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Developmental and sex differences in tetramethylenedisulfotetramine (TMDT)-induced syndrome in rats

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Abstract

Tetramethylenedisulfotetramine (TMDT) is a synthetic neurotoxic rodenticide considered a chemical threat agent. Symptoms of intoxication include seizures leading to status epilepticus and death. While children and women have been often the victims, no studies exist investigating the neurotoxic effects of TMDT in developing individuals or females. Thus, we performed such an investigation in developing Sprague-Dawley rats of both sexes in order to identify potential age- or sex-dependent vulnerability to TMDT exposure. Subcutaneous injection was chosen as the preferred route of TMDT exposure. EEG recordings confirmed the seizure activity observed in both postnatal day 15 (P15) and adult rats. Additionally, P15 rats displayed greater sensitivity to TMDT than postnatal day 25 or adult animals. Seizures were generally more severe in females compared to males. Barrel rotations accompanied convulsions in P25 and adult, but sparsely in P15 rats. Adults developed barrel rolling less frequently than P25 population. Neuronal cell death was not present in 24-hr TMDT survivors at any age or sex tested. A seizure rechallenge with flurothyl 7 days following TMDT exposure demonstrated longer latencies to the first clonic seizure but a faster progression into the tonic-clonic seizure in P15 and adult survivors as compared to their vehicle-injected counterparts. In conclusion, the youngest age group represents the most vulnerable population to the TMDT-induced toxidrome. Females appear to be more

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Conflicts of interest

The authors declare no conflicts of interest.

vulnerable than males. TMDT exposure promotes seizure spread and progression in survivors. These findings will help to establish sex- and age-specific treatment strategies for TMDT-exposed individuals.

Keywords

TMDT; seizures; rat; development; sex

1 Introduction

Tetramethylenedisulfotetramine (TMDT, C₄H₈N₄O₄S₂, CAS Number: 80-12-6) is a synthetic neurotoxic rodenticide used primarily in mainland China despite a ban on its production and use worldwide. TMDT action upon the central nervous system originates from binding to the GABA_A chloride ionophore complex (GABA_AR) and impeding the chloride ion entry through this channel. This process interferes with GABAergic inhibitory actions in the brain and leads to unchecked depolarizations and excessive influx of Ca²⁺ into neurons (Ticku and Olsen, 1979; Zhao et al., 2014). As a result of the exposure, agitation, seizures with refractory status epilepticus, cardiac abnormalities, coma and death due to multiorgan failure are commonly observed (Chau et al., 2005; Lu et al., 2008; Poon et al., 2005; Zhang et al., 2011). TMDT is one of the most potent seizure-inducing chemicals known, with low selectivity for rodents as evidenced by an LD₅₀ for humans of ~0.1 mg/kg (Croddy, 2004). This agent meets criteria for inclusion in the list of extremely hazardous pesticides maintained by the World Health Organization (WHO) since it is more lethal than WHO's most toxic registered pesticide, fluoroacetate ((CDC), 2003). Because of the severity of poisoning, TMDT was also a subject of a great attention by the CDC and the New York Poison Control Center when a poisoning occurred in the United States ((CDC), 2003). Moreover, the easy synthesis, water solubility, and lack of odor and taste makes it an effective adulterant of food and drink and a desirable tool for terroristic acts, contributing to frequent misuse of TMDT. It was reported that there were about 3,500 persons, including 225 fatalities, involving TMDT poisoning in mainland China from 2001 to 2012 (Li et al., 2014). Despite of numerous cases of TMDT-induced intoxication, no standardized and effective treatment currently exists against TMDT syndrome. Clinical studies recommend gastro-intestinal tract decontamination, charcoal haemoperfusion and haemodialysis to decrease the levels of the toxin, accompanied by aggressive treatments with available anticonvulsant drugs (Deng et al., 2012; Li et al., 2012; Wang et al., 2016) together with supportive therapy (Wang et al., 2016). Experimental data in adult male mice indicate that a combined treatment with GABA_AR agonist, diazepam, either with an N-methyl-D-aspartate (NMDA) receptor antagonist, MK801 (dizocilpine maleate) (Shakarjian et al., 2012) or allopregnanolone (Bruun et al., 2015) can effectively prevent from TMDT-induced seizures.

Despite these experimental successes in treatment of TMDT syndrome in adult male animals, there are no reports investigating neurotoxic effects of TMDT in developing animals or in females. Yet, children in particular have been often direct targets of TMDT poisoning, such as instances described as “kindergarten wars” when multiple preschoolers were intentionally poisoned because of rivalry between competing kindergartens (Bloom,

2014). Despite its worldwide ban, TMDT is still used as an illegal constituent of numerous rodenticides. Due to their powdery consistency, these rodenticides can be easily sprinkled around the living area and subsequently intoxicate toddlers by hand-to-mouth delivery (Barrueto et al., 2003). Additionally, reports show that men and women are equally likely to be victims of TMDT poisonings (Zhang et al., 2011). In the event of mass exposure, civilians of all ages, men, women and children, could be affected, as exemplified by an incident in a restaurant in China (Xinhua, 2015).

The purpose of this study was therefore to examine convulsive and lethal effects of TMDT in developing male and female rats in order to identify potential age- or sex-dependent vulnerability. Rats were chosen to expand our previous studies done in mice to an additional rodent model to confirm general validity of the syndrome because (neuro)physiological differences may exist between rats and mice (Ellenbroek and Youn, 2016). Furthermore, we investigated whether there were persisting effects of TMDT intoxication that could alter susceptibility to acutely induced generalized seizures.

Results of this study will assist in predicting broader dangers and consequences of TMDT intoxication in humans in case of individual or mass poisonings. Detailed characterization of the TMDT toxidrome as a function of age and sex will further help in evaluating the most effective countermeasures as requested by NIH CounterAct program, and aid in establishing tailored treatments for affected subjects (Jett, 2016).

2 Material and methods

2.1 Chemicals

TMDT 98% purity (CAS 80-12-6, MW=240.26) was kindly provided by Dr. Lowri S. deJager, Center for Food Safety and Applied Nutrition, US Food and Drug Administration (FDA). TMDT was stored in a secure location at -80°C . Multiple small aliquots (1–2 mg) were weighed out in one session to minimize hazards of the compound handling using appropriate safety precautions. Stock solutions of TMDT were prepared in dimethylsulfoxide(DMSO) at a concentration of 10 mg/ml and remained stable for 30 days when stored at -80°C . Prior to the experiment, TMDT stock solution was diluted in sterile water for injection to a final concentration 0.02 mg/ml (for rat pups) or 0.2 mg/ml (for prepubertal and adult rats) and the amount of solution was adjusted for the each animal and each dose based on the animal weight. DMSO was obtained from Sigma-Aldrich (St. Louis, MO). FluoroJade C powder was purchased from Histo-Chem Inc. (Jefferson, AR), while potassium permanganate and cresyl violet were obtained from Aldrich Chemical Company (Milwaukee, WI); these were dissolved in distilled water shortly before their usage. Ethyl alcohol was obtained from Pharmaco-AAPER (Shelbyville, KY) and flurothyl (bis(2,2,2-trifluoroethyl) ether) of 99% purity from SynQuest Labs (Alachua, FL). Permunt was purchased from Thermo Fisher Scientific.

2.2 Animals

The experiments were performed on male and female Sprague-Dawley rats obtained from Taconic Farms (Germantown, NY). Rats were kept in our AAALAC-accredited animal

facilities. Adult rats were housed four per cage while rat pups (10–12 individuals) were kept with lactating dam in a single cage until the day of the experiment. Animals were given food and water ad libitum on a regular light cycle (light on at 07:00 and off at 19:00), and were allowed to acclimate at least 48 hrs prior to the experimentation. All experiments were approved by the Institutional Animal Care and Use Committee of the New York Medical College and conformed to the Revised Guide for the Care and Use of Laboratory Animals (Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011). All efforts were made to minimize pain and the number of animals used while maintaining sufficient statistical power.

Rats of three different ages were selected: postnatal day 15–16 (P15), postnatal day 25–26 (P25), and adult rats (125–150 g at arrival, >60 days old; P60). We used 11 litters for the P15 and 10 litters for the P25 rats to generate the data. Each litter consisted of 10–12 pups. To eliminate any litter effect, we randomly distributed the animals into groups treated with different doses of TMDT. Thus, no more than two pups from the same litter were used in a single TMDT dose-group to minimize the „litter“ effect. In brain development, these groups roughly correspond to infant/toddler (P15 pups), prepubertal (P25 rats), and young adult (P60 rats) humans (Gottlieb et al., 1977; Scantlebury et al., 2007; Velísková et al., 1999; Velísková and Velíšek, 1992). P25 rats were previously housed with the lactating mother until the postnatal day 21 when they were weaned. Seizure testing took place between 10:00 and 12:00. On the day of the experiment, animals were weighed and randomly divided into experimental groups representing specific TMDT doses. Each group consisted of 8–12 rats of each sex based on the sample size analysis as previously described (Shakarjian et al., 2012). Prior to TMDT injection, the female estrous cycle was monitored using changes in vaginal epithelium impedance (Estrous Cycle Monitor EC40; Fine Science Tools, Foster City, CA) (Velíšek et al., 2006; Velísková and Velíšek, 2013) because individual cycle stages may alter seizure susceptibility (Velísková, 2007).

2.3 TMDT administration

In a preliminary experiment we compared subcutaneous (s.c.) with intraperitoneal (i.p.) route of TMDT administration in adult rats. For i.p. injection, the following doses were used: 0.20, 0.30, 0.45, 0.60 mg/kg while for s.c. administration, 0.30, 0.40, 0.50 and 0.60 mg/kg were used because the lower doses did not induce any convulsions. Based on the comparison of dose response curves, we chose the s.c. injection as preferred route of administration for subsequent experiments. Rats were distributed randomly into experimental groups, injected with TMDT and placed into individual plexiglass cages. P15 rat pups were kept on an electric heating pad to maintain their body temperature. For evaluation of age and sex differences we used s.c. injection. P15 animals received TMDT in the following doses: 0.10, 0.15, 0.20, 0.25 and 0.30, while the P25 rats received 0.30, 0.35, 0.40, 0.50 and 0.60 mg/kg and the adults 0.30, 0.40, 0.50, 0.60 and 0.70 mg/kg of TMDT. After TMDT administration, rats were observed continuously for 60 min for latency to onset of clonic and tonic-clonic seizures. Since we observed an age-dependent appearance of barrel rotation behavior, we scored the occurrence of barrel rotations, as well. We also monitored the number of clonic and tonic-clonic seizures, as well as incidence of and latency to death. There were additional observation points at 2, 3, 6 and 24 hrs following

TMDT administration. Each animal was also assigned a severity score between 1 and 5, which represented the maximal severity of the TMDT syndrome (Shakarjian et al., 2015): 1, a single clonic seizure; 2, multiple clonic seizures; 3, one or more tonic-clonic seizures; 4, lethality within 24 hrs; and 5, for lethality within 1 hr. The median lethal dose (LD50) for 24-hrs lethality and convulsive dose 50 (CD50) for tonic-clonic seizure induction with 95% confidence intervals were calculated for each age and sex group using the JFlash Calc Program (Dr. Ossipov, University of Arizona, Tucson, AZ). The dose response curves for 24-hr lethality and tonic-clonic seizures were established for each sex and age using CompuSyn software (ComboSyn, Inc, Paramus, NJ).

2.4 EEG recordings

Recording of EEG was performed using two P15 and two adult rats of both sexes as described previously (Shakarjian et al., 2012; Velíšek et al., 2010). Cortical electrodes were implanted under combined ketamine/xylazine anesthesia (70/30 mg/kg, i.p.). A screw placed in the nasal bone served as the reference electrode; a second screw serving as a ground was positioned behind lambda. Four silver ball epidural electrodes were placed bilaterally above the frontal and occipital cortices. Electrodes and screws were covered with dental acrylic. Rats were returned to their home cages to recover for two days following surgery. Pinnacle Technology (Lawrence, KS) EEG/videomonitoring system was used to record the TMDT syndrome. Baseline EEG was recorded for 10 minutes prior to s.c. injection with either 0.3 mg/kg (P15) or 0.6 mg/kg TMDT (adult).

2.5 Evaluation of neurodegeneration in TMDT survivors

The ability of TMDT to induce neuronal cell death was investigated in 24-hr survivors of each developmental stage and sex that had experienced tonic-clonic seizures. Under deep anesthesia, one male and one female rat of each age group (together 6 animals) were perfused transcardially with phosphate buffered saline followed by 4% paraformaldehyde. Brains were removed and cryoprotected with ascending sucrose (10–30%) prior to cutting using a cryostat into 30 µm sagittal sections to ensure coverage of entire brain. Sections were mounted on 2% gelatinized slides and stained with FluoroJade C. To ensure that lack of positive staining in TMDT-exposed animals is not related to a technical problem, we always processed in parallel a slide with coronal sections from an adult animal experiencing kainic acid-induced status epilepticus (kainic acid 12.5 mg/kg i.p.) for one hour and sacrificed 24 hrs later. These sections containing the well established vulnerable hilar region of the dentate gyrus were used as a positive control to validate the staining protocol. Adjacent brain sections from TMDT-exposed rats were stained with cresyl violet to examine tissue integrity. After the staining, slides were gradually dehydrated in ethanol, incubated in xylene and mounted with Permount. Neurodegeneration was analyzed by fluorescence (FluoroJade C) and bright field microscopy (cresyl violet) using a Nikon Eclipse E400 microscope.

2.6 Flurothyl exposure

Rats from the dose response experiments that survived TMDT-induced seizures or their respective vehicle controls were evaluated for alteration in seizure threshold to flurothyl one week later. Since P25 animals displayed similar sensitivity to TMDT as the adult rats, we used only P15 and adult TMDT survivors for this experiment. Flurothyl (bis(2,2,2-

trifluoroethyl) ether; CAS 333-36-8) is a volatile convulsant liquid acting primarily as a noncompetitive GABA_AR antagonist. Flurothyl was delivered on a filter paper placed at the top of the airtight chamber (25cm×25cm×45cm) using infusion pump with a constant rate of 30 µl/min, as described previously (Velísková et al., 2005). Flurothyl evaporated immediately and the animal breathed its vapors. Administration continued until the onset of a tonic-clonic seizure. We measured latency to onset of a first clonic and tonic-clonic seizures as well as the number of clonic seizures.

2.7 Statistical analysis

All the statistical analyses were calculated using StatView 5.0.1 for Windows (Abacus, SAS) or GraphPad Prism Software version 6.07 (GraphPad Software, Inc., CA, USA). Initially, the data were subjected to the distribution and variance tests. If the distribution was normal (Gaussian) and there were no major differences in the group variances, the data were tested either using ANOVA (multigroup protocol) with post hoc Fisher Protected Least Square Degree (PLSD) test or two-tailed Student's t test (two group protocol). Vaginal impedance was used as a covariate in the ANOVA for adult female rats to determine the contribution of individual cycle stages to the seizure susceptibility. If the data did not follow Gaussian distribution or their variances were unequal, corresponding non-parametric tests were used (Mann–Whitney and Kruskal–Wallis, respectively). Mortality was evaluated by Kaplan–Meier survival statistical analysis with a censored variable and Mantel–Cox log rank test. Finally, for incidence comparisons, we used chi-square (multiple groups) or Fisher's exact (two groups) test. Level of significance was preset to $p < 0.05$ and corrected to multiple comparisons.

3 Results

3.1 Choice of route of administration and confirmation of seizure activity by EEG

In a preliminary experiment, we compared intraperitoneal (i.p., dose range 0.2–0.6 mg/kg) and subcutaneous (s.c., dose range 0.3–0.6 mg/kg) routes of TMDT administration in adult male rats. Following i.p. injection, the dose-response curve was very steep compared to s.c. injection (Figure 1). Thus, we chose s.c. administration for subsequent examination of the TMDT poisoning syndrome to allow clearer delineation of the dose-response relationship.

The EEG recordings were performed in P15 and adult rats, sampling the youngest and oldest age groups tested in this study, to determine whether TMDT-induced seizure-like behavior is indeed associated with EEG ictal activity. Prior to TMDT administration (0.3 mg/kg for P15 and 0.6 mg/kg for adults), all channels displayed a low-amplitude asynchronized fast activity corresponding to the awake state of the animals (Figure 2). Similarly to our previous reports in mice (Shakarjian et al., 2015; Shakarjian et al., 2012). EEG activity became more synchronized following TMDT injection and included occurrence of high-amplitude discharges corresponding behaviorally to myoclonic twitches, which transitioned into a polyspike- and spike-and-wave rhythm associated behaviorally with a clonic seizure. The amplitude of EEG spike-and-wave discharges later decreased; this part behaviorally corresponded to a tonic-clonic seizure (Figure 2).

3.2 Age-specific features of TMDT-induced seizures

In all age and sex groups, the first sign of TMDT poisoning was tail curving associated with whip-like movements, and followed by several myoclonic twitches of entire body. The twitches progressed into unilateral or bilateral clonic seizures associated with tail erection (Straub tail) and preserved righting reflex. Clonic seizures were brief, less than minute-lasting seizure episodes, in all age groups in both sexes. Depending on the TMDT dose, the rats developed tonic-clonic seizures starting as a wild run followed by a loss of righting reflex, and tonic flexion and extension of all limbs followed by clonus involving all limbs. The tonic-clonic seizures were either short lasting similarly as the clonic seizures, and the animals recovered from these short seizure episodes in all age-groups. Higher doses of TMDT induced long-lasting tonic-clonic convulsions that were always terminal regardless of age or sex. Salivation, as well as bloody nose and mouth often accompanied these convulsions. In P15 rats, usually a single, sometimes two clonic seizures preceded the tonic-clonic seizure while P25 and adult rats usually developed two or more clonic seizures prior to the tonic-clonic convulsion. One or two clonic seizures occurred generally with lower TMDT doses while additional clonic seizures occurred after higher TMDT doses. Clonic seizures were usually followed by a single tonic-clonic seizure. P25 animals developed several barrel rotations during the forelimb clonus as well as during tonic-clonic seizures. These rotations were characterized by fast turning along the animal's long body axis. In adult rats, barrel rotations were less frequent and occurred only in association with tonic-clonic seizures.

3.3 Age- and sex-specific sensitivity to TMDT poisoning

In P15 pups, latencies to the first clonic and tonic-clonic seizures were dose-dependent (dose range 0.1–0.3 mg/kg, ANOVA, $F_{(4,69)}=28.665$ and $F_{(3,71)}=14.664$, respectively, both $p<0.0001$; Figure 3 A,B). Similarly, number of clonic and tonic-clonic seizures displayed dose dependence (ANOVA, $F_{(4,91)}=6.877$, and $F_{(4,90)}=42.055$, respectively, both $p<0.0001$, Figure 3 C,D). The CD50 for tonic-clonic seizure induction was 0.13 mg/kg (95% CI 0.12–0.15). There was a significant sex difference in the number of TMDT-induced clonic seizures (higher in females than in males; ANOVA, $F_{(1,91)}=7.854$; $p=0.0062$; Figure 3 C). Barrel rotations were only sparsely observed accompanying tonic-clonic seizures in P15 pups (data not shown due to insufficient number). Survival analysis demonstrated an accelerated occurrence of TMDT lethal effects with an increasing dose ($n=81$, Kaplan-Meier survival analysis with Mantel-Cox log-rank test $p<0.0001$, data not shown). The LD50 for P15 was 0.13 mg/kg (95% CI 0.12–0.15).

In P25 rats, the latency to a first clonic seizure showed dose dependence (dose range 0.3–0.6 mg/kg, ANOVA, $F_{(4,69)}=9.470$, $p<0.0001$, Figure 3 E) while the onset of tonic-clonic seizures did not (Figure 3 F). The number of clonic and tonic-clonic seizures depended upon the TMDT dose (ANOVA, $F_{(4,71)}=9.620$ and $F_{(4,71)}=28.384$, respectively, both $p<0.0001$, Figure 3 G,H). CD50 for the induction of tonic-clonic seizures was 0.40 mg/kg (95% CI 0.38–0.42). Incidence of barrel rotations was substantial in the P25 population (Figure 4 A) ranging from approximately 12–90 % (0.3–0.6 mg/kg of TMDT, respectively) with a significant effect of TMDT dose (ANOVA, $F_{(4,71)}=9.686$; $p<0.0001$) and sex (higher occurrence in females, ANOVA, $F_{(1,71)}=4.815$; $p=0.0315$). The effect of TMDT dose on

lethality was also significant ($n=48$; Kaplan-Meier survival analysis with Mantel-Cox log-rank test $p=0.0012$, data not shown). The LD50 for P25 rats was 0.42 mg/kg (95% CI 0.40–0.43).

In adult rats, similarly to P25 rats, clonic but not tonic-clonic seizure onset was dose dependent (dose range 0.3–0.7 mg/kg, ANOVA, $F_{(4,91)}$, $p=0.0019$, Figure 3 I,J), while the number of both clonic and tonic-clonic seizures was dose-dependent (ANOVA, $F_{(4,93)}$, $p=0.004$ and $F_{(4,94)}=16.474$, $p<0.0001$, respectively, Figure 3 K,L). CD50 for the induction of tonic-clonic seizures was 0.46 mg/kg (95% CI 0.43–0.48). The effect of sex was evident in the onset of the first clonic seizure with a shorter latency in females (ANOVA, $F_{(1,91)}=5.457$; $p=0.0217$; Figure 3 I). Vaginal impedance in females showed no correlation with the seizure manifestations or seizure severity following TMDT administration. There was a significant effect of TMDT dose on the occurrence of barrel rotations (ANOVA, $F_{(4,94)}=2.523$; $p=0.046$, Figure 4 B), which, however, were generally lower (10–32%) than the P25 population. No effect of sex on barrel rotation occurrence was observed in adult rats. The effect of TMDT dose on lethality was significant also in the adults ($n=84$; Kaplan-Meier survival analysis with Mantel-Cox log-rank test $p<0.0001$, data not shown). The LD50 for adult rats was 0.50 mg/kg (95% CI 0.46–0.53).

A detailed analysis of dose-response curves did not reveal any significant sex differences in 24-hr lethality and tonic-clonic seizure induction at any developmental stage tested (Figure 5). However, in the adult population, a steeper dose response curve was observed for tonic-clonic seizure induction in females as compared to males (Figure 5 F). LD50 and CD50 for tonic-clonic seizures together with corresponding 95% confidence intervals were calculated for males and females separately in all developmental stages tested and also did not show any marked sex differences (Table).

Severity score, which encompasses number and type of seizures as well as the lethality was calculated for each sex, age and dose (Figure 6 A). The data clearly demonstrate that P15 pups were more sensitive to TMDT-induced seizures and lethality compared to other age groups since they required much lower doses of TMDT to reach the same score. In pups, the severity score reached plateau at only about 0.25 mg/kg of TMDT compared to P25 animals (~0.50–0.60 mg/kg). The plateau for the adult rats was yet even higher (0.7 mg/kg), reflecting their greater resistance to the lethal effects of TMDT. The P25 and adults displayed similar thresholds to the TMDT effects, but the dose response of P25 rats was steeper than that of the adults. Interestingly, the P15 animals experienced much longer tonic-clonic seizures prior to the death (over one hour), while P25 and adult rats perished within couple of minutes after the onset of this terminal tonic-clonic seizure (ANOVA, $F_{(2,161)}=64.85$, $p<0.0001$, Figure 6 B). Comparison of occurrence of several TMDT-induced symptoms between all three age groups after administration of 0.3 mg/kg of TMDT, the only dose used in all populations, is shown in Figure 6 C. While all three age groups displayed comparable occurrence of clonic seizures at the same dose received, the P15 pups showed markedly higher incidence of tonic-clonic seizures (100%, Chi-square, $df\ 49.59$, 2, $p<0.0001$) and 24-hr death (95.5%, Chi-square, $df\ 49.91$, 2, $p<0.0001$) than P25 and adult rats (Figure 6 C), from which only 5–10% of subjects developed tonic-clonic seizures and

only 1 of 20 adult animals died within 24 hrs after TMDT administration. P25 and adult rats did not significantly differ from each other in this regard.

3.4 Neurodegeneration in TMDT survivors

We examined the entire brain of TMDT-exposed animals to screen for neuronal death in any brain region 24 hrs after TMDT intoxication. We did not observe any significant neuronal degeneration or morphological alterations by FluoroJade C staining in any brain region in the survivors (representative images of cortex, hippocampus and brainstem in Figure 7). Age and sex did not play any role in this effect or lack of thereof. Positive staining with FluoroJade C was observed in coronal brain sections obtained from the adult rat after kainic acid-induced status epilepticus serving as a positive control.

3.5 Seizure sensitivity to flurothyl in TMDT survivors

Adult and P15 rats that developed tonic-clonic seizures and survived were exposed to flurothyl one week later and compared to age-matched vehicle-injected animals. In both age groups, lower amounts of flurothyl initially induced agitation and increased exploratory activity. Thereafter with increasing doses of flurothyl, animals developed several myoclonic twitches followed by one or several separated clonic seizures of face and forelimb muscles with preserved righting ability. Clonic seizures further culminated into a single tonic-clonic affecting all four limbs, resulting in loss of righting reflex and persisting with continuous flurothyl administration. Following removal from the chamber, the animal recovered in the home cage.

In P15 animals, challenged one week after TMDT exposure (P22) to flurothyl, the mean latency to the first clonic seizure was shorter in vehicle controls compared to TMDT survivors (vehicle: 295 ± 25 s; $n=6$; TMDT: 380 ± 17 s; $n=9$; Student's *t* test, $p=0.0115$, Figure 8 A). Similarly, the adults administered the vehicle displayed shorter latencies to clonic convulsions than TMDT survivors (vehicle: 300 ± 11 s; $n=12$; TMDT: 418 ± 21 s; $n=26$; Student's *t* test, $p<0.0001$, Figure 8 B). There was no significant difference in the onset of tonic-clonic seizure between vehicle controls and TMDT survivors. On the other hand, the lag time between the clonic and tonic-clonic seizure onsets was longer in vehicle controls while it was significantly shorter in TMDT-exposed rats at P22 (vehicle: 142 ± 14 s; TMDT: 53 ± 16 s; Mann-Whitney test, $p=0.0116$, Figure 8 C) and in adults (vehicle: 241 ± 29 ; TMDT: 109 ± 28 s; Mann-Whitney test, $p=0.0044$, Figure 8 D). Control rats had in average two clonic seizures preceding the tonic-clonic seizure while only a single clonic seizure appeared in TMDT survivors of both ages (Mann-Whitney test, $p=0.0051$ for P22 and $p=0.0002$ for the adults, Figures 8 E,F).

4 Discussion

Our data demonstrate that TMDT-induced seizure behavior is accompanied by electrographic ictal activity in both developing and adult rats. We also found that infant rats are more vulnerable to developing seizures following TMDT exposure compared to prepubertal or adult rats. Females appear to develop more severe seizures than males, though the aspects of seizure severity (clonic seizure number and onset, barrel rotation frequency)

vary at different developmental stages. Interestingly, while P25 and adult rats developed barrel rotations during the TMDT-evoked seizures, this type of convulsion rarely occurred in P15 population. P25 females showed higher occurrence of barrel rotations than P25 males. This difference diminished in adult rats, which generally displayed fewer barrel rotations than P25 animals. No neuronal cell death was detected in 24-hr TMDT survivors in any age or sex tested. A second seizure challenge with flurothyl seven days after TMDT exposure showed that although the TMDT survivors displayed elevated seizure threshold for clonic seizure as compared to vehicle controls, the seizure progression from the clonic into the tonic-clonic seizure was significantly enhanced.

We demonstrate that the onset of convulsions and seizure severity following TMDT administration depend on the dose in all age groups. This is consistent with clinical data reporting that the latency time and severity of symptoms are closely related to the amount of poison received (Zhang et al., 2011). The severity of poisoning in these patients depended on the plasma concentration of TMDT. Accordingly, in mild poisonings (<50 ng/ml of TMDT), symptoms included headache, dizziness, nausea, vomiting, fatigue, twitching, and agitation. Generalized seizures occurred in moderate poisonings (50–100 ng/ml), while in severe poisonings (>100 ng/ml) status epilepticus and coma developed (Lu et al., 2008). Thus, haemoperfusion and haemofiltration, intended to reduce plasma TMDT levels, attenuated the severity of symptoms and reduced mortality and morbidity in some patients. This approach was especially effective when commenced within 12 hrs of TMDT ingestion (Dehua et al., 2006). In patients, the latent period for the effects of TMDT ranged from 10 to 30 min. The time interval between the onset of symptoms and fatal outcome lasted from 1–2 hrs (Chau et al., 2005). Our experimental data indicate similar timing: The first clonic seizure (the first serious symptom of intoxication) occurred within 8–30 min depending on the dose of TMDT. Lethality usually followed the tonic-clonic seizures and appeared within 1 hrs in P25 and adult rats following the higher doses. P15 animals were considerably more sensitive to TMDT poisoning compared to P25 and adult rats and displayed prolonged tonic-clonic seizures.

Our results are consistent with previous studies showing that sensitivity to seizures induced by GABA_AR blockade is highest in immature brain (Baram and Snead, 1990; Haut et al., 2004; Velísková et al., 2004; Velísková and Velísek, 1992; Vergnes et al., 2003). Similarly to picrotoxin- or bicuculline-induced seizures, TMDT did not induce long-lasting seizures (status epilepticus, SE) in the adult or P25 rats. Tonic-clonic convulsions lasted only couple of minutes in P25 and the adult animals, however, in P15 rats, they progressed into SE in most cases. Despite the higher susceptibility to TMDT poisoning, P15 rats showed delayed mortality (within 6 hrs) depending on the dose of TMDT. Clinical studies also report lower mortality in children with SE compared to adults (Boggs, 2004; DeLorenzo et al., 1995).

Interestingly, we did not observe barrel rotations previously in adult mice as part of TMDT-induced seizure syndrome (Shakarjian et al., 2012). In rats, barrel rotations typically represent a hallmark of hypoglycemia-associated convulsions but occur also following application of quinolinic acid, scorpion venom poisoning or intracerebroventricular administration of neuropeptides (somatostatin, vasopressin, endothelin-1) and are considered a severe seizure behavior (Chew et al., 1994; Gastaut et al., 1968; Marrannes and Wauquier,

1988; Nencioni et al., 2000; Velíšek et al., 2008). Regional brain glucose utilization studies suggest that barrel rotations mainly involve brainstem structures, i.e., inferior colliculus, pontine nuclei and vestibulo-cerebellar pathways (Chew et al., 1994; Chew et al., 1995; Doriat et al., 1998; Würpel et al., 1988). Thus, it is possible that TMDT poisoning, especially in prepubertal (P25) and also to some extent adult rats, preferentially involves brainstem structures. Further studies are needed to investigate this possibility.

Our data suggest that distinct features of TMDT neurotoxicity syndrome are sex dependent. Compared to males, P15 females developed more clonic seizures, P25 females developed barrel rotations more frequently and adult females displayed shorter latency to clonic seizure onset. These effects likely depend on sex differences in the brain (Ngun et al., 2011; Zaidi, 2010). Sex differences have been reported in other GABA_AR antagonist-induced seizures, e.g. bicuculline and picrotoxin. Bicuculline-induced seizures are associated with blood brain barrier disruption, which was present in 85% of female compared to 61% of male rats (Ozta et al., 1992). Increased blood vessel permeability, subarachnoid and cerebral haemorrhages were also found in TMDT patients (Zhang et al., 2011). Thus, we cannot rule out possible involvement of sex-specific TMDT-induced disintegration of the blood brain barrier, although clinical studies do not specifically focus on such sex differences. In studies with picrotoxin, adult female rats were also more sensitive than males (Perici and Bujas, 1997; Perici et al., 1986; Perici et al., 1985; Schwartz-Giblin et al., 1989) and this effect was dose dependent (Thomas, 1990). Females were more vulnerable to clonic and tonic-clonic seizures after a low dose of picrotoxin. Accordingly, 2.5 mg/kg of picrotoxin was subconvulsive in males but 92% convulsive in females. On the other hand, males developed picrotoxin-induced clonic seizures faster than females. A higher picrotoxin dose (4 mg/kg) did not induce death in the males, but it was 75% lethal for females. Some of the literature demonstrated a certain similarity in the response of the rats to TMDT and picrotoxin (Cao et al., 2012; Zolkowska et al., 2012); our study suggests that there are sex-specific differences in the neurotoxic activity of these two agents. Namely, with picrotoxin the sex specific effects reported were dose dependent (Thomas, 1990), with TMDT poisoning, we demonstrated rather qualitative differences in seizure severity, which varied during development. Moreover, no study so far has reported any barrel rotations associated with picrotoxin intoxication compared to our observations with TMDT.

No neuronal cell death was found throughout the developing brain, including the brainstem, confirming previous reports in adult rodents (Vito et al., 2014; Zolkowska et al., 2012). However, development of neurodegeneration requires several hours (3–24 hrs) of prolonged seizures as demonstrated in temporal lobe epilepsy models (Covolan and Mello, 2000). Despite the difficulties with obtaining the animals surviving TMDT-induced tonic-clonic seizures, those that survived developed only short episodes of tonic-clonic seizure(s) but never SE. Thus, we did not expect to find any neuronal damage from seizures per se. Nevertheless, the animals surviving TMDT poisoning showed alterations in susceptibility to acute generalized seizures induced by flurothyl seven days later. Both age groups tested displayed increased seizure threshold for clonic seizures. Our data are consistent with those of Zolkowska and colleagues (Zolkowska et al., 2012) who observed that repeated TMDT exposure did not lead to a persistent increase in seizure susceptibility. This observation differs from that demonstrated with other GABA_AR antagonists, which show long-lasting

increases in seizure susceptibility (Corda et al., 1991; Grecksch et al., 1990; Shandra et al., 1996). Yet, we found that progression from clonic to tonic-clonic seizures was more rapid in TMDT survivors compared to vehicle controls. This suggests an accelerated seizure spread from forebrain to the brainstem structures (Browning and Nelson, 1986), possibly as a result of TMDT-induced seizure activity remodeling of the neurocircuitry in structures responsible for endogenous seizure control, i.e. the substantia nigra network (Velísková and Moshé, 2006).

In conclusion, our data demonstrate that P15 rats (juveniles) represent the most vulnerable population to the neurotoxic effects of TMDT compared to the prepubertal (P25) and adult populations. Females displayed greater seizure severity than males, thus appearing the more vulnerable sex to TMDT-induced convulsions. TMDT also induces long-lasting alterations in the brain neurocircuitry in both, juveniles and adults, manifested by increased susceptibility to seizure generalization and progression when challenged by a different convulsant. Further studies are needed to shed more light on the broader long-term neurological impairments induced by this agent primarily in developing individuals and females. Overall, our data should aid in establishing more focused and tailored treatment strategies and thus better management of TMDT exposed individuals, especially the juvenile population.

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Figure 1.

Comparison of severity score after intraperitoneal (i.p.) and subcutaneous (s.c.) administration of various doses of TMDT in adult male Sprague-Dawley rats. Animals were injected with several doses of TMDT per body weight (4–10 rats per each dose) and assigned severity score 1–5.

Figure 2.

Representative image of EEG recordings from P15 (A) and adult rat (B) administered with TMDT (0.3 mg/kg for P15, 0.6 mg/kg for the adult). Upper panels display a typical baseline EEG for an awake state prior to administration of TMDT. Three channels were recorded simultaneously. LF and RO (signals from the left frontal and right occipital cortices) were recorded in unipolar mode (each versus reference electrode). The signal from right frontal (RF) and left occipital (LO) cortices was recorded as bipolar RF-LO. Synchronous EEG discharges of spike-and-wave character associated behaviorally with whole body twitches (a). Typical EEG recordings during a clonic seizure. Individual high-amplitude discharges preceded the occurrence of the clonic seizure, after which multiple spikes and spike-and-wave complexes developed. Clonic motor seizure started at the arrow (b). From a preceding clonic seizure, the rat progressed to a tonic-clonic seizure (onset of motor seizure marked by an arrow (c). The arrow points to the onset of wild run followed by a fall during the tonic phase of the seizure and a long clonus of all four limbs exceeding the duration of this sample recording associated with relatively low-amplitude spike-and-wave rhythm. (calibrations in μ V, time mark 1s).

Figure 3.

Latencies to the first clonic and tonic-clonic seizure (in seconds) and number (n) of clonic and tonic-clonic seizures in P15 (A–D), P25 (E–H) and the adult rats (I–L) as a function of TMDT dose administered (s.c.) and sex. Data for each value are displayed as mean $\pm$ SEM, n=8–12 per each dose, sex and age group. *p<0.05 represents a statistically significant difference between the groups.

Figure 4.

Barrel rotations in P25 (A) and adult (B) male and female rats. Barrel rotations are shown as a percentage of occurrence for each TMDT dose and sex. n=8–12 per each dose, sex and age group.

Figure 5.

Dose response curve analysis for 24-hr lethality in P15 (A), P25 (B), and adult (C) male and female rats, and for tonic-clonic seizure induction in P15 (D), P25 (E) and adult (F) males and females.

Figure 6.

Severity score calculated for P15, P25 and adult male and female rats (A). The average lag time between the onset of the first tonic-clonic seizure and death in the P15, P25 and adult rats (B). The occurrence of clonic and tonic-clonic seizures and 24-hr lethality following administration of 0.3 mg/kg TMDT in P15, P25 and the adult rats (C). For severity score, the data are displayed as mean $\pm$ SEM, $n=8-12$ per each dose, sex and age group. * $p<0.05$ represents a statistically significant difference relative to P15 group.

Figure 7.

Representative images from FluoroJade C and cresyl violet staining of rat brain sections from TMDT- and kainic acid-treated (12.5 mg/kg) adult 24-hr survivors. Representative images from the cortex, hippocampus and brainstem are shown. Positive FluoroJade C staining was observed only in kainic acid- but not in TMDT-treated rat. Cresyl violet staining is shown only for TMDT-exposed animal. Magnification for cresyl violet staining 40× and for FluoroJade C staining 100× (except the brainstem, both 20×). Scale bar for cresyl violet 200 μ m and for FluoroJade C staining 50 μ m (except the brainstem, both 200 μ m). n=1 rat per each sex and age group.

Figure 8.

Latencies to the first clonic and tonic-clonic seizure induced by flurothyl 7 days after exposure to TMDT or vehicle in P22 (A) and the adult survivors (B). Lag time between clonic and tonic-clonic seizure in P22 (C) and adult rats (D), and number of clonic seizures in P22 (E) and the adults (F). Data for each value are displayed as mean $\pm$ SEM, $n=6-9$ for P22, $n=12-26$ for the adults. * $p<0.05$ represents a statistically significant difference between the TMDT treatment and corresponding control group.

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Table

Median lethal dose (LD50) and convulsive dose 50 for tonic-clonic seizure induction (CD50) and 95% confidence intervals (CI) for both sexes within each age group

		P15	95% CI	P25	95% CI	Adults	95% CI
LD50	males	0.131	0.1113–0.1544	0.417	0.3936–0.4412	0.495	0.4529–0.5398
	females	0.150	0.1343–0.1685	0.417	0.3948–0.4403	0.500	0.4419–0.5646
CD50	males	0.123	0.1041–0.1449	0.404	0.3757–0.4333	0.470	0.4274–0.5173
	females	0.132	0.1178–0.1467	0.403	0.3737–0.4343	0.442	0.4090–0.4768