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Triclosan alters adult zebrafish behavior and targets acetylcholinesterase activity and expression

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Highlights

- Triclosan induces anxiety-like behavior in adult zebrafish.

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- Triclosan exposure elevates freezing behavior and erratic movements in zebrafish.
- Triclosan reduces acetylcholinesterase activity in the brain and skeletal muscle.
- Triclosan lowers *ache* and *mbp* gene expression in the skeletal muscle of zebrafish.

Abstract

Triclosan is widely used in consumer products as an antimicrobial agent. Epidemiological studies have reported the association of triclosan with adverse birth outcomes. The toxic effects of triclosan on the developing stages of zebrafish are reported, however, its role as behavioral modifier is limited. In the present study, adult zebrafish were exposed to triclosan (0.3 and 0.6 mg/L) for 48 hrs and the exploratory behavior was analyzed using ZebraTrack. Triclosan exposed group showed significantly reduced locomotion concomitant with increased freezing duration. They also showed erratic movements suggesting that triclosan induced anxiety-like behavior in adult zebrafish. Next, we tested the hypothesis that the anxiety-like behavior is linked to altered acetylcholinesterase activity. We found that the triclosan exposure decreased acetylcholinesterase activity in the brain and skeletal muscle but acetylcholinesterase (*ache*) gene was significantly down-regulated only in the skeletal muscle of the adult zebrafish exposed to triclosan. In addition, we also observed a down-regulation of

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myelin basic protein (*mbp*) gene in the skeletal muscle of adult zebrafish treated with triclosan. Thus, our data indicates that even short exposure of triclosan is potent enough to induce behavioral anomalies in adult zebrafish that appear to involve acetylcholinesterase and other structural proteins especially in the skeletal muscle.

Keywords: Triclosan; Open field test; Anxiety; Acetylcholinesterase; *mbp* gene; Zebrafish.

1. Introduction

Triclosan (5-chloro-2-(2,4-dichlorophenoxy) phenol, TCS), owing to its anti-bacterial and anti-mould properties, is present in a wide range of personal care products, cleaning supplies, shoes as well as household products and medical devices (Bhargava and Leonard, 1996; Dann and Hontela, 2011). Of late, due to its extensive use and incomplete removal by wastewater treatment plants, TCS and its derivatives including methyl-triclosan, chloroform and dioxins are detected in the biosolids, soils and surface waters of natural systems ranging from undetectable to 5160 ng/L (Santos et al., 2016). One of the major source for its environmental presence is its use in personal care products which contain 0.1 to 0.3 % of TCS (Rodricks et al., 2010). Alarmingly, TCS had been detected in different human tissues such as adipose tissue, brain, liver and also in fluids such as human plasma, urine and breast milk (Allmyr et al., 2006; Dirtu et al., 2008; Geens et al., 2012; Orvos et al., 2002; Toms et al., 2011), raising the concerns of its potential long-term adverse effects on human health (Weatherly and Gosse, 2017).

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Studies have reported toxic effects of TCS using zebrafish as an animal model (Falisse et al., 2017; Gaulke et al., 2016; Ho et al., 2016; Oliveira et al., 2009; Wirt et al., 2018). TCS has deleterious effects on zebrafish early-life stages by interfering with many developmental processes such as cartilage development, organogenesis, hatching and alterations of biomarker levels. A similar 96 hrs LC_{50} value was reported for zebrafish embryos and adults (0.42 and 0.34 mg/L respectively) (Oliveira et al., 2009). Another study reported that zebrafish embryos exposed to environmentally relevant concentrations of triclosan showed impaired foraging efficiency (Wirt et al., 2018). However, information on the behavioral anomalies induced by TCS on larval and adult zebrafish and its mechanisms is still limited.

Neurotoxicity potential of any compound can be evaluated by sensory, motor, autonomic and integrative neurological functions. Behavior is defined as “the internally coordinated responses (actions or inactions) of whole living organisms (individuals or groups) to internal and/or external stimuli, excluding responses more easily understood as developmental changes” (Levitis et al., 2009). Exposure to various compounds such as endocrine disrupting chemicals and pollutants may have an adverse effect on a wide range of behaviors including aggression, exploratory pattern, learning and cognitive abilities, locomotion etc. (Zala and Penn, 2004). Therefore, behavioral data obtained from animal models can provide valuable information as early indicators of adverse effects in higher organisms (Kalueff et al., 2013). Zebrafish is an established model to study behavior anomalies (Nguyen et al., 2013; Norton and Bally-Cuif, 2010). Behavior of zebrafish is sensitive towards the environmental toxicants and therefore behavioral

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tests can provide important toxicity data (Ašmonaitė et al., 2016; Huang et al., 2014; Kalueff and Cachat, 2011; Nema and Bhargava, 2018).

In the present study, we investigated whether exposure to TCS can induce behavioral anomalies using zebrafish animal model. We have investigated several behavioral endpoints in adult zebrafish upon short exposure to TCS using Open-Field Test (OFT) tank. The OFT offers simple yet powerful tool to investigate emotional and exploratory behavior in various species such as rodents, mice and zebrafish (Nema et al., 2016; Robinson, 2009; Stanford, 2007; Stewart et al., 2012). After concluding the behavioral toxicity potential of TCS, we also explored the possible mechanisms of such behavioral anomalies. In the past, behavioral changes such as anxiety-like behavior and loss of motor coordination have been linked to alterations in the Acetylcholinesterase (AChE) activity (Jensen et al., 1997; Tilton et al., 2011). Therefore, we explored the effects of short exposure of TCS on adult zebrafish brain and skeletal muscle AChE activity. AChE hydrolyses neurotransmitter acetylcholine at brain cholinergic synapses and neuromuscular junctions and affects motor function (Jensen et al., 1997; Nilsson and Björklund, 1992). Furthermore, we also investigated the effect of TCS on the expression of acetylcholinesterase (*ache),* myelin basic protein *(mbp)* and synapsin IIa *(syn2a)* genes. The *mbp* gene encodes for the myelin basic protein, a major component of myelin sheath of neurons, and typically serves as a biomarker of the myelination of the axons. Studies have reported that the gene expression, structure and synthesis of myelin sheath are highly conserved between mammals and zebrafish (Chung et al., 2013; Jung et al., 2010). The *syn2a* gene, widely used as a biomarker for synapse formation in mammals, plays a vital role in synaptogenesis and neurotransmitter release

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(Garbarino et al., 2014; Kao et al., 1998). Our results indicate that TCS can induce anxiety-like behavior in adult zebrafish by altering the AChE activity and its gene expression along with other structural proteins.

2. Materials and Methods

2.1 Chemicals

Triclosan (CAS 3380-34-5) of Pharmaceutical Secondary Standard grade was obtained from Sigma-Aldrich Co. A stock of 50 mg/mL of TCS was prepared in acetone and stored at 4 °C. Solvent (acetone) control tank contained 0.001 % of acetone, which matches with the highest concentration of acetone used in the TCS treatment group. 18 µL or 36 µL from the TCS stock solution was added to two separate tanks. Each tank contained 3 L of three-stage purified reverse osmosis (RO) water to make final concentrations of 0.3 mg/L TCS and 0.6 mg/L TCS respectively.

2.2 Zebrafish maintenance

Adult zebrafish of mixed gender were purchased from a local commercial supplier and were housed in a static water tank as described in (Gaur et al., 2018). Briefly, zebrafish were maintained in transparent rectangular tanks fitted with white light, aeration assembly, thermostat (for maintaining 28 °C) and stone-pellets for biological filtration. Three-stage purified RO water supplemented with E3 medium (2 mL 60X E3 per 5 liters of RO water) (60X E3 media containing 0.595 M NaCl, 0.021 M KCl, 0.039 M $CaCl_2.2H_2O$, and 0.048 M $MgCl_2.6H_2O$ and pH 7.2) was used for the zebrafish

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maintenance. A 14/10 hrs (light/dark) period was maintained using automatic switch-assembly. Fishes were manually fed *ad libitum* twice-a-day with commercial pellet based diet. All zebrafish underwent at least four weeks of acclimatization period in the laboratory environment before being used in the experiments. The zebrafish were approximately nine months old as estimated by their size.

All animal handling and experiments were conducted in accordance with the approved protocols and guidelines prescribed by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

2.3 Drug exposure of adult zebrafish

The overall study design is shown in supplementary fig. 1. In this study, we did 6 independent trials which were performed on different days and in each trial we used naive fishes. Once the fish was used in a trial, it was either sacrificed for biochemical assays at the end of the trial or discarded and not used further. The details of each trial used in this study are listed in the supplementary table 1.

For the drug treatment, adult zebrafish of mixed gender were randomly selected from their housing tank and gently transferred into three separate tanks containing 3 L of three-stage purified RO water supplemented with 1.2 mL of 60X E3 media (not more than 3 fishes per tank, per treatment group, per trial were used). These three separate tanks served as treatment chambers for solvent control, 0.3 mg/L TCS and 0.6 mg/L TCS groups. The first tank which served as the solvent control group, contained 36 µL of acetone without TCS (final concentration of acetone: ~0.001 % acetone). The second tank contained 0.3 mg/L TCS and the third tank contained 0.6 mg/L TCS. Each tank

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was aerated and a temperature of 28 °C was maintained. The exposure was done for a total of 48 hrs in a semi-static manner in which treatment solutions were changed completely with fresh solutions after every 24 hrs.

As, the reported LC_{50} value at 96 hrs of TCS exposure in adult zebrafish is ~0.3 mg/L (Oliveira et al., 2009), we used this concentration and its double concentration (0.6 mg/L) as our nominal TCS doses to study its dose dependence toxicity on adult zebrafish. We did not observe any mortality in 0.3 mg/L concentration group during 48 hrs exposure, but 5 out of 15 fishes could not survive the 0.6 mg/L TCS exposure for 48 hrs and hence the behavior data is reported for only 10 fishes for 0.6 mg/L concentration.

2.4 Behavioral acquisition

The behavior modulatory potential of TCS on adult zebrafish was determined using open-field tank test as described previously (Nema et al., 2016). Briefly, after 48 hrs of TCS exposure, one fish at a time from each treatment tank was gently transferred to the behavioral imaging setup and its exploratory behavior in brightly backlit OFT tank (approx. 1000 Lux) was recorded as AVI video files at 10 frames per second for 6 min. The behavior was recorded between 01:00 pm to 03:00 pm for each trial. The details of each trial are listed in the supplementary table 1and summary of all trials is provided in table 2. Water in the OFT tank was replaced with fresh three-stage purified RO water between every behavioral recording and maintained at 28 ± 2 °C. We have recorded behavior of total 13 fishes in solvent control, 10 fishes in 0.3 mg/L TCS and 10 fishes in 0.6 mg/L TCS groups. As mentioned above, 5 out of 15 fishes could not survive the 0.6

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mg/L TCS exposure for 48 hrs and hence these could not be assessed for behavior. After, the behavioral recordings, some of the fishes were used in determining AChE enzymatic activity and *ache, mbp* and *syn2a* gene expression from brain and skeletal muscle tissues of adult zebrafish.

2.5 Extraction of behavioral endpoints

Behavioral endpoints from the recorded videos were extracted as described earlier (Nema et al., 2016; Nema and Bhargava, 2018). Briefly, using automated tracking by the ZebraTrack method, we have determined the behavioral endpoints (Total distance travelled, speed, freezing bouts and angle related behavior) as described below.

2.5.1 Total distance travelled by the zebrafish and speed of locomotion

Automated tracking of each zebrafish for 6 min duration in the OFT tank resulted in a list of X and Y locations of their centroid, indicating their spatial location in all the video frames. Displacement of zebrafish centroid between successive video frames for 6 min duration was calculated and their cumulative values were used in obtaining the total distance travelled by the zebrafish for the said duration of time (Nema et al., 2016). Speed of the locomotion was calculated by taking the cumulative values of the distance traveled by each zebrafish for the total duration of 6 min.

2.5.2 Freezing behavior

A complete cessation of the movement by the fish (except eyes and gills) in the OFT tank is considered as freezing. In the present study, one freezing bout was considered if

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the displacement of zebrafish centroid was 3 mm or less for 1 s (i.e. in 10 consecutive frames in our video recording) during any period within the 6 min duration of the behavioral recording. For the total freezing duration, cumulative duration of freezing bouts during 6 min was calculated and plotted.

2.5.3 Assessment of angle based behavior

The turn angle of the zebrafish between successive frames was calculated as theta in degrees and the absolute turn angle was obtained by taking the sum of all the turn angle magnitudes for each zebrafish without considering the positive or negative angle sign for the 6 min duration of behavioral recording (Rosemberg et al., 2011). Angular velocity was calculated as the change in degree ($\Delta\theta$) per second. Meandering was calculated as the magnitude of the absolute turn angle taken by the zebrafish per meter of locomotion (θ/m) (Nema et al., 2016; Nema and Bhargava, 2018).

2.6 Acetylcholinesterase activity

AChE activity was determined for each treatment group in the supernatant of individual adult zebrafish brain and skeletal muscle tissue homogenates following a previously described protocol (Padilla et al., 1998). Briefly, after 48 hrs exposure to the respective solutions, each zebrafish brain and skeletal muscle tissue were isolated. The obtained tissues were individually homogenized in 500 µL of ice-cold sodium phosphate buffer (pH 8.0, 0.1 M) supplemented with 1 % Triton-X 100 using hand held motor driven mini-homogenizer. Homogenates were centrifuged twice (Initially at 6,000g for 8 min and then at 12,000g for 10 min at 4 ˚C) and supernatant was collected at each step. AChE

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activity was measured in 96-well plates using 5,5'-dithio bis-2-nitrobenzoic acid (DTNB) and acetylthiocholine iodide (ATCI) as assay reagents in the total volume of 200 µL/well. Each sample was assayed in triplicates in separate wells and averaged to obtain a single value. AChE activity was calculated as µM of ATCI hydrolyzed per hr per mg of total protein in the resultant supernatant of brain or skeletal muscle tissue homogenate of zebrafish and reported as normalized activity with respect to the control group. Protein assay was performed using Bradford method (Bradford, 1976).

2.7 RNA isolation, Reverse transcription and Real-Time PCR

Total RNA was isolated from the solvent control and TCS treated adult zebrafish brain or skeletal muscle tissue using TRIzol reagent following manufacturer instructions and quantified by NanoDrop™ 2000 spectrophotometer. Using Qiagen's QuantiTect Reverse Transcription kit, the first-strand of cDNA was synthesized by reverse transcription of 1 µg of total RNA and stored at -20 ˚C for further use. The expression of *ache, mbp* and *syn2a* was performed on QuantStudio 3 Real-Time PCR system (Applied Biosystems) using PowerUp SYBR Green Master Mix. The sequence of the oligonucleotide primers (*actb1*-Fw: 5'-TGGTATCGTGATGGACTCTG-3' and Rv: 5'-CTCTCGGTCAGGATCTTCAT-3'; *ache*-Fw: 5'-CATACGCACAATACGCTGCC-3' and Rv: 5'-TACACAGCACCATGCGAGTT-3'; *mbp*-Fw: 5'-AATCAGCAGGTTCTTCGGAGGAGA-3' and Rv: 5'-AAGAAATGCA CGACAGGGTTGACG-3'; *syn2a*-Fw: 5'-GTGACCATGCCAGCATTTC-3' and Rv: 5'-TGGTTCTCCACTTTCACCTT-3') was taken from previous studies (He et al., 2017; Velki et al., 2017) and were purchased from Integrated DNA Technologies, Belgium.

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The thermal cycling conditions were- UDG activation at 50 °C for 2 min, Dual-Lock DNA polymerase at 95 °C for 2 min followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 57 °C for 15 s and extension at 72 °C for 1 min. Immediately, dissociation step was also performed for checking non-specific amplification by using the conditions as 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s. The relative mRNA expression levels of *ache, mbp* and *syn2a* genes were normalized to *actβ1* gene and fold-changes were calculated as described before (Schmittgen and Livak, 2008). Each sample was run in triplicates and no-template reactions were used as control.

2.8 Statistics

All data were analyzed using GraphPad Prism version 5.0. The data were tested for normality distribution using normality test in GraphPad prism. If the obtained data was parametric, unpaired t-test was used between the two groups and for non-parametric data, Mann-Whitney test was used. Appropriate conversion of pixels to centimeter or meter was done prior to statistical analysis wherever applicable. Enzymatic activity and fold-change in expression were analyzed by one-sample t-test by assigning a hypothetical value of 100 and 1 respectively. $P \leq 0.05$ was considered significant. * denotes $P \leq 0.05$; ** denotes $P \leq 0.001$ and *** denotes $P \leq 0.0001$.

3. Results

3.1 TCS exposure reduced the total distance travelled by the adult zebrafish

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In our experiments, we observed that 100 % fishes survived in solvent control and 0.3 mg/L TCS group after 48 hrs of exposure. However, only 66 % fishes survived after 48 hrs exposure to 0.6 mg/L TCS. When their behavior was measured in the OFT tank, we observed that the total distance travelled and speed of locomotion of the treated fishes were reduced significantly at both the TCS concentrations when compared to the solvent control (Fig. 1A, 1B).

3.2 TCS induced freezing behavior in adult zebrafish

TCS exposed adult zebrafish showed an increase in the number of freezing bouts (Fig. 2A) and duration of freezing (Fig. 2B) in both the concentrations of TCS in the OFT tank. However, the data reached the level of significance only for 0.3 mg/L TCS. Qualitative representation of all the freezing events for each zebrafish in the OFT tank for the duration of 6 min showed that such events were higher in TCS treated adult zebrafish as compared to the control group (Fig. S2).

3.3 TCS altered temporal behavior in adult zebrafish

Novel environment such as the OFT tank has been reported to induce anxiety-like behavior in zebrafish. Therefore, we determined if the altered behavior observed above was due to the novel environment of the OFT or because of the TCS exposure. We characterized the temporal changes in the behavior of each adult zebrafish by comparing the behavior of first and last 6 s of the overall 6 min in the OFT duration. In the solvent control group, the distance travelled by the zebrafish was found to be reduced significantly in the last 6 s when compared to its own first 6 s exploration in the

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OFT tank (Fig. 3). Interestingly, there was no difference observed in the distance travelled in the first and last 6 s by the zebrafish in both the TCS treated groups (Fig. 3).

3.4 TCS altered the angle based endpoints in adult zebrafish

In the OFT, TCS exposed zebrafish showed a dose-dependent decrease in the angular velocity (Fig. 4A). Also, significantly enhanced meandering was observed in TCS treated zebrafish (Fig. 4B) when compared to the control fishes. We did not observe any changes in the mean absolute turn angle in the adult zebrafish exposed to TCS in the OFT tank for 6 min duration (Fig. S3).

3.5 TCS reduced AChE activity in the brain and skeletal muscle tissue of adult zebrafish

To determine if AChE could act as a molecular target of TCS induced toxicity, we determined its activity in the brain and skeletal muscle of the zebrafish in various treatment groups. We observed a dose-dependent decrease in both, brain (Fig. 5A) and skeletal muscle AChE activity (Fig. 5B).

3.6 TCS downregulated the gene expression of *ache* and *mbp* in the skeletal muscle tissue of adult zebrafish without affecting *syn2a* gene expression

To get further insights into the behavioral anomalies induced by TCS exposure, we studied the expression of genes involved in maintaining the structure and function of nerve cells and in turn the overall motor coordination. We studied the expression profile of *ache*, *mbp* and *syn2a* genes in the brain and skeletal muscle tissue of the adult

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zebrafish exposed to 0.3 and 0.6 mg/L of TCS for 48 hrs. A significant, almost two-fold down regulation of the *ache* and *mbp* genes was observed in the skeletal muscle tissue of adult zebrafish exposed to 0.6 mg/L TCS for 48 hrs, without affecting the *syn2a* gene expression (Fig. 6). We did not observe any change in the expression levels of the above three genes in the brain tissue of adult zebrafish exposed to TCS for 48 hrs (Fig. S4).

4. Discussion

In the present study, we aimed to determine if the widely used chemical, TCS can induce behavioral anomalies. For this, we used zebrafish animal model which has been validated as a behavioral animal model (Ašmonaitė et al., 2016; Kalueff and Cachat, 2011; Nema and Bhargava, 2018). Locomotion behavior in zebrafish reflects the motor coordination especially related to its swimming behavior (Canavello et al., 2011). Previously, environmental toxicants such as endosulfan, acrylamide, copper or chlorpyrifos have been shown to alter locomotion behavior (Dutta and Arends, 2003; Faria et al., 2018; Li et al., 2017; Tilton et al., 2011). Our results indicate that, similar to other environmental toxicants, TCS can also reduce locomotion as is evident by reduced distance travelled and reduced speed (Fig. 1), thus showing behavioral toxicity. More importantly, even the short TCS exposure of 48 hrs induced anxiety-like behavior in the exposed zebrafish as indicated by an increase in the freezing bouts and freezing duration (Fig. 2). Though, TCS exposed adult zebrafish showed an increase in the number of freezing bouts in both the concentrations of TCS, however, the data reached the level of significance only for 0.3 mg/L TCS. This could be due to 66.6 % mortality in

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0.6 mg/L TCS, meaning severely affected fishes could have been eliminated in the higher concentration. This increase in the freezing duration correlated well with the decreased distance travelled by the exposed zebrafish (Fig. 1).

It is reported that the novel environment of OFT tank can itself induce anxiety-like behavior (Cachat et al., 2010; Nema et al., 2016), therefore, it was important to dissect the role of TCS and novel environment in the observed anxiety-like behavior in the TCS treated groups. For this, we compared the distance travelled by the zebrafish in each treatment group to its first and last 6 s of 6 min OFT duration. The idea behind such comparison is that the first 6 s is too less for the fish to explore the complete OFT arena to exhibit anxiety-like behavior due to the novel environment. However, if the anxiety-like behavior is developed gradually due to the novel environment of that tank, we should see an altered behavioral profile only during the last 6 s of its 6 min exploration in the OFT tank. But, if the fish is already anxious due to TCS exposure, it should exhibit no such change in the behavior between the first and last 6 s of 6 min behavioral acquisition. This is because the novel field of the OFT would not temporally develop anxiety in the already anxious zebrafish within the 6 min behavioral measurement (Nema and Bhargava, 2018). We observed a significant reduction in the distance travelled by the zebrafish in control group but not in TCS treated groups (Fig. 3). The behavior pattern we observed in the control fishes, when placed in a novel tank, is reported for healthy fishes (Nema and Bhargava, 2018). Also, this suggests that the anxiety-like behavior we observed in our study was indeed due to TCS treatment and not due to the novel environment of the OFT tank in TCS treated fishes.

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Further, TCS treated zebrafish also showed erratic movements such as change in the angular velocity and meandering (Fig. 4) without any observable changes in the mean absolute turn angle (Fig. S3). Anxious zebrafish are known to display a sudden change in the swimming angles. Any change in angular velocity and meandering is indicative of anxiety-like behavior (Li et al., 2017; Tilton et al., 2011). We also speculate that any difference in the mean absolute turn angle is abrogated by increased freezing events in both the treated groups of zebrafish.

In mice, it has been established that an increase in the anxiety-like behavior decreases AChE activity (Luo et al., 2013; McCloskey et al., 2017). This prompted us to evaluate AChE activity and its expression in brain and neuromuscular junctions at skeletal muscle in the TCS treated adult zebrafish. AChE inhibition leads to accumulation of acetylcholine at synapses and leads to abnormal nervous system functions including erratic movements and spatial orientation of the species (Dutta and Arends, 2003; Glusczak et al., 2006; Haverroth et al., 2015). We observed a significant decrease in the brain and skeletal muscle AChE activity (Fig. 5) indicating the potential neurotoxic effects of TCS on the cholinergic system. In addition to that, we also observed a significant decrease in the gene expression of *ache* only in the skeletal muscle tissue (Fig. 6) unlike brain tissue which indicates that apart from anxiety-like behavior TCS may be inducing defects in motor function. TCS is linked to enhanced production of reactive oxygen species (Aboul Ezz et al., 2015; Parenti et al., 2019; Zhou et al., 2017). Few studies reported the link between the AChE activity and oxidative stress where down-regulation of the AChE activity is reported with an increase in the oxidative stress (den Hartog et al., 2002; Tsakiris et al., 2000; Wyse et al., 2004). Therefore, it is

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possible that, in our study the decreased AChE activity in the brain tissue was due to the mild oxidative stress induced by TCS and not due to changes in *ache* gene expression unlike skeletal muscle.

Because defective swimming behavior may also indicate defective motor function, to assess the structural integrity of neurons which may relate to motor function, we also measured *mbp* and *syn2a* gene expression in the brain and skeletal muscle tissue of adult zebrafish. We only observed a reduction in the gene expression of *mbp* in the skeletal muscle tissue (Fig. 6). Down regulation of the *mbp* gene in the skeletal muscle tissue of adult zebrafish exposed to TCS indicates the potential toxic effect of TCS that might affect the function of neurons by affecting the function of myelin sheath on axons. It is surprising that the gene expression of all the genes in the brain was not affected by TCS. It may be due to different mechanisms in brain and skeletal muscle tissue.

Overall, our study provides significant evidence that TCS exposure can impair behavioral function in adult zebrafish by interfering with the AChE activity and expression.

Author Contribution Statement

Idea and conceptualization by A.B, Y.B and N.P; Experiments by N.P and S.N; data analysis by A.B, Y.B, N.P and S.N; data visualization by A.B, Y.B and N.P; original draft preparation by N.P, review and editing by all authors; supervision, project administration, and funding acquisition by A.B. and Y.B.

Declaration of Interest

The Authors have nothing to declare.

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Figure legends

Fig. 1. **TCS treatment affected distance-based endpoints.** (A) Total distance travelled and (B) Speed of the locomotion of zebrafish for control (Ctrl) and TCS treated groups when tested in the OFT tank for the duration of 6 min. The data is plotted as mean ± SEM of n = 13 zebrafish in Ctrl; n = 10 zebrafish in 0.3 TCS and n = 10 zebrafish in 0.6 TCS. * denotes $P \leq 0.05$. Ctrl = Solvent control; 0.3 TCS = 0.3 mg/L TCS; and 0.6 TCS = 0.6 mg/L TCS.

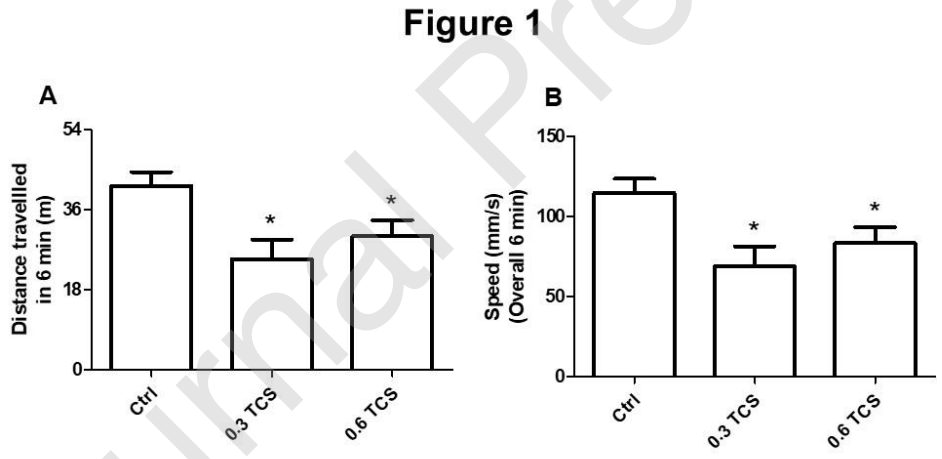

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Fig. 2. TCS treatment affected freezing based endpoints. (A) Total freezing bouts and (B) Freezing duration for control (Ctrl) and TCS treated groups for the duration of 6 min. The data is plotted as mean ± SEM of n = 13 zebrafish in Ctrl; n = 10 zebrafish in 0.3 TCS and n = 10 zebrafish in 0.6 TCS. * denotes $P \leq 0.05$. Ctrl = Solvent control; 0.3 TCS = 0.3 mg/L TCS; and 0.6 TCS = 0.6 mg/L TCS.

Figure 2

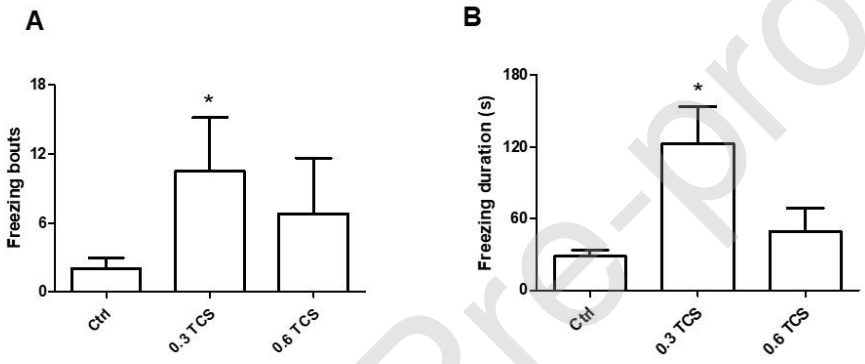

Fig. 3. Temporal behavior based on the distance travelled by control (Ctrl) and TCS treated groups for first 6 (F6) and last 6 (L6) s. The data is plotted as mean ± SEM of n = 13 zebrafish in Ctrl; n = 10 zebrafish in 0.3 TCS and n = 10 zebrafish in 0.6 TCS. *** denotes $P \leq 0.0001$. Ctrl = Solvent control; 0.3 TCS = 0.3 mg/L TCS; and 0.6 TCS = 0.6 mg/L TCS.

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Figure 3

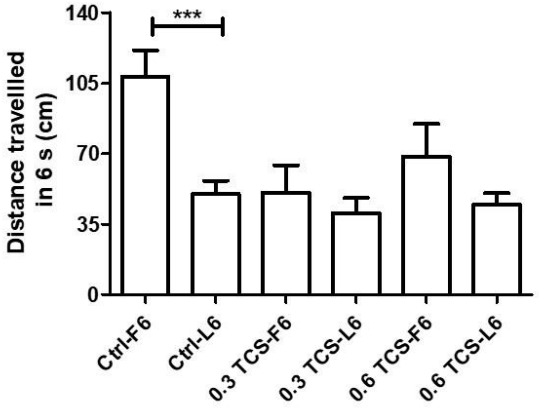

Fig. 4. Effect of TCS treatment on angle based endpoints for control (Ctrl) and TCS treated groups for the duration of 6 min. (A) Angular velocity of zebrafish in Ctrl and TCS treated groups. (B) Erratic movements induced by the exposure of TCS as measured by meandering. The data is plotted as mean ± SEM of n = 13 zebrafish in Ctrl; n = 10 zebrafish in 0.3 TCS and n = 10 zebrafish in 0.6 TCS. * denotes $P \leq 0.05$ and *** denotes $P \leq 0.0001$. Ctrl = Solvent control; 0.3 TCS = 0.3 mg/L TCS; and 0.6 TCS = 0.6 mg/L TCS.

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Figure 4

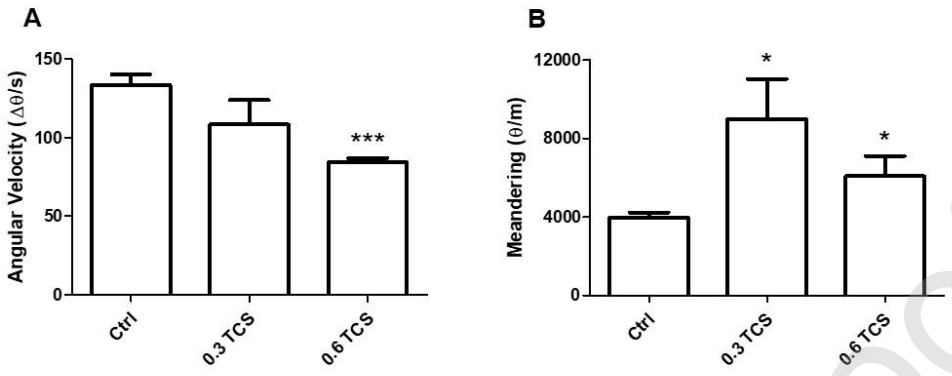

Fig. 5. AChE activity in brain and skeletal muscle tissue. (A) Normalized AChE activity in brain homogenates of adult zebrafish exposed to 0.3 mg/L and 0.6 mg/L TCS treatments. The data is plotted as mean ± SEM of n = 12 zebrafish in Ctrl; n = 10 zebrafish in 0.3 TCS and n = 8 zebrafish in 0.6 TCS. (B) Normalized AChE activity in skeletal muscle homogenates of adult zebrafish exposed to 0.3 mg/L and 0.6 mg/L TCS treatments. The data is plotted as mean ± SEM of n = 9 zebrafish in Ctrl; n = 8 zebrafish in 0.3 TCS and n = 6 zebrafish in 0.6 TCS. * denotes $P \leq 0.05$; and *** denotes $P \leq 0.0001$. Ctrl = Solvent control; 0.3 TCS = 0.3 mg/L TCS; and 0.6 TCS = 0.6 mg/L TCS.

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Figure 5

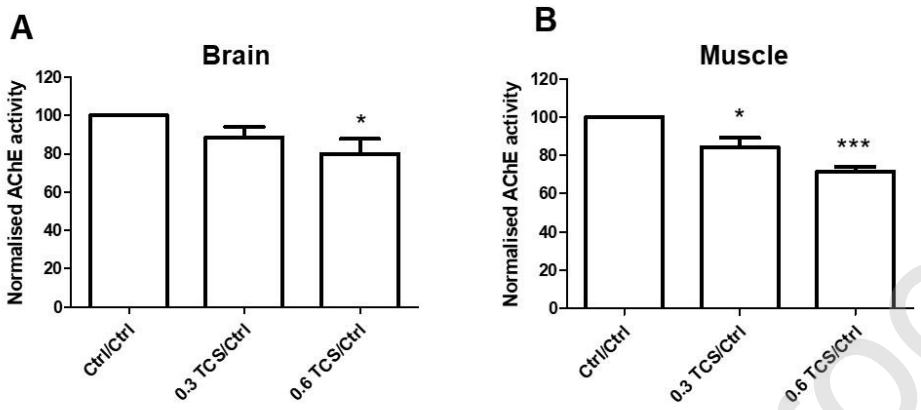

Fig. 6. The mRNA expression levels in skeletal muscle tissue of the TCS treated adult zebrafish. The expression levels of *ache* and *mbp* genes were significantly down-regulated in 0.6 TCS but *syn2a* gene expression was not altered. Each sample was run in triplicates. The data is plotted as mean ± SEM of n = 6 zebrafish in control; n = 6 zebrafish in 0.3 TCS; n = 7 zebrafish in 0.6 TCS. * denotes $P \leq 0.05$. 0.3 TCS = 0.3 mg/L TCS; and 0.6 TCS = 0.6 mg/L TCS.

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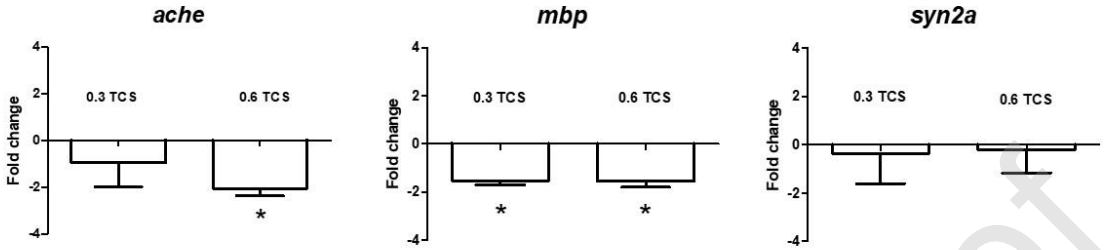
Figure 6



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