

Flaxseed Oil Attenuates Trimethyltin-induced Neurodegeneration via Down-regulation of Inflammatory Activity of Astrocytes

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Abstract

Trimethyltin chloride (TMT) is an organotin neurotoxicant that selectively targets the hippocampus, and induces selective and progressive neuronal loss, gliosis, neuroinflammation and cognitive deficits, thus resembling critical features of Alzheimer's diseases (AD). Flaxseed oil (FSO) is anti-inflammatory agent with potent neuroprotective properties. Therefore, the presented study was designed to evaluate the protective effects of flaxseed oil (FSO) continuous pretreatment to alleviate TMT- (8 mg/kg) induced neurodegeneration. Ovariectomized (OVX) female rats were pretreated with FSO (1 ml/kg, orally) for two weeks. At day 14, part of animals received single dose of TMT (8 mg/kg, i.p.) and application of FSO continued for seven more days. Data have convincingly shown that FSO counteracted TMT effects. Specifically, daily administration of FSO improved TMT- induced behavioral manifestations manifested as hyper-excitability, and hyper-responsiveness, reduced neuronal loss, ameliorated expression of pro-inflammatory cytokines (tumor necrosis factor (TNF)- α , interleukin (IL)-10, and IL-6), alleviated astrogliosis and A1-like astrocytes conversion which was related to down-regulation of aromatase/estrogen receptor α signaling, and microgliosis. Together, these findings support beneficial neuroprotective properties of FSO against TMT-induced neurotoxicity and hint at a promising preventive use of FSO in hippocampal degeneration and dysfunction.

Introduction

A number of basic research studies and clinical trials have shown that inflammatory response mediated by glial cells may be one of the mechanisms by which common neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease (PD) etc. occur or worsen (Nordengen et al. 2019; Kwon and Koh 2020). In general, neurodegenerative diseases are characterized by an initial acute neuroinflammatory response that is followed by a self-reproductive cascade of pathogenic events that cause chronic neuritis. Trimethyltin chloride (TMT) is an organotin neurotoxicant that selectively targets the limbic region, particularly the hippocampus (Lee et al. 2016b; Pompili et al. 2020). Compiling evidence show that TMT intoxication induces selective and progressive hippocampal neurodegeneration and triggers an immediate reactive response of astrocytes, followed with sustained astrogliosis (Haga et al. 2002; Röhl and Sievers 2005; Geloso et al. 2011; Lee et al. 2016b; Pompili et al. 2020; Dragić et al. 2021), resulting in cognitive impairments, mental confusion, memory defects, and seizures (Geloso et al. 2011; Lee et al. 2016b; Pompili et al. 2020). Accordingly, TMT represents a useful tool to recapitulate critical features of most common neurodegenerative disorders such as AD or temporal lobe epilepsy (Piacentini et al. 2008; Geloso et al. 2011; Lee et al. 2016b; Pompili et al. 2020).

In response to injury, astrocytes assume reactive states that may be discriminated based on the proliferation, and induction of pro-inflammatory mediators and reactive oxygen species organized to forms borders around areas of tissue damage or inflammation, and non-proliferative astrocytes that retain the basic cell structure, tissue architecture, and functional interactions established in healthy tissue (Sofroniew 2020). With the analogy to reactive states of microglia, based on their molecular signature these two broad reactive astrocyte subtypes are also classified as A1-like pro-inflammatory and A2-like

anti-inflammatory astrocytes (Zamanian et al. 2012). We previously found that astrocytes change their morphology early after TMT-intoxication (Dragić et al. 2019), up-regulate transcription factors and inflammatory mediators (Dragić et al. 2019, 2021), typical for neurotoxic A1-like astrocytes (Zamanian et al. 2012; Liddelow and Barres 2017). Specifically, TMT induces up-regulation of NF-κB and its target pro-inflammatory genes, such as interleukin (IL)-1 β , IL-10, IL-6, tumor necrosis factor- α (TNF- α) that was limited to activated astrocytes (O'Callaghan et al. 2014; Haim et al. 2015; Dragić et al. 2020, 2021). Also, TMT robustly elevate complement C3 (C3) which is highly up-regulated in A1-like reactive astrocytes (Zamanian et al. 2012; Liddelow and Barres 2017; Dragić et al. 2020, 2021). Furthermore, such activated astrocytes probably precedes microglial activation (Röhl and Sievers 2005; Dragić et al. 2019, 2020).

Another characteristic of injury- induced activation of astrocytes is up-regulation of aromatase (Arom; estrogen synthase) (Duncan and Saldanha 2019), followed by local increase in 17 β -estradiol (E2) levels as well as expression of estrogen receptor-alpha (ER α) (Blurton-jones and Tuszyński 2001; García-Ovejero et al. 2002; Arimoto et al. 2013; Morgan and Finch 2015; Duncan and Saldanha 2019; Wang et al. 2020), which play key role in the regulation of reactive astrogliosis (Wang et al. 2020). Collectively, astrocyte activation and neuroinflammation contribute to the pathogenesis of TMT-induced cognitive decline and neurotoxicity, and specific prophylactic/therapeutic strategies actively targeting activated/pro-inflammatory astrocytes might be of great relevance.

Since their broad spectrum of pharmacological and biological activities, natural products may be promising alternatives for prevention/amelioration of neuroinflammation accompanying neurodegenerative diseases. Flaxseed oil (or linseed oil, FSO), derived from the seeds of the flax (*Linum usitatissimum L.*, FS) is used worldwide as a functional food. It contains the highest amounts of an essential omega-6 (ω -6/n-6) and omega-3 (ω -3/n-3) polyunsaturated fatty acids (PUFAs) obtained from plants such as linoleic acid (LA) and -linoleic acid (ALA), respectively, but also lignans, proteins, and vitamin E (Parikh et al. 2019a). FSO consumption improve cardiovascular and metabolic diseases, and decrease risk of mammary and prostate gland cancers (Kajla et al. 2015; Parikh et al. 2019a). Moreover, FS/FSO are beneficial for menopausal women (Parikh et al. 2019a) who experience an increased risk of cardiovascular diseases, neurological and psychiatric disorders, osteoporosis, and other sequel (Shuster et al. 2010). It was shown that FS/FSO are a potent neuroprotective agents through their properties to ameliorate depressive-like behavior (El Tanbouly et al. 2017; Han et al. 2020), improve functional motor recovery after ischemic stroke (Bagheri et al. 2017), reduce oxidative stress (Bhatia et al. 2006; Badawy et al. 2015; Ismail et al. 2016), and neurotoxicity (Abdel Moneim 2012). Furthermore, as components of FSO, individually PUFAs have a potent anti-inflammatory effect after different central nervous system (CNS) injuries. Thus, n-3 PUFAs restored spatial memory loss and protected neurons by suppressing up-regulation of pro-inflammatory cytokines, selectively targeted pro-inflammatory microglia and/or astrocytes and reduced their reactivity reverting activated cells to the physiological state (Crupi et al. 2012; Labrousse et al. 2012; Park et al. 2012). Given that anti-inflammatory effects of individually PUFAs, as major components of FSO, are selectively directed to attenuation of pro-inflammatory activity of glial cells, anti-inflammatory effects of FSO in CNS, as a complex of a number of PUFAs, are scarce. Therefore, we hypothesized that supplementation with FSO may affect astrocytes as the main target of TMT

intoxication (Aschner and Aschner 1992; Dragić et al. 2021) and consequently reduce neuroinflammation, neuronal loss and microglial activation thereby attenuating TMT-induced behavioral alterations.

Methods

Animals

All experiments were conducted on 10-week-old female Wistar rats (200–220 g) obtained from local colony maintained in the animal facility of the VINČA Institute of Nuclear Sciences - National Institute of Republic of Serbia, University of Belgrade.

Appropriate actions were taken to alleviate the pain and discomfort of the animals in accordance with the compliance with European Communities Council Directive (2010/63/ EU) for animal experiment, and the research procedures were approved by the Ethical Committee for the Use of Laboratory Animals of VINČA Institute of Nuclear Sciences - National Institute of Republic of Serbia, University of Belgrade, Belgrade, Republic of Serbia (Application No.

02/11; 323-07-02057/2017-05). Animals were housed (3–4 /cage) under standard conditions: 12-h light/dark regime, constant ambient temperature ($22 \pm 2^\circ\text{C}$), and free access to food and water.

Surgical procedure and treatments

A total of 40 animals were used for the experiments. To avoid fluctuations of sex hormones during the estrus cycle, the experiments were performed on female rats submitted to the bilateral ovariectomy (OVX) under ketamine/xylazine anesthesia (50 mg/kg and 5 mg/kg), as previously described (Mitrović et al. 2016; Grković et al. 2019). One week after the surgery, control OVX females (Ctrl group) received as pretreatment (Fig. 1): flaxseed oil (Granum®, commercial, cold pressed, 1 ml/kg, orally, FSO group) for two weeks. At day 14 (Fig. 1), part of untreated Ctrl animals and animals pretreated with FSO received single dose of TMT (8 mg/kg, i.p.; TMT, FSO + TMT, respectively) (Corvino et al. 2015; Dragić et al. 2019) and application of FSO continued for seven more days. A Ctrl group received the same volume of saline. Thus, animals were sacrificed after total of 4 weeks on the study protocol. Schematic illustration of the experimental design is shown in Fig. 1. The dose for FSO was chosen based on literature data showing its beneficial effects in various organ systems in males and females (Gorriti et al. 2010; Kaithwas and Majumdar 2012; Singh et al. 2012; El Makawy et al. 2018; Youness et al. 2019). The fatty acid composition of FSO was provided by manufacturer and is presented in Table 1. All animals were decapitated by a small animal guillotine (Harvard apparatus) 24 h after the last injection, and brains were isolated for tissue processing.

Table 1
Fatty acid composition of Flaxseed oil

Fatty acid	g/100g FSO
Linoleic acid	42.93
α-linoleic acid	28.78
Oleic acid	16.73
Palmitic acid	6.16
Stearic acid	4.36
Arachidic acid	0.16
Behenic acid	0.14
Eicosenoic acid	0.13
γ-linoleic acid	0.11
Margaric acid	0.09
Palmitoleic acid	0.08
Eicosadienoic acid	0.05

^aFatty acid content was analyzed by Gas Chromatography with Flame-Ionization Detection

Hyperactivity/tremor assessment

TMT induces specific behavioral pattern, which includes hyper-excitability, tremors, and seizures (Trabucco et al. 2009; Geloso et al. 2011; Dragić et al. 2019). The behavioral assessment was performed daily by scoring all animals in brightly lit arena (40 × 40 cm, 250 lux), during 5-min interval using the arbitrarily defined scale for a hyperactivity/tremor, as following: (0) normal motor activity, (1) hyperactivity and hyper-responsiveness (2) mild tremor with normal motor activity, (3) systemic tremor (Trabucco et al. 2009; Dragić et al. 2019).

Tissue processing for histology, immunohistochemistry and immunofluorescence

For histology and immunohistochemistry, brains (n = 5 animals /group) were carefully removed from a skull and fixed in 4 % paraformaldehyde/0.1 M phosphate buffer (pH 7.4). After cryoprotection in graded sucrose solutions (10–30 % in 0.2 M phosphate buffer) at 4°C, 18-μm thick hippocampal coronal sections were mounted on gelatin-coated slides, air-dried for about 2 hours and stored at – 20 °C until use. Sections of the dorsal hippocampus were taken starting from level Bregma 2.6 to 3.6 mm, according to the rat stereotaxic atlas of Paxinos and Watson (Paxinos and Watson 2004). One series of coronal sections was stained with 0.1 % thionin blue (Thionin powder, T-409, Fisher Chemicals), and the digital

images were acquired using LEITZ DM RB light microscope (Leica Mikroskopie& Systems GmbH, Wetzlar, Germany), a LEICA DFC320 CCD camera (Leica Microsystems Ltd., Heerbrugg, Switzerland) and LEICA DFC Twain Software (Leica, Germany).

Fluoro-Jade C (FJC) staining was performed as previously described (Dragić et al. 2019). The FITC filter system was used for visualization of FJC staining at 20 x magnifications and examined under Zeiss Axiovert microscope (Carl Zeiss GmbH, Vienna, Austria).

Immunohistochemistry was performed as previously described (Dragić et al. 2019). Sections were probed with goat anti-rat ionized calcium-binding adapter molecule-1 (IBA-1) antibody (1:500 dilution, Abcam, UK, ab5076) overnight at 4 °C. After washing in PBS, sections were incubated with goat anti-mouse HRP-conjugated secondary antibody (1:150 dilutions, R&D Systems, HAF007). The signal was visualized with the use of 3, 3'-S-diaminobenzidine-tetrahydrochloride kit (DAB, Abcam, UK) as a chromogen for HRP-conjugated secondary antibodies. Digital images of DAB-stained microglia were acquired using LEITZ DM RB light microscope (Leica Mikroskopie & Systems GmbH, Wetzlar, Germany), a LEICA DFC320 CCD camera (Leica Microsystems Ltd., Heerbrugg, Switzerland) and LEICA DFC Twain Software (Leica, Germany).

Double Immunofluorescence labeling was performed as described previously (Dragić et al. 2019, 2020). The sections were immunostained for ERα by using rabbit anti-rat ERα antibody (1:50, Santa Cruz Biotechnology, TX, USA sc-543, 4 °C over night) and donkey anti-rabbit Alexa Fluor 555 antibodies (1:400, Invitrogen, CA, USA ab162543, 2 hours at RT). The sections were counterstained for IBA1 by using goat polyclonal anti- IBA1 antibodies (1:500 dilution, Abcam, UK ab5076), and donkey anti goat Alexa Fluor 488 antibodies (1:400 dilution, Invitrogen, CA, USA, ab 142672).

Triple immunofluorescence labeling was performed with ERα by using rabbit anti-rat ERα antibody (1:50, Santa Cruz Biotechnology, TX, USA sc-543, 4 °C over night) and donkey anti-rabbit Alexa Fluor 555 antibodies (1:400, Invitrogen, CA, USA ab162543, 2 hours at RT), mouse anti-rat GFAP antibodies (1:100 dilution, UC Davis/NIH NeuroMab Facility 73–240, ab10672298) and donkey anti-mouse Alexa Fluor 647 antibodies (1:400 dilution, Invitrogen, CA, USA, A-31571), anti- complement C3 antibody (1:100 dilution, Thermo Fisher Scientific, USA, PA1-29715) and donkey anti-goat Alexa Fluor 488 (1: 400 dilution, Invitrogen, USA, A-11055). The primary and secondary antibodies were applied separately. Sections were mounted in Mowiol (Calbiochem, CA, USA) and digital images were taken on confocal laser-scanning microscope (LSM 510, Carl Zeiss GmbH, Germany), using Ar Multi-line (457, 478, 488 and 514 nm) and HeNe (543 nm) lasers, under 63x, 40x or 20x magnification (x2 digital zoom), equipped with AxioCam ICm1 camera (Carl Zeiss GmbH, Germany). FSO did not affect examined proteins in Ctrl animals (Ctrl + FSO) (data not shown).

Gene expression analysis by RT-qPCR

Total RNA from the whole hippocampal formation (n = 5 animals/group) was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's protocol. The concentration and the

purity of the RNA were assessed using OD260 and OD260/OD280 ratio, respectively. Complementary DNA (cDNA) species were synthesized using High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific, MA, USA), as previously described (Grković et al. 2019; Dragić et al. 2020). Quantitative real-time PCR was performed using Power SYBR™ Green PCR Master Mix (Applied Biosystems, MA, USA) and ABI Prism 7000 Sequence Detection System (Applied Biosystems, MA, USA) as described previously (Grković et al. 2019; Dragić et al. 2020). Primer sequences used for the amplification are given in Table 2. Relative quantification was performed using the comparative $2^{-\Delta Ct}$ method, using peptidylprolyl isomerase A, also known as cyclophilin A (CycA), as a reference gene. Samples obtained from 5 animals for each experimental group were run in duplicate. In each run, internal standard curves were generated by several fold dilutions of generated cDNA to check amplification efficacy. Melt curve analysis was performed at the end of every experiment to confirm the formation of a single PCR product.

Table 2
Primer sequences used for RT-qPCR

Gene	Sequence (5'-3')	Length (bp)
Caspase-3 (<i>Casp3</i>)	GATGTCGATGCAGCTAACCT TGTCTCAATACCGCAGTCC	321
Bax (<i>Bax</i>)	TGCTACAGGGTTTCATCCAG CCAGTTCATGCCAATTG	135
Bcl-2 (<i>Bcl2</i>)	TGGAAAGCGTAGACAAGGAGATGC CAAGGCTCTAGGTGGTCATTGAG	88
GFAP (<i>Gfap</i>)	CGGCTCTGAGAGAGATTGCG GGTCTGCAAACCTGGACCGA	120
<i>C3</i> (<i>C3</i>)	GCGGTACTACCAGACCATCG CTTCTGGCACGACCTTCAGT	166
Aromatase (<i>Cyp19a1</i>)	CTCAACCTCACCAACGGATGT-3' GGCTCTCTGGATGGATGCTC-3'	93
ERα (<i>Esr1</i>)	GCGCAAGTGTACGAAGTGG AGTGCCCATTTCATTTCGGC	121
<i>TNF-α</i> (<i>Tnf</i>)	CCCCCATTACTCTGACCCCT CCCAGAGCCACAATTCCCTT	88
<i>IL-10</i> (<i>Il10</i>)	GCTCAGCACTGCTATGTTGC GTCTGGCTGACTGGGAAGTG	106
<i>IL-6</i> (<i>Il6</i>)	GCCCACCAGGAACGAAAGT GGCAACTGGCTGGAAGTCTC	86
CycA (<i>Ppia</i>)	GGCAAATGCTGGACCAAACAC TTAGAGTTGTCCACAGTCGGAGATG	196

Data analysis

qPCR data were analyzed in Prism – GraphPad software package with two-way ANOVA followed by non-parametric Kruskal-Wallis test. The values of $p < 0.05$ or less were considered statistically significant.

Results

FSO pretreatment alleviated TMT-induced behavior symptoms

The behavioral score have changed over the 7-day assessment period as early stages of TMT intoxication. Behavioral severity score exhibited a bell-shaped curve which peaked at day 4 and decreases at day 7 (Fig. 2) as shown previously (Dragić et al. 2019). Animals also develop aggressive behavior after TMT poisoning, peaking at day 4: avoids hand by running and/or struggles when captured, or leaps, struggles, and bites when captured (Lee et al. 2016b; Dragić et al. 2019). The FSO-treated animals exhibited significantly milder behavioral symptoms which resulted in a flattened behavioral score curve (Fig. 2), and reduced aggressive behavior.

FSO pretreatment attenuated TMT-induced neurodegeneration

The pattern of TMT-induced neuronal death was consistent with our previous study (Dragić et al. 2019). Since the differences between Ctrl (OVX) and Ctrl + FSO (OVX treated with FSO) group were insignificant (data not shown), comparisons were made in respect to Ctrl. Dying and/or necrotic neurons, apoptotic cells bodies and significant gliosis were observed in the *pcl* of CA1, in the hilus of DG and proximal part of CA3 region (hilus/pCA3) (Fig. 3a). On the contrary, in the FSO + TMT group, regular laminar organization and the absence of necrotic cells were observed in the hippocampal CA1 and the hilus, although with some level of gliosis in the hilar/pCA3 subfield of DG (Fig. 3a).

Further, TMT-induced neurodegeneration was analyzed by FJC staining (Fig. 3b). Ctrl and Ctrl + FSO (data not shown) sections did not show the presence of FJC-positive cells, while in the TMT group strong fluorescence staining suggested significant neuronal degeneration and cell loss in *pcl* of CA1 and in the hilar/pCA3 subfield of DG (Fig. 3b). Prolonged treatment with FSO ameliorates the effects of TMT and prevents the neuronal cell loss (Fig. 3b).

Since FSO treatment induced significant neuroprotective effect and prevented TMT-induced neurodegeneration, we further analyzed the cell death signaling pathway by qRT-PCR (Fig. 3c), while the statistical significance was determined by two-way ANOVA. Significant effects of TMT intoxication ($F(1, 15) = 18.56; P < 0.001$), FSO treatment ($F(1, 15) = 6.094; P < 0.05$) and their interaction ($F(1, 15) = 8.401; P < 0.05$) were observed for the expression of Caspase3 (Casp3)- mRNA. Similarly, significant effect of TMT intoxication ($F(1, 15) = 135.7; P < 0.001$), FSO treatment ($F(1, 15) = 160.9; P < 0.001$) and their interaction ($F(1, 15) = 125.7; P < 0.001$) were observed for Bax-mRNA expression. For Bcl-2-mRNA expression, significant effect of FSO treatment ($F(1, 16) = 9.717; P < 0.05$) and the interaction ($F(1, 16) = 19.69; P < 0.01$) were observed, without statistical significance of TMT ($F(1, 16) = 2.234; P = 0.1544$). Post hoc analyses revealed that TMT induced up-regulation of Casp3- ($p < 0.01$) and Bax- mRNAs ($p < 0.01$), and down-regulation of Bcl-2 mRNA ($p < 0.01$) in respect to Ctrl (Fig. 3c), while the treatment with FSO counteracted the alterations of target genes expression relative to TMT. Namely, the abundances of

Casp3- ($p < 0.01$) and Bax- mRNA ($p < 0.001$) decreased, while the Bcl-2- mRNA ($p < 0.001$) significantly increased in TMT animals treated with FSO compared to TMT animals (Fig. 3c).

FSO pretreatment attenuated TMT-induced inflammation and astrocytes reactivity

Early astrocyte activation precedes TMT-induced neuronal loss (Dragić et al. 2019). Since we previously reported that reactive astrocytes are the main source of pro-inflammatory cytokines (Dragić et al. 2020, 2021), we further investigated whether FSO pretreatment attenuates TMT-induced astrocytes pro-inflammatory response. Figure 4 shows the effect of FSO pretreatment on the expression of main pro-inflammatory cytokines (TNF- α , IL-10, and IL-6). For TNF α -mRNA - the effect of TMT ($F(1, 15) = 12.45; P = 0.0030$), treatment ($F(1, 15) = 114.9; P < 0.0001$) and interaction ($F(1, 15) = 12.70; P = 0.0028$) was observed, while for IL-10 mRNA significant effect of TMT ($F(1, 16) = 28.784; P < 0.0001$) and FSO treatment ($F(1, 16) = 21.17; P = 0.0003$) was observed, but without interaction ($F(1, 16) = 4.391; P = 0.0524$). For IL-6- mRNA – the effect of TMT ($F(1, 196) = 26.17; P = 0.0001$), FSO treatment ($F(1, 16) = 51.05; P < 0.0001$) and the interaction ($F(1, 16) = 28.92; P < 0.0001$) was observed. Post hoc analyses showed that TMT induced significant up-regulation of TNF α -, IL-10-, IL-6- mRNA ($p < 0.001$ significance for all examined genes) relative to Ctrl as we reported previously (Dragić et al. 2020, 2021), which is fully prevented with FSO treatment ($p < 0.001$ for TNF α and IL-6, $p < 0.01$ for IL-10) (Fig. 4), suggesting that FSO may attenuate astrocyte expression of pro-inflammatory cytokines.

Furthermore, we examined expression of astrocyte marker (GFAP) and marker of pro-inflammatory A1-like reactive astrocytes (C3) at both gene and cellular levels (Fig. 5). For GFAP and C3 target genes significant effects of TMT, FSO treatment and the interaction were obtained: GFAP- mRNA- significant effects of TMT ($F(1, 15) = 87.49; P < 0.0001$), treatment ($F(1, 15) = 61.21; P < 0.0001$) and the interaction ($F(1, 15) = 142.8; P < 0.0001$) and for C3-mRNA- the effect of TMT ($F(1, 14) = 56.26; P < 0.0001$), FSO treatment ($F(1, 14) = 46.49; P < 0.0001$) and the interaction ($F(1, 14) = 23.46; P = 0.0003$). Post hoc analyses showed that TMT induced significant up-regulation of GFAP mRNA ($p < 0.001$) (Fig. 5a) and C3 mRNA ($p < 0.001$) (Fig. 5a) relative to Ctrl, while FSO pretreatment attenuated GFAP-, and C3- mRNA ($p < 0.001$ for both GFAP and C3) in respect to TMT, suggesting that FSO may attenuate pro-inflammatory, A1-like astrocyte activation.

Since E2 and ER α are direct regulators of GFAP transcription and ultimately major contributors to astrocyte activation (Arimoto et al. 2013; Wang et al. 2020), the possible modulation of hippocampal local E2 production was also explored through the analysis of the expression levels of Arom (the key enzyme involved in E2 biosynthesis) and ER α , which acts as transcription factor towards GFAP promotor sequence (Arimoto et al. 2013). For all target genes, significant effects of TMT, FSO treatment and the interaction were obtained: Arom-mRNA – the effect of TMT ($F(1, 15) = 8.52; P = 0.0106$), FSO treatment ($F(1, 15) = 9.28; P = 0.0081$) and the interaction ($F(1, 15) = 8.065; P = 0.0124$); for ER α -mRNA - the effect of TMT ($F(1, 16) = 6.61; P < 0.05$), treatment ($F(1, 16) = 10.92; P < 0.01$) and interaction ($F(1, 16) = 5.962; P < 0.05$). Post hoc analyses showed that TMT induced significant up-regulation of transcripts coding for

aromatase ($p < 0.01$) and ER α ($p < 0.01$) relative to Ctrl (Fig. 5a), while treatment of TMT animals with FSO significantly decreased relative abundance of Arom- ($p < 0.01$) and ER α - mRNA ($p < 0.01$) compared to TMT.

To confirmed cellular localization of harmful astrocytic marker C3 as well as ER α , we performed triple immunofluorescence labeling. In Ctrl group, GFAP $^+$ astrocytes were labeled throughout the entire structure, displaying normal, stellate morphology with numerous fine-branched processes radiating in all directions (Fig. 5b, c). No GFAP $^+$ /C3 $^+$ cells were observed in the hippocampus of OVX rats. C3-ir was present in the perikaryal membrane (Fig. 5b, c) in accordance with literature data (Kumar and Dhar 2020). As already reported in our previous work (Dragić et al. 2019), seven days after TMT intoxication two distinct astrocyte morphotypes were observed; hypertrophied astrocytes in CA1 region (Fig. 5b), and atrophied-like astrocytes in hilar/pCA3 subfield (Fig. 5c). The response of astrocytes to TMT resulted in an increase of GFAP $^+$ /C3 $^+$ cells in CA1 (Fig. 5b) and hilar/pCA3 subfields (Fig. 5c). After FSO pretreatment, GFAP $^+$ /C3 $^+$ cells were no longer observed in investigated hippocampal regions (Fig. 5b, c).

At Ctrl sections, ER α immunofluorescence (ir) was primarily localized at the hippocampal neurons in *pc/l* of CA1 region (Fig. 5b), and *gcl* of DG (Fig. 5c). TMT intoxication induced complete disappearances of ER α -ir from disorganized neuronal layer in CA1 (Fig. 5b) and the hilar/pCA3 subfield of DG and ER α -ir was only observed in glial cells (Fig. 5b, c). All GFAP $^+$ /ER α $^+$ astrocytes were also C3 $^+$. FSO pretreatment prevented loss of ER α -ir in neurons from CA1 and hilar/pCA3 subfield of DG. However sporadic astrocytes retain ER α -/GFAP- ir in hilar/pCA3 subfield (Fig. 5b, c).

The effects of FSO pretreatment on TMT-induced microgliosis

Given that astrocytes activate microglial cells and precedes their activation (Röhl and Sievers 2005; Dragić et al. 2020), we examined IBA-1 imunoreactivity as an indicator of microgliosis, and weather FSO pretreatment prevents activation of microglia. A homogeneous distribution of IBA-1-immunoreactive (ir) microglial cells throughout the hippocampus of Ctrl group was noticed (Fig. 6). Higher magnification image of CA1 (Fig. 6) and hilar/pCA3 subfield of DG (Fig. 6) showed that IBA-1 $^+$ microglia cells had a typical ramified morphology with small cell bodies, highly branched and elongated processes. Seven days post-TMT intoxication, total IBA-1-ir intensity increased delineating mostly rod-shaped, bushy and amoeboid microglial cells in CA1 (Fig. 6). Also, microglial cells of mixed morphology gradually populated DG (Fig. 6). Repeated FSO pretreatment prevented overall increase in IBA-1-ir cells observed after TMT (Fig. 6). Although occasional microglia with enlarged cell bodies in CA1 subfield (Fig. 6) as well as sporadic clusters of these cells in the hilar/pCA3 subfield of DG might be observed (Fig. 6). FSO noticeably attenuated microglia activation.

Discussion

Flaxseed oil is abundant in many nutrients, such as polyunsaturated fatty acid, protein, vitamins, and lignans, and it is eminent by α-linolenic acid in high content, which recently has been found as chiefly vital for human organism (Parikh et al. 2019a). To our knowledge, this is the first study that investigates dietary flaxseed oil in the animal model of TMT-induced neurodegeneration and neuroinflammation. The data demonstrate that dietary flaxseed oil given to animals before and over the course of TMT- induced neuropathology (a) reduced hippocampal neuronal loss, (b) exerted an anti-inflammatory effect by preventing the activation of astrocytes and microglia and thus (c) prevented the occurrence of TMT- induced behavioral signs manifested as hyper-excitability, and hyper-responsiveness. Since FSO was administered before and over the course of TMT- induced neuropathology, it is not possible to determine if the primary beneficial effect was preventative or therapeutic. Although there is a growing body of literature that indicates the pernicious effects of TMT, there are still no specific antagonists or clinical strategies for patients poisoned with TMT. Furthermore, TMT induces a spectrum of neurological deficits, including seizures, behavioral alterations and cognitive impairment (Trabucco et al. 2009; Geloso et al. 2011; Corvino et al. 2015; Pompili et al. 2020) resembling behavioral and neurological symptoms, and memory dysfunction similar to AD and temporal lobe epilepsy (Lee et al. 2016b; Pompili et al. 2020).

Trimethyltin chloride induces behavioral signs manifested as hyper-excitability and hyper-responsiveness causing behavioral signs typical for TMT intoxication, when mild tremor progressed into the systemic one during the first week of intoxication, as previously reported (Lee et al. 2016a; Dragić et al. 2019).

Application of FSO exerts a remarkable neuroprotective effect against TMT-induced neurobehavioral changes. Therefore, pre-TMT administration of FSO that continuous over the course of TMT- induced neuropathology was used and proven to be effective in our study. Data from our histopathological observations indicate that TMT induces remarkable neuronal loss in the hippocampal *pc*/*l* of CA1 and the hilar/pCA3 subfield of DG. These changes were consistent with those observed in previously reported studies (Geloso et al. 2011; Corvino et al. 2015; Dragić et al. 2019). FSO pretreatment mitigates neuronal loss and ameliorates morphological injuries in the hippocampus of TMT-treated rats as well as apoptotic markers. These results suggested that FSO could protect against TMT neurotoxicity by preserving neurobehavioral function and ameliorating histopathological changes and cell death. Based on available literature data, it is most likely that TMT disturbs excitatory/inhibitory synaptic balance critical for hippocampal information processing (Geloso et al. 2011; Corvino et al. 2015; Lee et al. 2016b), causing behavioral signs typical for TMT intoxication. It is known that observed neurodegenerative changes occur as a result of mitochondrial dysfunction, which stimulate hippocampal neuronal death probably through activation of caspases 8/caspase 3 death pathway (Kuramoto et al. 2011; Lee et al. 2016b). It is known that treatment with component of FSO - ALA, resulted in a down-regulation of caspase-3 expression, reduced Bax translocation to mitochondria and cytochrome c release into the cytosol thereby lessening the neuronal injury after traumatic brain injury (Wei et al. 2015). Conjugated LA (18:2 n-6) also protected neurons from mitochondrial dysfunction and demise (Hunt et al. 2010). Specifically, treatment of cortical neurons with this fatty acid following excitotoxic glutamate exposure resulted in decreased glutamate- induced loss of mitochondrial function, increased Bcl-2 expression, and prolonged neuronal survival. Flaxseed is the richest plant source of n-3 and n-6 PUFAs, especially ALA and LA, respectively (Parikh et

al. 2019a). Therefore, the mechanism responsible for the healing effects of dietary flaxseed on the TMT-induced hippocampal neuronal loss, likely involves, directly or indirectly, this rich content of PUFAs. Our study complemented the knowledge of the anti-apoptotic actions of FSO by demonstrating that the oil pretreatment reduced the early TMT-induced cell death in the hippocampus, most probably by turning down caspase-3 signaling pathway, reducing expression of pro-apoptotic factor Bax, and enhancing expression of Bcl-2, suggesting that the oil preserved mitochondrial integrity and function.

Neuroinflammation after TMT intoxication is mainly characterized by activation of astrocytes and increased production of pro-inflammatory cytokines (Haga et al. 2002; Little et al. 2002; Lattanzi et al. 2013; Lee et al. 2016b; Dragić et al. 2020, 2021). We previously found that TMT induces an increase in number of reactive astrocytes (Dragić et al. 2019) and elevates expression of pro-inflammatory cytokines, including TNF- α , IL-10, and IL-6 in reactive astrocytes (Dragić et al. 2020, 2021). FSO pretreatment induced a significant reduction in investigated inflammatory cytokines. Several studies have shown that FSO provides a wide array of health benefits including protection against inflammation in peripheral tissues. For example, FSO inhibited expression of TNF- α , and IL-6, expression during cardiovascular disease (Dupasquier et al. 2007; Parikh et al. 2019b). However, previous studies have not specifically examined the effects of FSO on the TMT-induced neuroinflammation and thus underlying mechanism remains unclear. Together with pro-inflammatory cytokines, FSO also suppressed TMT-induced up-regulation of C3 on astrocytes, thus preventing harmful, pro-inflammatory A1-like astrocytes conversion. The present study indicates that FSO could induce the beneficial inhibition of neuroinflammation and could provide another path for the study of the anti-neuroinflammatory actions of FSO.

In response to injury, aromatase is primarily expressed in astrocytes and locally synthesized E2 regulates/modulates astrocyte activation via ER α (Arimoto et al. 2013; Duncan and Saldanha 2019; Wang et al. 2020). Up-regulation of aromatase and ER α in activated astrocytes as well as GFAP expression observed after TMT intoxication, may be a generalized response to injury, since this phenomenon is observed in different types of CNS pathologies, like AD, penetrating brain injury, but also kainic acid model of temporal lobe epilepsy (Blurton-jones and Tuszynski 2001; Dubal et al. 2001; Lu et al. 2003). Thus, by preventing aromatase/ER α signaling, FSO might prevent astrocyte activation and its conversion towards pro-inflammatory A1-like phenotype, probably through inactivation of ER α which acts as a transcription factor over GFAP and significantly decreased downstream inflammatory cascade, ultimately lessening the neuronal damage.

TMT directly affect astrocytes which in turn activate microglial cells (Röhl and Sievers 2005), thus we may assume that by preventing TMT-induced astrogliosis, FSO exhibits indirect effects toward activated microglial cells and prevent their activation. However, a portion of activated ER α^+ /GFAP $^+$ astrocytes and microglia remained in the hilar/pCA3 subfield of DG despite FSO pretreatment. This observation may imply that FSO could not completely protect hippocampus from primary injury caused by strong neurotoxin such as TMT, but prevents from secondary injury which contributes to degeneration of CA1 sub-region (Trabucco et al. 2009).

Conclusion

In conclusion, the current findings indicate that administration of FSO may have anti-inflammatory and neuroprotective potential as a nutrient that may prevent neuronal injury and development of neurodegenerative disorders, such as AD. Results obtained here represent a model to further investigate the efficacy of using flaxseed as a dietary supplement in population predisposed to neurodegenerative disorders. However, research has cautioned that the diet may depress body growth due to an imbalance between omega-3 and omega-6 fatty acid levels (Parikh et al. 2019a), thus additional experiments are in need to address the beneficial doses without side effects.

Declarations

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Conflicts of interest/Competing interests: The authors declare no conflict of interest.

Availability of data and material: Data are available from corresponding author upon reasonable request.

Authors' contributions: NM- conceptualization/design, methodology, investigation, formal analysis, writing – original draft preparation; MD- investigation and data collection, writing – review and editing; MZ- investigation, data collection; NN- Resources, writing – review and editing; IG- conceptualization, formal analysis, writing – review and editing; supervision. All authors read and approved the final manuscript.

Consent to participate: not applicable

Consent for publication: not applicable

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Figures

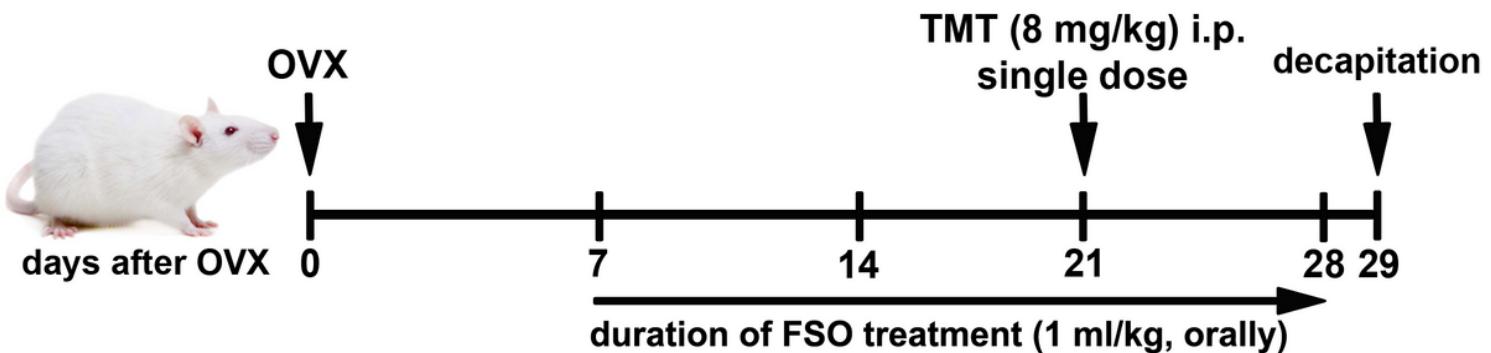


Figure 1

Schematic illustration of the experimental design. One week after the surgery, OVX females (Ctrl group) were treated with FSO (1 ml/kg) for two weeks prior to TMT intoxication (8 mg/kg, i.p., single dose). The treatment was continued for seven more days and animals were decapitated 24h after the last injection

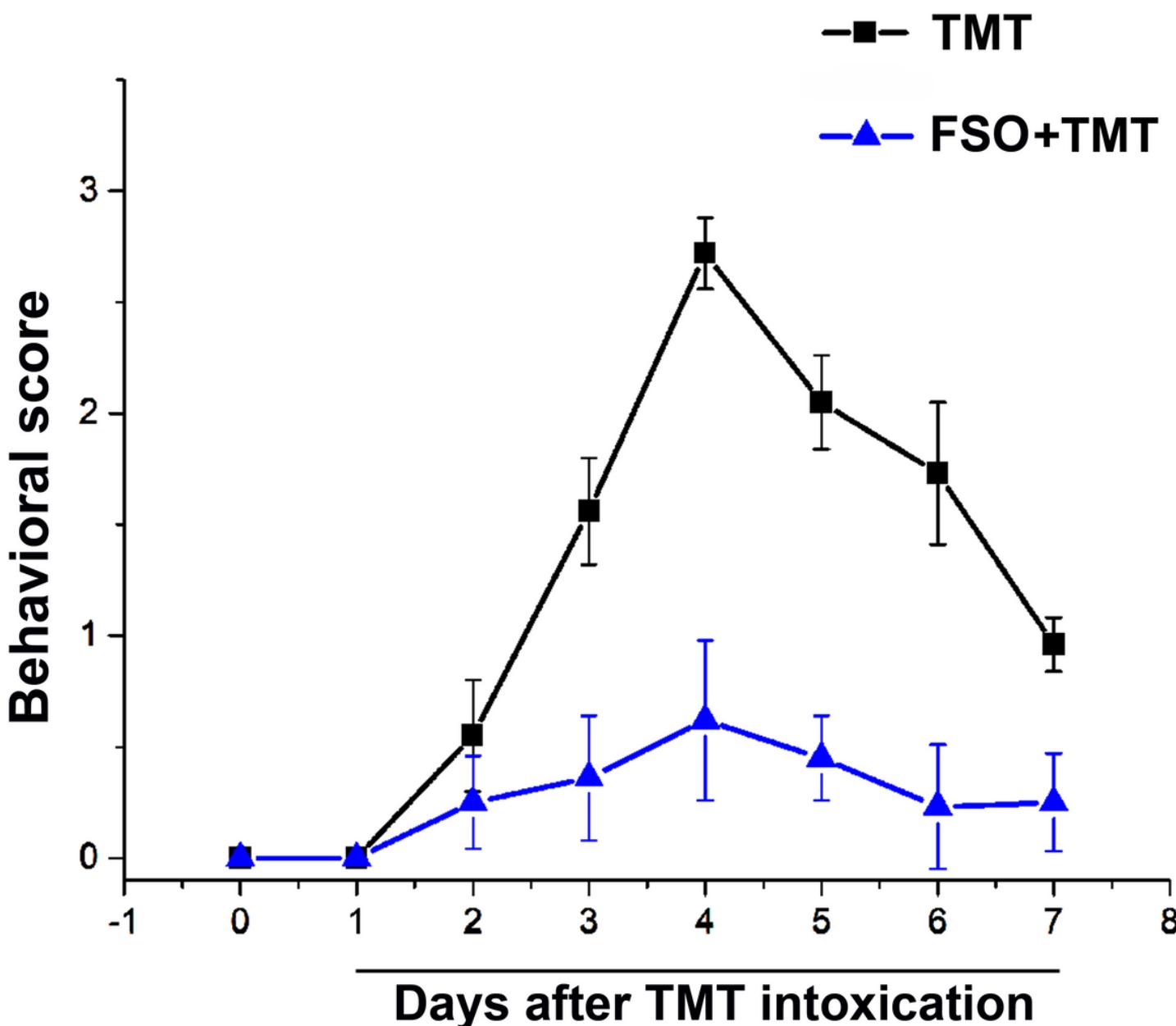
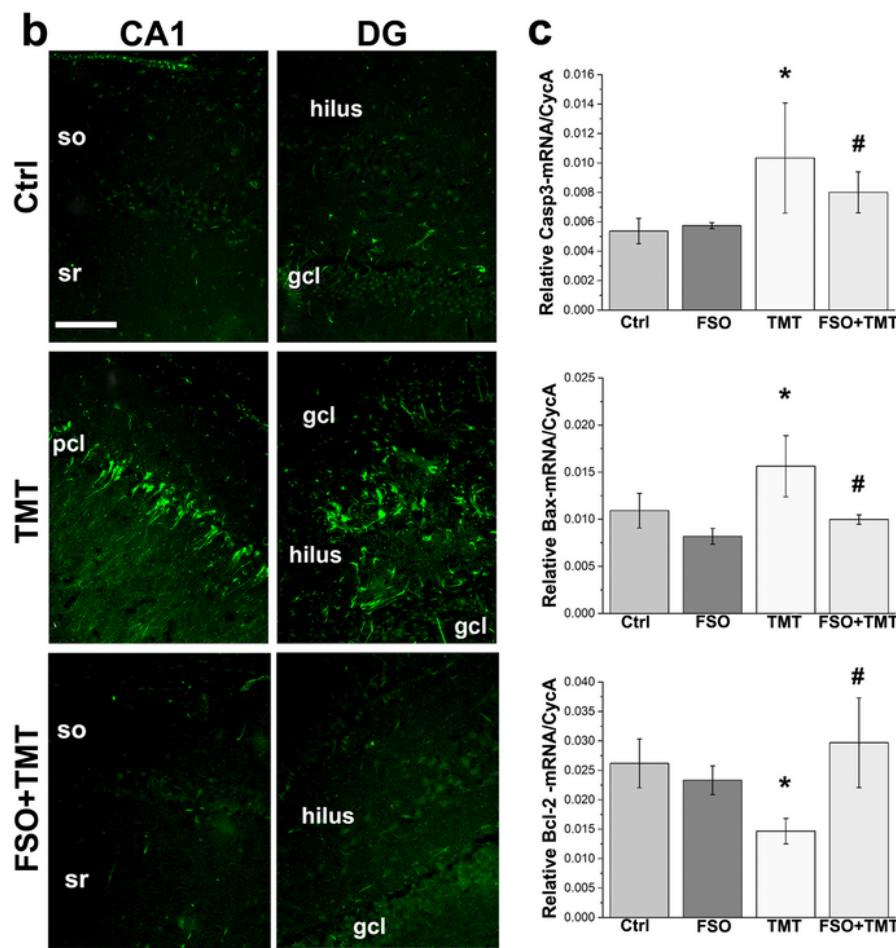
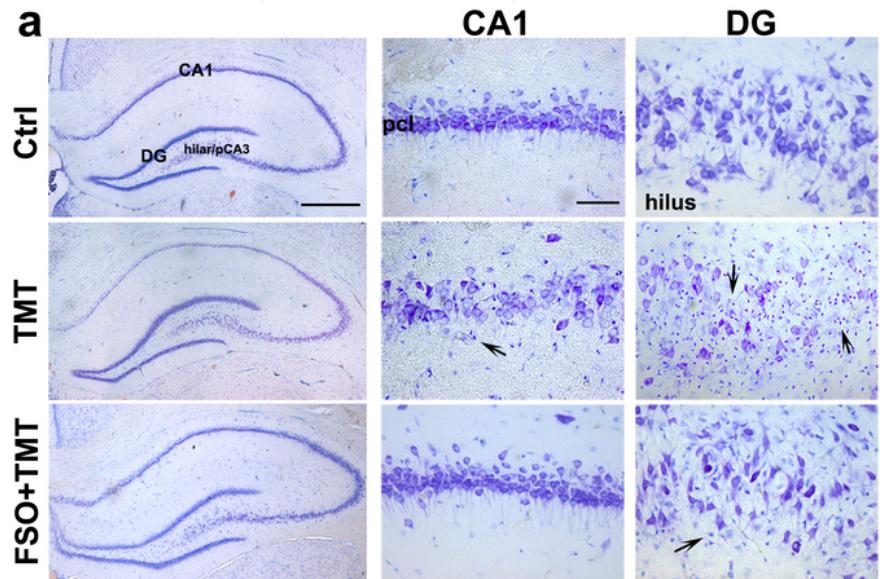


Figure 2

Assessment of behavior severity. Behavioral signs were scored according to 0–3 scale (0, without symptoms; 1, hyperactivity and hyper-responsiveness, 2, mild tremor with normal motor activity; 3, systemic tremor). The data are reported as the mean \pm SEM. square- TMT treated group, triangle- FSO+TMT group (Dragić et al. 2019)

**Figure 3**

FSO pretreatment prevented TMT-induced neuronal loss. (a) Representative thionin-stained hippocampal sections showing CA1 or the hilar/pCA3 subfield of DG of Ctrl animals, TMT animals, and animals treated with FSO+TMT. Dying neurons were observed in the pcl of CA1, and in the hilar/pCA3 subfield of DG seven days after TMT intoxication, accompanied with small, darkly-stained cells, suggesting gliosis (black arrows). In the FSO+TMT group, regular laminar organization and the absence of cell death were observed in the hippocampal CA1 and hilus, although with some level of gliosis in the hilar/pCA3 subfield of DG. Scale bar applicable to lower magnifications – 500 µm and 20µm applicable to higher magnifications. (b) Representative images of hippocampal CA1 and the hilar/pCA3 subfield of DG stained with FJC. After TMT, a marked neurodegeneration is observed in both, pcl of CA1 and the hilar/pCA3 subfield of DG. In FSO+TMT group, no FJC staining was observed in either hippocampal subregions. Scale bar applicable to all - 100 µm. (c) Quantitative real time-PCR data for Casp3-, Bax- and Bcl-2- mRNA involved in neuronal cell death pathway. Bars represent mean target gene- mRNA abundance relative to CycA (\pm SEM). * p < 0.05 or less relative to Ctrl; # p < 0.05 or less relative to TMT. so – stratum oriens; sr – stratum radiatum; pcl – pyramidal cell layer; gcl – granular cell layer (Dragić et al. 2019)

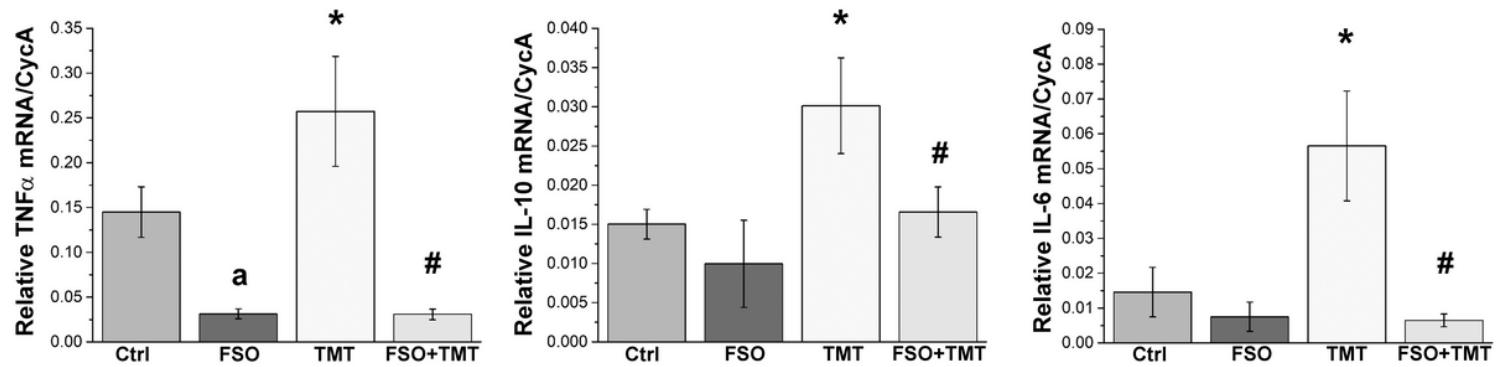


Figure 4

FSO pretreatment prevented neuroinflammation. qRT-PCR expression analyses of proinflammatory TNF- α -, IL-10-, IL-6- mRNA abundance relative to CycA (\pm SEM). * p < 0.05 relative to Ctrl; # p < 0.05 relative to TMT

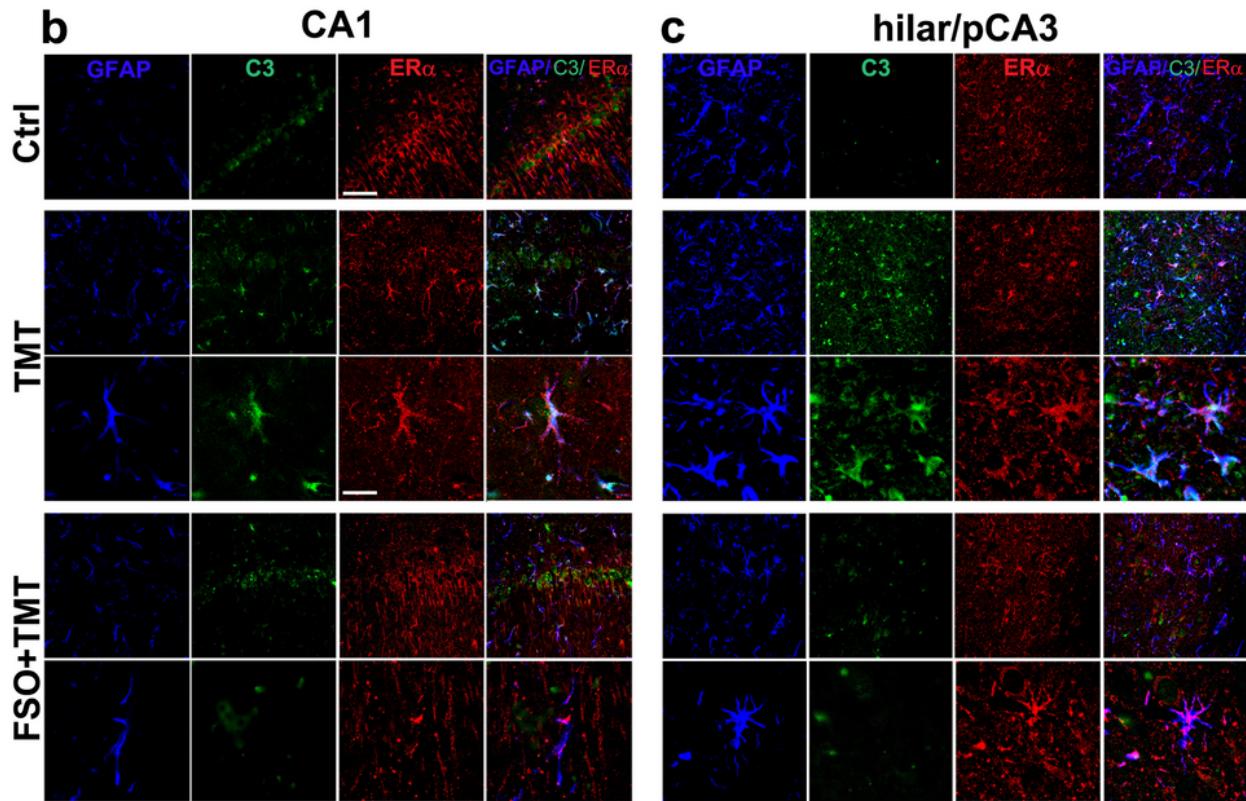
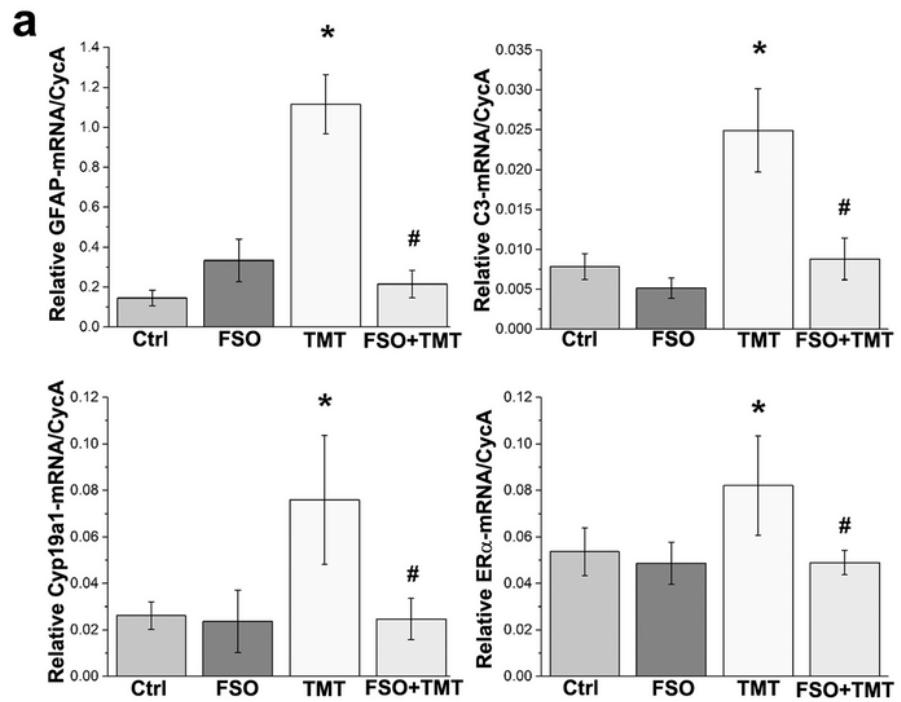


Figure 5

FSO pretreatment prevented TMT-induced astrogliosis. (a) qRT-PCR expression analyses GFAP-, C3-, Arom-, and ER α - mRNA. Bars represent mean target- mRNA abundance relative to CycA (\pm SEM). * p < 0.05 relative to Ctrl; # p < 0.05 relative to TMT. (b, c) Representative images of triple immunofluorescence labeling directed to GFAP (blue), C3 (green), and ER α (red) in CA1 (b) and hilar/pCA3 subfield of DG (c) in Ctrl, TMT and FSO+TMT experimental groups. The high magnification images represent ER α /GFAP/C3

colocalization in CA1 and hilar/pCA3 subfield of DG in TMT and FSO+TMT group. At Ctrl sections, ERα-ir is primarily localized at hippocampal neurons. Seven days after TMT ERα-ir was only observed in GFAP+/C3+ astrocytes in CA1 and in the hilar/pCA3 subfield of DG. FSO pretreatment prevented the loss of neuronal ERα- ir in CA1 and in the hilar/pCA3 subfield of DG, while sporadic GFAP+ astrocytes retain ERα- ir in the hilar/pCA3 subfield of DG. Scale bar 50 µm applicable to lower magnifications and 20µm applicable to higher magnifications

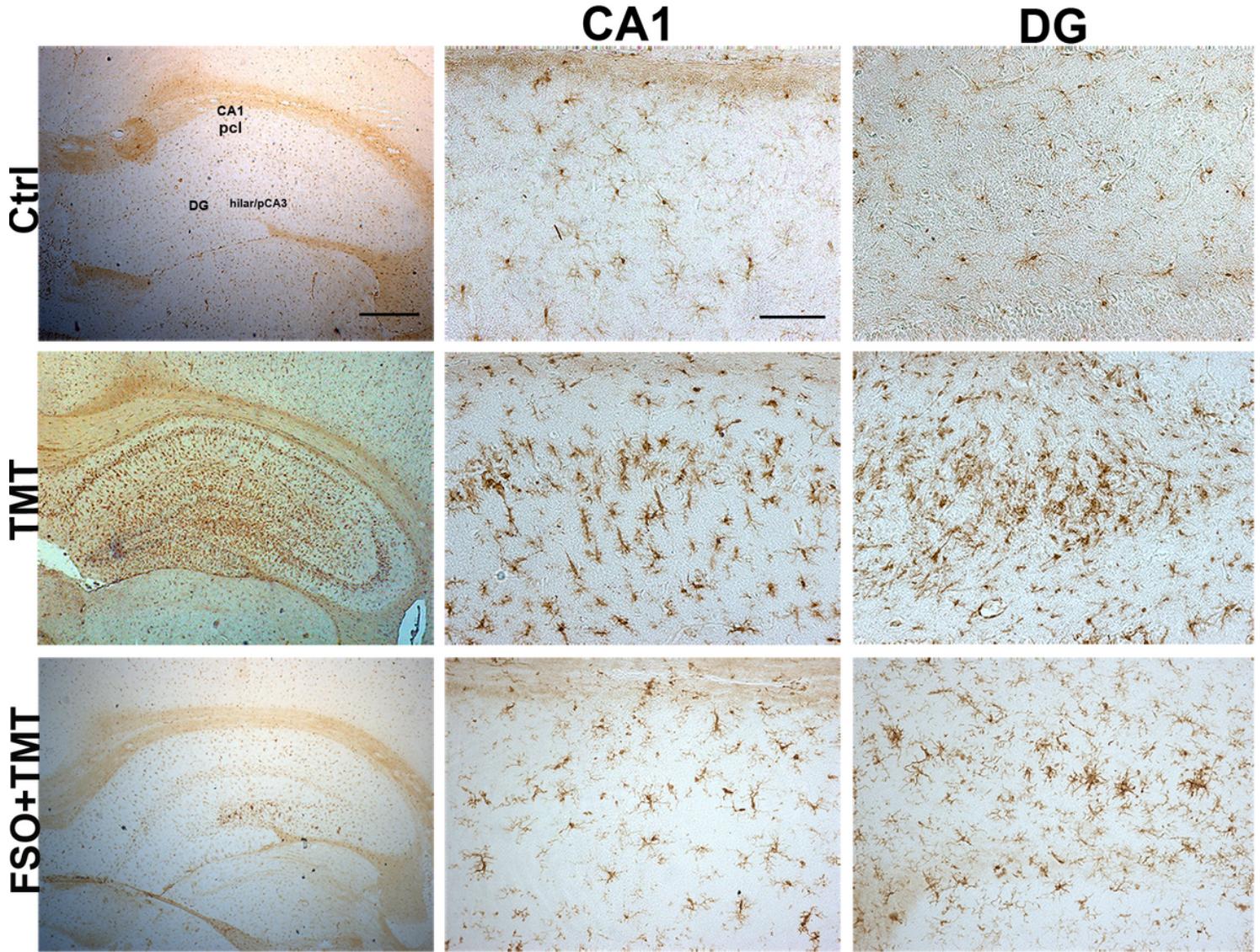


Figure 6

FSO pretreatment prevented TMT-induced activation of microglia. Representative IBA-1-stained hippocampal sections showing CA1 or the hilar/pCA3 subfield of DG of Ctrl, TMT and FSO+TMT experimental groups. Seven days after TMT intoxication microglial cells of mixed morphology gradually populated CA1 and hilar/pCA3 subfield of DG. In FSO+TMT group occasional microglia with enlarged cell bodies in CA1 subfield as well as sporadic clusters of these cells in the hilar/pCA3 subfield of DG were observed. Scale bar applicable to lower magnifications – 500 µm and 20µm applicable to higher

magnifications. DG – dentate gyrus; pcl – pyramidal cell layer; hilar/pCA3 - hilus of DG and proximal part of CA3 region