. 7. Time to onset of clonic seizures following various intraventricular doses of TETS (anhydrous batch) and picrotoxin. Bars indicate mean ± S.E.M. of the interval between the initiation of the

infusion and the onset of clonic seizure activity. When no clonic seizure was observed during the 1 h observation period, the time was taken as 3600 s. Number of animals tested at each dose is as given in Table 1.

Fig. 8. Comparison of the effects of repeated administration of sublethal doses of PTZ (A) and TETS (hydrated batch) (B) in mice. The doses were given on a 48 h (alternate day) schedule. In each case, a group of 8 animals was treated with vehicle (sterile water in A or 10% DMSO in 0.9% sterile saline in B). Seven days after the last dose, all animals received a PTZ challenge dose of 35 mg/kg PTZ in A or 30 mg/kg in B. Animals were observed for 60 min in the case of TETS or 30 min in the case of PTZ for the occurrence of seizure signs, which were rated as follows: 0 = no response; 1 = immobility; 2 = brief myoclonic body jerks; 3 = clonic seizures. Data points indicate the mean ± S.E.M. of the composite seizure score values calculated as the sum of the scores (maximum score is 6). Three of the 8 animals in the TETS-treated group died, 1 after the 7th treatment and 2 after the 8th treatment; 1 of the 8 animals in the PTZ-treated group died after the 5th treatment.

Fig. 9. Repeated administration of a sublethal dose of TETS (hydrated batch). The doses were given on a 2 h schedule to 8 mice. The same dose was administered 24 h after last injection of TETS ("Following day"). Data points indicate the mean ± S.E.M. of the composite seizure score values determined as described in the caption for Fig. 8.

Fig. 10. Representative photomicrographs of Fluoro-Jade B (FJB), H&E, and DAPI staining in the cortex of a control mouse and a mouse 7 days after intraperitoneal injection of a sublethal dose of TETS (0.11 mg/kg; hydrated batch). The control and treated groups consisted of 3–4 animals. An

image of a section of rat brain from an animal treated with DFP that was stained concurrently with Fluoro-Jade B is shown as a positive control. Scale bar, 50 μm.

Fig. 11. GFAP immunoreactivity in the cortex (*left four images and bar graphs*) and hippocampus (*right four images and bar graphs*) of mice 24 h or 2, 3 or 7 days after treatment with vehicle (control) or TETS (hydrated batch) at sublethal (0.11 and 0.15 mg/kg) or lethal (0.20 mg/kg) doses. Representative photomicrographs for control and sublethal TETS treatment at 24 h and 2 days are shown. Scale bars, 50 μm. To assess the levels of GFAP expression and the number of GFAP-immunopositive cells in the two brain regions, GFAP fluorescent intensity (*top bar graphs*) and total area of GFAP immunoreactivity (*bottom bar graph*s) were quantified. In both brain regions, there was a significant increase in mean GFAP staining area at 2 and 3 days after sublethal TETS exposure compared with control. There was a small, but significant increase in mean GFAP fluorescence intensity at 2 days. *, $p<0.05$; ***, $p<0.001$; one way ANOVA and Student's *t*-test. Bars represent mean ± S.E.M. of values from 4–10 animals per dose.

Fig. 12. Iba1 immunoreactivity in the cortex (*left 4 images and bar graphs*) and hippocampus (*right 4 images and bar graphs*) of mice 24 h or 2, 3 or 7 days after treatment with vehicle (control) or TETS (hydrated batch) at sublethal (0.11 and 0.15 mg/kg) or lethal (0.20 mg/kg) doses. Representative photomicrographs for control and sublethal TETS treatment at 24 h and 2 days are shown. Scale bars, 50 μm. The total number of microglia and the levels of Iba1 expression were assessed by quantifying the total area of Iba1 staining (*top bar graphs*) and mean fluorescence intensity (*bottom bar graphs*), respectively. In both brain regions, there was a significant increase in the mean total area of Iba1 immunoreactivity at 2 days in animals exposed to a sublethal dose of TETS. There was also an increase in the mean total area of Iba1 immunoreactivity at 24 h in animals exposed to a lethal dose of TETS although in the case of the hippocampus the change was only

significant when assessed with respect to the sublethal dose and not with control. There was a small, but significant increase in mean GFAP fluorescence intensity at 2 days in the hippocampus. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; one-way ANOVA and Student's *t*-test. Bars represent mean ± S.E.M. of values from 4–10 animals per dose.

Fig. 13. Microglial activation at 24 h or 2, 3 or 7 days after treatment of mice with vehicle (control) or TETS (hydrated batch) at sublethal (0.11 and 0.15 mg/kg) or lethal (0.20 mg/kg) doses. *Top*, Representative photomicrographs of resting and activated microglia from the hippocampus of an animal treated with TETS. Activated microglia were distinguished by an enlarged cell body and fewer, shorter hypertrophic processes extending from the soma. Scale bars, 25 μm. Activated microglia in cortical and hippocampal sections were counted and the mean counts per section are presented in the bar graphs. At 24 h, there was an increase in the number of activated microglia in the cortex of mice exposed to the lethal dose of TETS compared to vehicle control. There was also an increase in the number of activated microglia in the 2 day sublethal dose group compared to vehicle control. *, $p<0.05$; one-way ANOVA and Student's *t*-test. Bars represent mean ± S.E.M. of averaged counts per section; 3–4 animals per dose.

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TABLE 1

Incidence of seizure signs and lethality following intraventricular TETS and picrotoxin in rats

Treatment	Number of rats	Clonic seizures	Tonic seizures	Lethality
TETS				
5 μg	5	1 (20%)	0 (0%)	0 (0%)
10 μg	6	4 (66.7%)	0 (0%)	0 (0%)
25 μg	6	4 (66.7%)	0 (0%)	1 (16.6%)
50 μg	4	4 (100%)	1 (25%)	3 (75%)
100 μg	4	4 (100%)	1 (25%)	4 (100%)
Picrotoxin				
5 μg	2	1 (50%)	0 (0%)	0 (0%)
10 μg	2	2 (100%)	0 (0%)	1 (50%)
25 μg	2	2 (100%)	0 (0%)	1 (50%)
50 μg	2	2 (100%)	1 (50%)	2 (100%)

TETS or picrotoxin (10 mg/ml DMSO) was administered intraventricularly and the animals were observed for 1 h. Values shown are the number (percent) of animals exhibiting clonus, tonus and lethality during the observation period.

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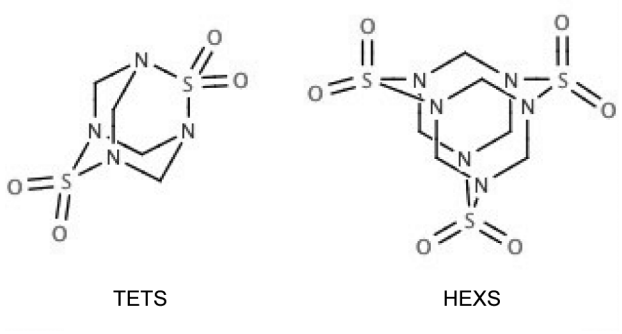

Fig. 1

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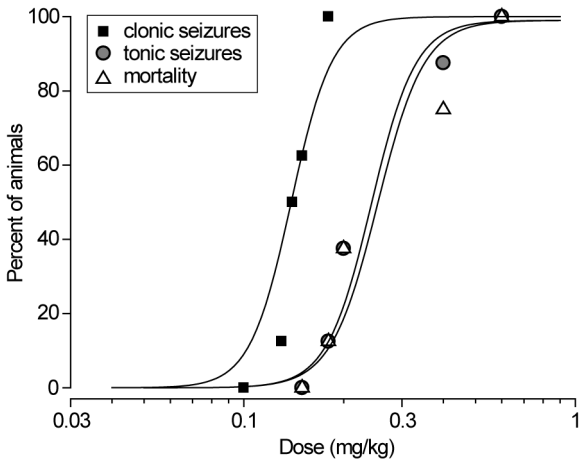

Fig. 2

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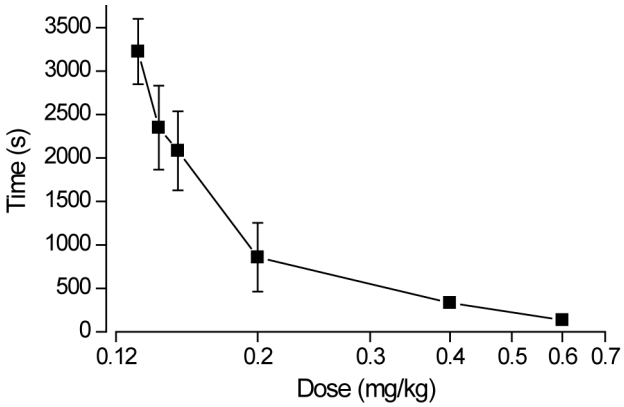

Fig. 3

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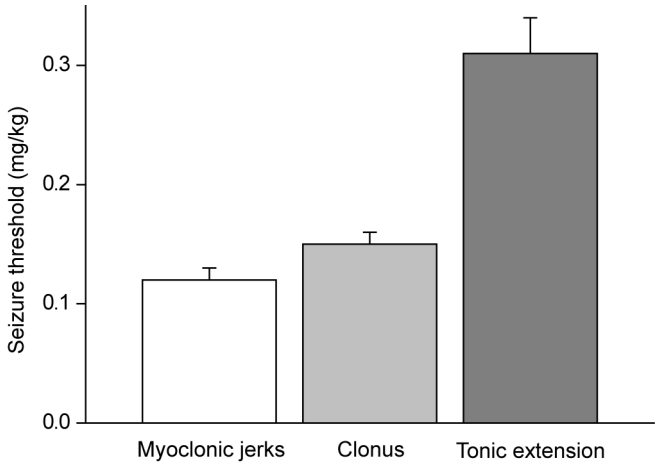

Fig. 4

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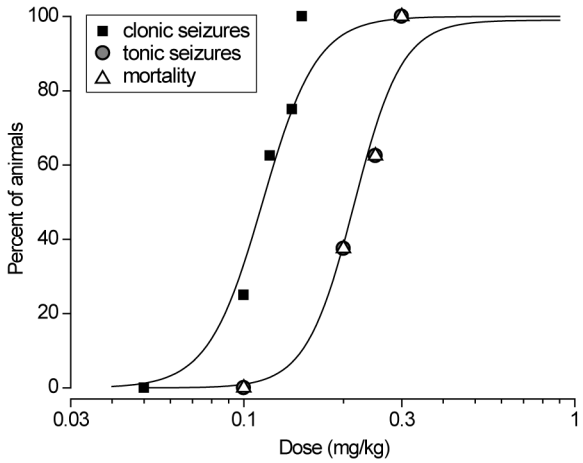

Fig. 5

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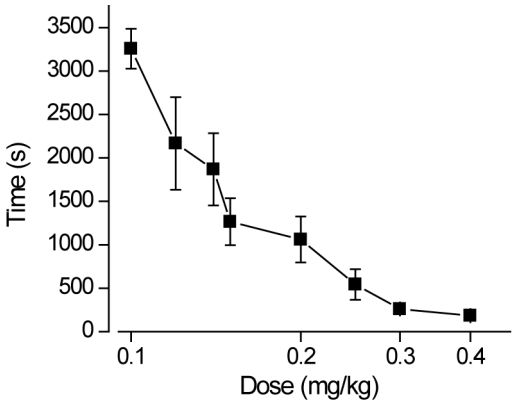

Fig. 6

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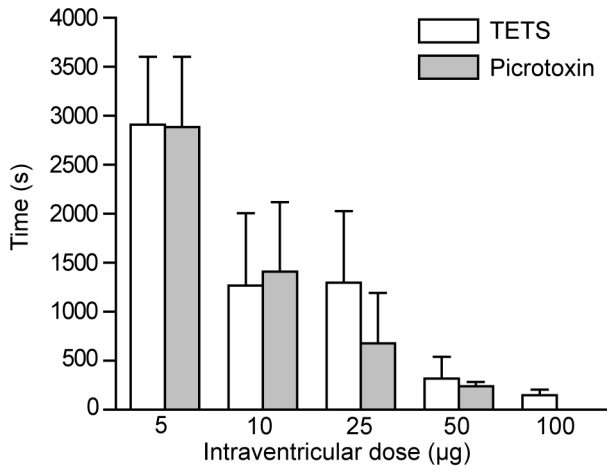

Fig. 7.

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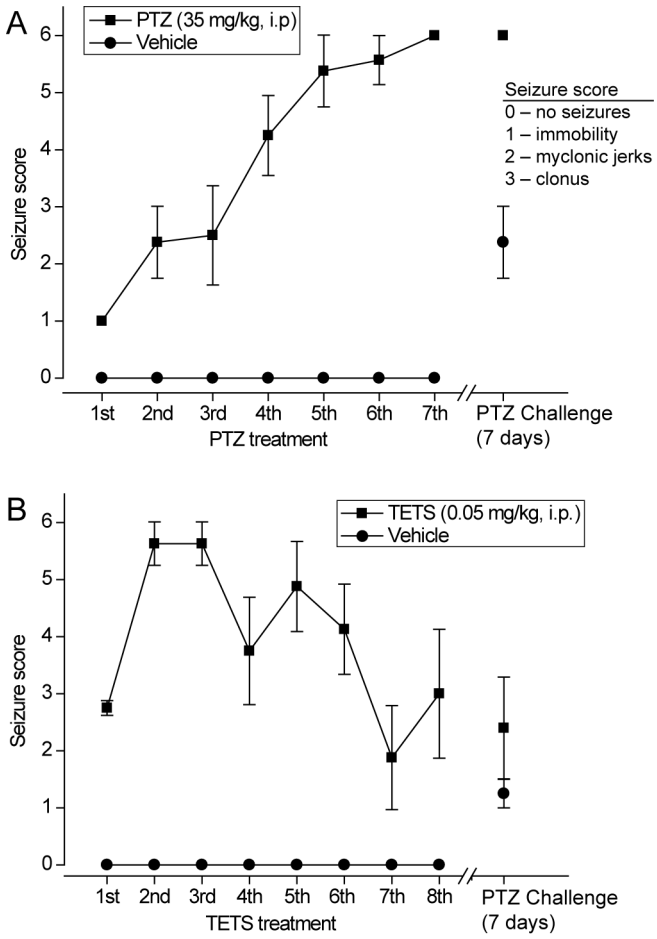

Fig. 8

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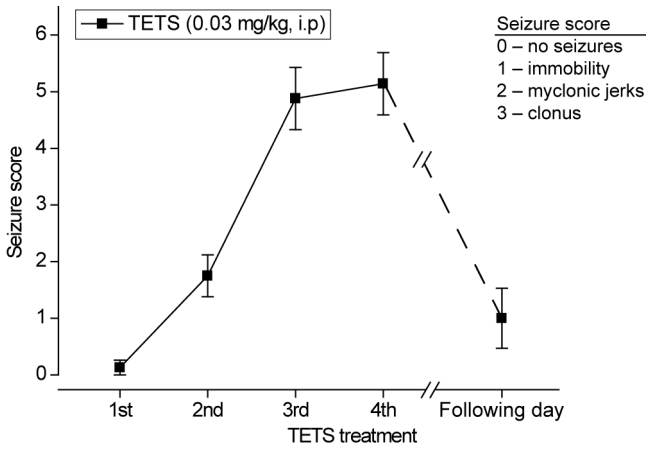

Fig. 9

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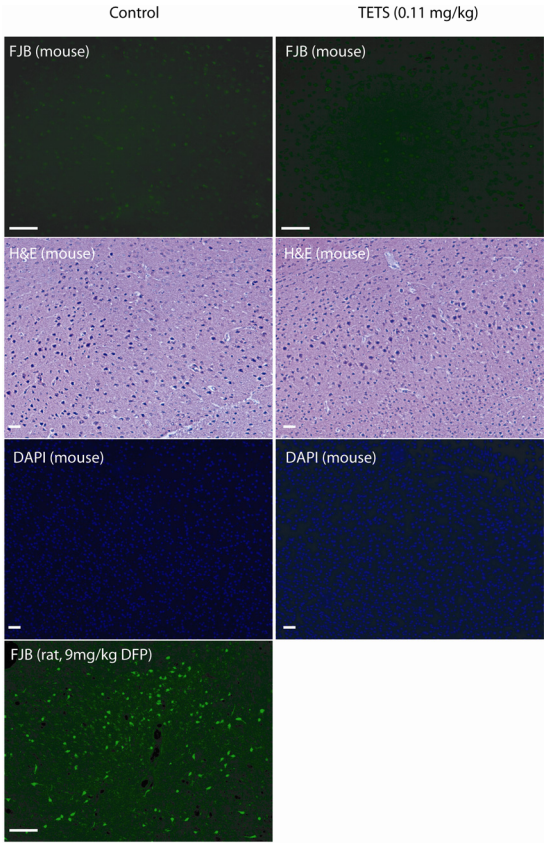

Fig. 10.

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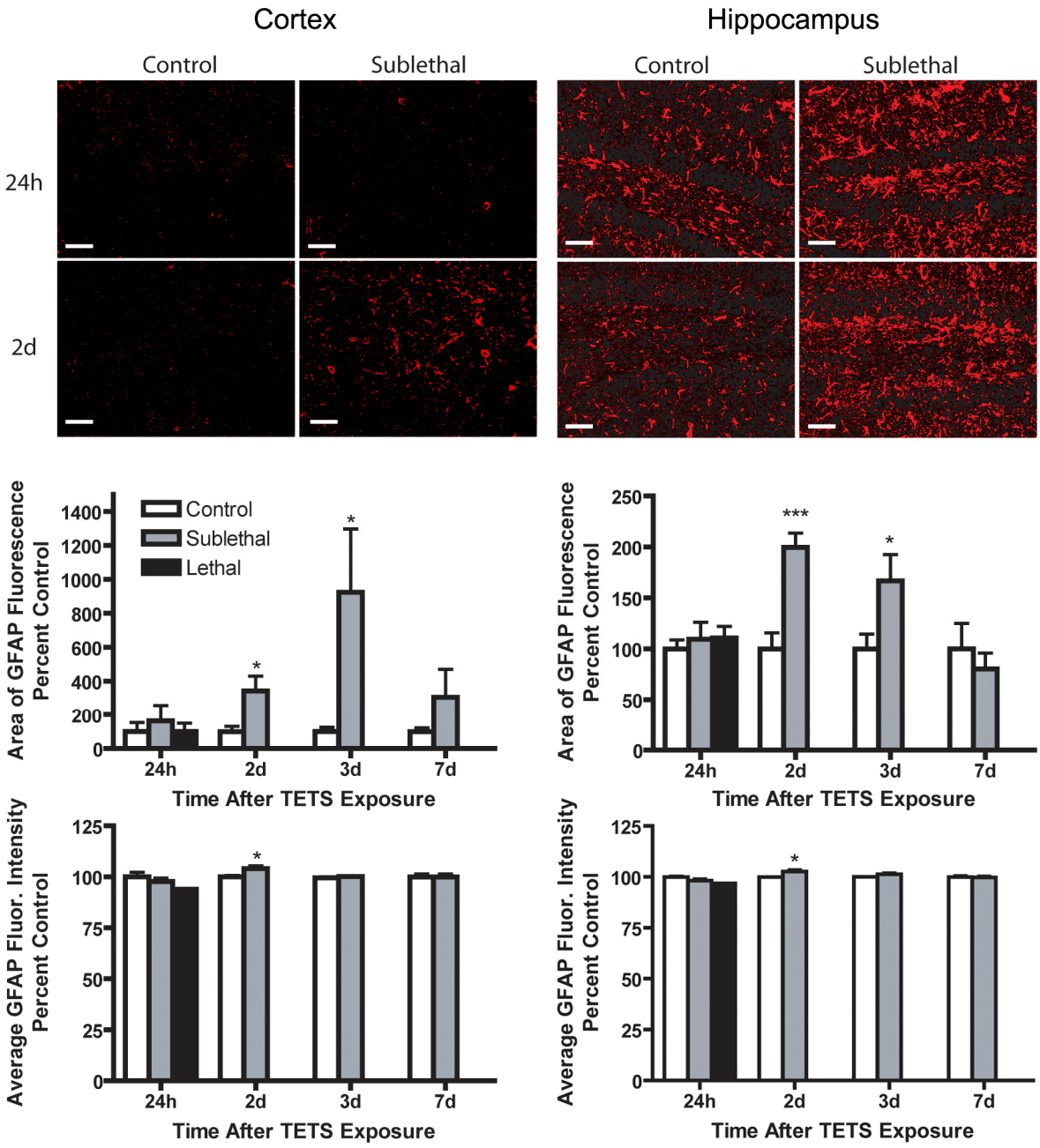

Fig. 11

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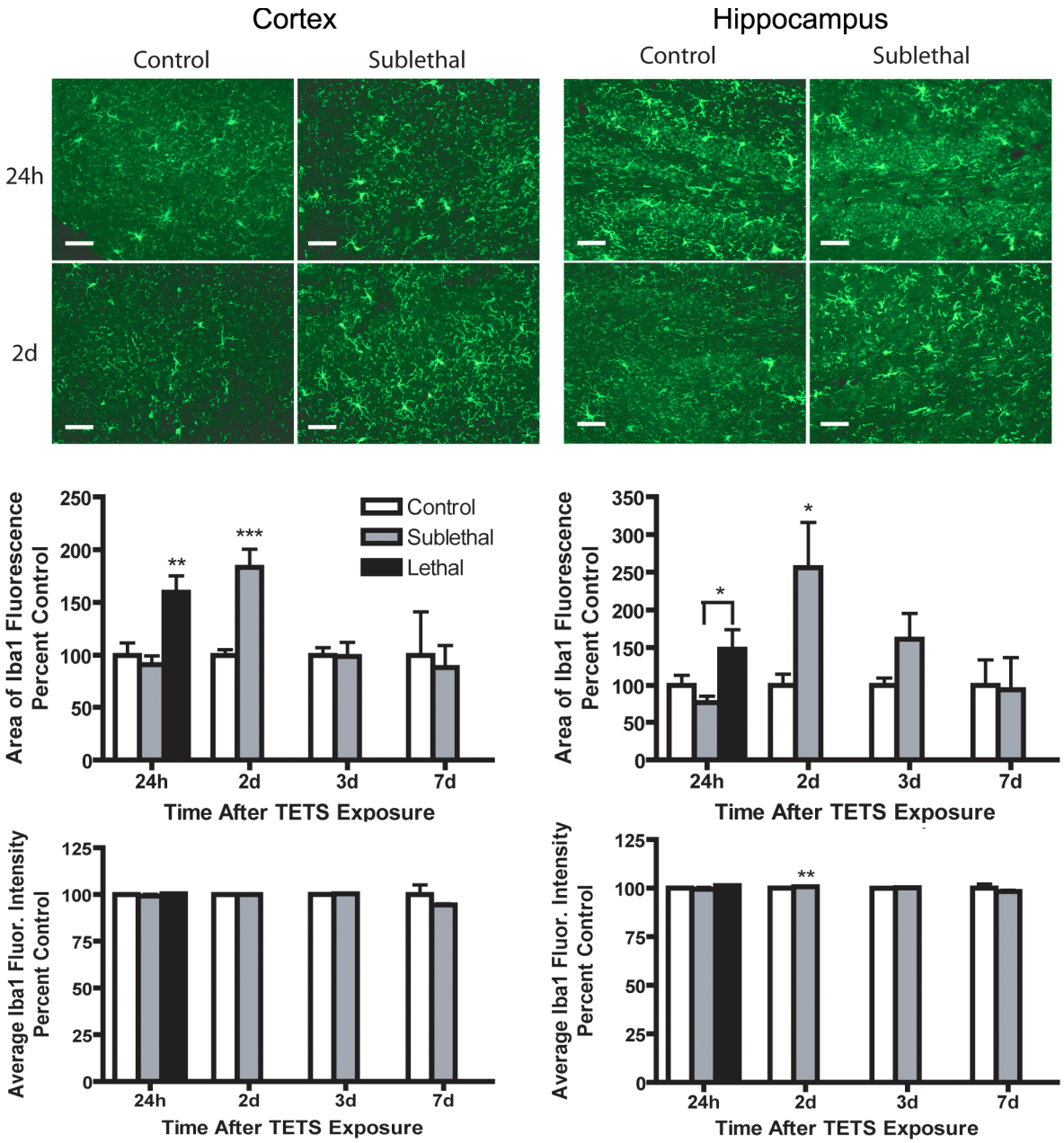

Fig. 12

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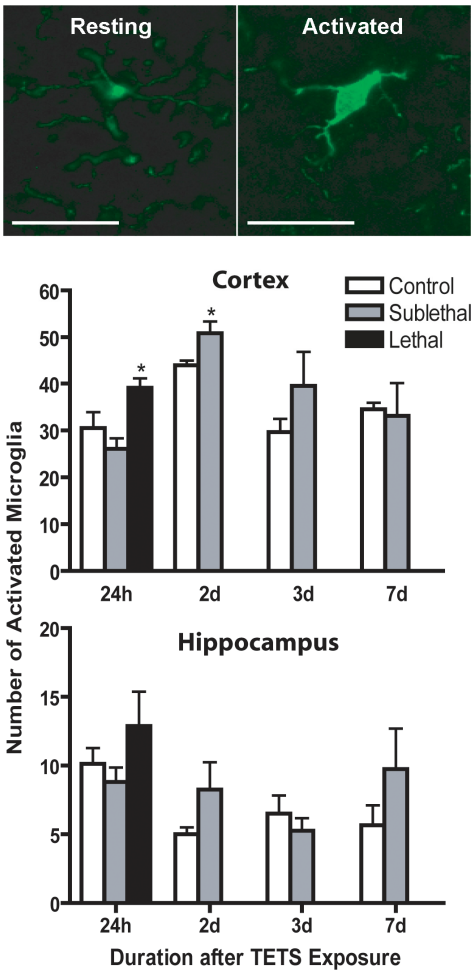

Fig. 13