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Developmental exposure to the A6-pesticide causes changes in tyrosine hydroxylase gene expression, neurochemistry, and locomotor behavior in larval zebrafish

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Abstract

In recent years, the increase in the synthesis of biopesticides for alternative agricultural uses has necessitated the study of their impacts. Among these compounds, several of them are known to exert endocrine-disrupting effects causing deregulation of a variety of physiological functions affecting cell signaling pathways involved in neural cell differentiation leading to developmental neurotoxicity. In this current paper, we thus determined the impact of the biopesticide A6 on zebrafish larvae, which is structurally linked to estrogenic endocrine disruptors. The objective of this study was to define the toxicity of A6, the mechanisms responsible, and to evaluate its effects on the locomotor activity at nanomolar concentrations (0, 0.5, 5, and 50 nM). We show through its blue fluorescence properties that A6 accumulates in different parts of the body as intestine, adipose tissue, muscle, yolk sac and head. We display also that A6 disrupt the development and affects the function of the central nervous system, especially the expression of tyrosine hydroxylase (TH) in dopaminergic neurons. We studied whether A6 disturbs the target genes expression and recorded that it downregulated genes embroiled in TH expression, suggesting that A6's neurotoxic effect may be the result of its binding propinquity to the estrogen receptor.

Keywords: zebrafish exposure, malformations, A6 accumulation, tyrosine hydroxylase, locomotor behavior

1. Introduction

The exponential growth of the world's population has necessitated the use of intensive production systems in agriculture globally (Köhler and Triebkorn 2013). The over the top use of synthetic pesticides and improve crop yields places great strain on natural resources (Liu et al. 2014). The use of chemical pesticides has led to the appearance of several impacts in the environment, including toxic effects on non-target organism (Abdelkhalek et al. 2015). The arrival of new technologies such as high throughput screening and mass spectrometry in the 1990s boosted “green” chemistry, i.e., the identification of new molecules extracted from plants, and the production of derived molecules with improved properties (Benelli et al. 2016). This “green” chemistry aims to design industrial processes that are more respectful of the environment, and to generate products that are more harmless to non-target organisms (Campos et al. 2016). These pesticides of plant origin or biopesticides are currently being explored as promising alternatives to synthetic pesticides (Benelli et al. 2016), because they are considered less harmful to non-target organisms since they are of natural origin (Shao and Zhang. 2017). A6 is a biopesticide derived from the molecule α -terthienyl which was originally isolated from plants such as Asteraceae (Nivsarkar et al. 2001) for its blue fluorescent properties (Zechmeister and Sease 1947; Nasri et al. 2016a), as well as being described to have herbicidal activity (Friedman and Friedman 1995).

The use of biological origin pesticides in agriculture presents more and more a risk due to their gradual accumulation in aquatic environments (Tejada et al. 2017). In addition, an increasing number of these compounds are now considered endocrine disruptors (EDCs) (Jiang et al. 2016; Nasri et al. 2021a). Estrogenic EDCs act by modulating the interaction between 17β -estradiol (E2) and estrogen receptors (ER) that deregulate physiological functions (Sonnenschein and Soto 1998). Nasri et al. (2016a) showed that A6 exercised anti-estrogenic activity and down-regulate cell proliferation in zebrafish brain after decrease of

gene transcription of estrogen receptors (ER). It has been demonstrated also that α -terthienyl has the capacity to inhibit several enzymes such as superoxide dismutase (SOD) and acetylcholinesterase (AChE) both in vitro and in vivo leading to important consequences in mosquito larvae nervous system (Nivsarkar et al. 2001). New data have shown that A6 at 5 mg/kg accelerates the prion pathology in mice after treatment (Lafon et al. 2018). Generally, biopesticides classified as endocrine disruptors (EDCs) have shown harmful effects on the nervous system development in aquatic organisms (Legradi et al. 2018). Neurotoxicity caused by these environmental pollutants is responsible for changes in locomotor behavior and an alteration in the regulation of the dopaminergic systems (Paiva et al. 2020; Xin et al. 2020).

Tyrosine hydroxylase is used as biomarker dopaminergic neurons since it is a dopamine synthesis enzyme (Hokfelt et al. 1976; Pickel et al. 2006). Dopamine is considered is a neurohormone and neurotransmitter that plays a crucial role in regulating animal life by acting in both the central and peripheral nervous systems (Ugrumov et al. 2012). Dopamine in the peripheral nervous system has been reported in the kidneys, carotid bodies, peripheral arteries, and parts of the gastrointestinal, genitourinary, and endocrine systems (Pivonello et al. 2007). The monoaminergic neurons use dopamine as a transmitter, and it contributes to a variety of functional processes in the vertebrates (Zagrean 2014). The very wide distribution of dopaminergic cells in the central nervous system illustrates its involvement in a varied range of central functions, such as motivation (Schultz 2002), learning and memory (Goldman-Rakic 1997), affective and emotional processes (Diehl and Gershon, 1992), control of body temperature (Cox et al. 1978), locomotion (Mok and Munro 1998), and behaviors (Dufour et al. 2010).

Because the multiple advantages (small size, high fecundity, short life cycle, and facile breeding in the laboratory), zebrafish was used for behavioral and developmental studies (Grunwald and Eisen 2002), genetics research (Eisen 1996), and toxicology bioassays (Hill

and Janz 2003). In our study, it was used to determine the acute toxicity of the biopesticide A6 at low concentrations. The aim was to evaluate the associated physical malformations, the body accumulation, and locomotion behavior as well as to determine the mechanism responsible in relation with the toxicity by evaluation the TH expression after exposure.

2. Materials and Methods

2.1. Fish strains and embryos treatment

The pyrimidino bithiophene or biopesticide A6 and Dimethyl sulfoxide (DMSO) were obtained from Maybridge (Friedman and Friedman 1995). Animals were treated in the laboratory Inserm U1198 following the National and European ethic guidelines under permit number (agreement #A34-172-37). Fish were fed twice daily and handled according to standard procedures in the laboratory (Westerfield 2007). Adult fish were maintained at 28 °C, with a circadian rhythm (14 h of light and 10 h of darkness), in a recirculation, flow-through system where tank water is constantly filtered. Embryos were obtained from pair matings and exposed in groups in an incubator, which was staged according to a previously published method (Nasri et al. 2021b). Embryos of wild-type AB line were dechorionated at 24 hpf, and then kept in the incubator (28 °C) in six-well plates, with 10 to 20 embryos in two milliliters (per well) of solution for A6 tissue accumulation study, body malformations and locomotion behaviors, or in glass bottles, with 80 embryos in one hundred milliliters of solution for gene expression and immunochemical study. For all experiments, the A6 concentrations tested were 0.5 nM, 5 nM, and 50 nM when compared to a DMSO control.

2.2. Response of zebrafish larvae to the effects of A6

Embryos of wild-type AB were treated with all concentrations of A6 or to solvent control (DMSO) for 6 or 9 days. Morphological abnormalities were identified using a stereomicroscope (Olympus Corporation). Three separate experiments were effectuated and

10 larvae were used for each condition. Individual malformation was expressed as the percentage of embryos with at least one malformation, in comparison to the control.

2.3. Measurement of A6 accumulation in tissues

Tricaine was used for anesthetizing embryos of wild-type AB (Nasri et al. 2016a) and put on slides using agar, at the end of treatment to A6 or to control DMSO. Using confocal microscopy and ImageJ software, we were capable to follow fluorescence labeling in contaminated and un contaminated embryos and process the associated images. The number of zebrafish embryos observed for each condition was more than 10 for three distinct experimentations.

2.4. Locomotor behavior measurement

Behavioral swimming was conducted using an automated behavior recording system, Zebrabox (ViewPoint Life Science, France), after zebrafish larvae treatment to A6 or control DMSO. Wild-type embryos were placed individually in a monitoring area in Zebrabox mounted with a camera system and their free-swimming activities were continuously recorded for the succeeding ten minutes (Nasri et al. 2016a). The swimming of more than 2 mm is represented by red lines; small movements (0.5–2 mm) are symbolized by green lines, and trips below 0.5 mm indicate inactivity. The number of animals followed for a given concentration was more than fifteen for three distinct experiments.

2.5. Gene expression measurement by q-PCR

Fertilized wild-type embryos were exposed from 1 to 6 dpf with all concentrations of A6 or to control DMSO. Each experimental group consisted of 80 embryos treated in 100 ml of solution. A set of sixty animal brains were used to extract total RNA using RNeasy Mini Kit (Qiagen, Courtaboeuf, France) (Diotel et al. 2011). Extracts were treated with RNase free DNase to purify the RNA from DNA contamination. Total RNA concentration was quantified using a nanodrop spectrophotometer, and quality was verified by measuring the 260/280 nm

ratio (1.8–2.0) (Nasri et al. 2016a). A Reverse-transcription followed by real-time PCR amplification was applied for mRNAs. The relative target genes transcription was performed with SYBR green detection, as previously described (Nasri et al. 2016a). QRT-PCR was performed with six biological replicates (three technical replicates) using a LightCycler 480 Instrument and the protocol consisted of the following phases: denaturation of PCR reaction mixture at 95 °C for 10 min followed by 45 cycles of amplification cycle profile (denaturation for 10 s at 95 °C; annealing for 10 s at 60 °C; and elongation for 10 s at 72 °C). Real-time detection of the SYBR green fluorescence effect (reflecting the amount of PCR produced) was determined at the end of each elongation part. Construction of standard curves using DNA standards was performed in order to display the consistency and efficiency of each qRT-PCR test. Expression of the target gene was quantified based on the comparative cycle threshold (Ct) method (Nasri et al. 2016a). Normalized expression was calculated for quantification according to the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak 2008) with the internal control gene, *ef1a*. Elongation factor 1 alpha (*ef1a*) was chosen as a reference (McCurley and Callard 2008) because of its stability during zebrafish development and after treatment to environmental pollutants (Tang et al. 2007). For the amplification of cDNA, oligonucleotide primers were used as described in Table 1.

2.6. Immunolabelling TH

After exposure to A6 or control DMSO for 6 days, zebrafish larvae were put in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) + 0.5% Triton at ambient temperature for 2.5 h with agitation and then washed with PBS. Then, the samples were fixed with PFA using Triton for 2 h for permeabilization, followed by blocking using a solution (PBS 1×/BSA 1×/DMSO 1×) and incubating with TH primary antibodies (TH, 1:500, ThermoFisher # PA1-4679) overnight at 4°C for immunofluorescence studies. The following day, the zebrafish were laved with PBST while changing the solution regularly (every 1–2 h).

Fluorescence-conjugated secondary antibody Alexa-488 (1/1000) was added to the primary antibody and left overnight (at 4°C). The next morning, larvae were washed in PBST (at least four times for 30 min each), ideally all day. Hoechst dye (stock 7.5 mg/ml; 1/1000) was added after the last wash, followed by incubation for 40 min and then washing with PBST (three times).

2.7. Imaging analysis and fluorescence quantification

Zebrafish larvae were placed in depressed slides in a drop of agarose (1%) and were immersed in system water and subjected to imaging using a water immersion objective from a confocal microscope (Leica, SPE). Stacks of images were processed and quantified with ImageJ (<http://rsbweb.nih.gov/ij/>). The nomenclature used for the brain nuclei and regions was in accordance with that of the zebrafish atlas (Rink and Wullimann 2002).

2.8. Data analysis

When our data showed approximate normality, analysis of variance (1-ANOVA) was chosen for global search of differences, and Tukey's HSD test was eventually used to compare treatment effects of the biopesticide A6 and control groups for evaluation of the malformation rate, swimming behavior, and gene expression. An α of 0.05 assesses the significance of differences for all measures.

3. Results

3.1. A6 caused morphological abnormalities in zebrafish larvae

The treatment of embryos from 24 hpf with the highest nanomolar concentration of A6 caused some abnormalities in zebrafish larvae, including yolk sac edema (YSE), spinal curvature (SC), tail deformity (TD), and pericardial edema (PE) (Fig. 1A). PE, TD, and SC were observed after 6 days, and YSE was detected only after 9 days of exposure at the same concentration (Fig. 1A). The total malformation rates in the group treated with 50 nM A6

were 16.66% ($p=0.0006$) after 6 days and 36.66% ($p < 0.0002$) after 9 days of experience (Fig. 1B).

3.2. Measurement of tissue accumulation with A6

We examined whether the biopesticide A6 might accumulate in the body of zebrafish larvae after 6 days of exposure at all of the considered concentrations. Zebrafish larvae were placed in depressed slides in a drop of agarose (1%) and were immersed in system water, followed by imaging using a water immersion objective from a confocal microscope (Leica, SPE). The results showed that A6 accumulated in the intestine, adipose tissue, muscle, yolk sac and head based on its blue fluorescence and the ability to detect this by confocal microscopy, when compared with control DMSO (Fig. 2).

3.3. A6 changed the locomotor behavior

The results showed that A6 significantly altered the free-swimming distance of zebrafish larvae in the center and periphery at all concentrations. Figure 3 displays that the swimming behavior of zebrafish larvae was affected by treatment with all concentrations of A6. At the center, the duration of inactivity (distance less than 0.5 mm), as well as the time spent for medium and long trips, was long. At the periphery, the idle time was short, as was the time spent moving medium and long distances.

3.4. A6 downregulates gene expression

The level of mRNA of each of the TH isoforms, *th1* and *th2*, showed a clear dose-dependent decrease following exposure to the biopesticide A6. Exposure to A6 or to the control DMSO from 1 dpf until 6 dpf caused significant decreases in the mRNA level of *th2* in the range of 0.3- ($p=0.005$), 0.32- ($p=0.006$) and 0.51- ($p=0.037$) (Fig. 4B). In addition, the mRNA level of *th1* was decreased in order 0.69- ($p=0.040$) only with 50 nM of A6 (Fig. 4A). These results reveal that the biopesticide A6 could directly or indirectly disturb the synthesis of enzymes regulating dopamine synthesis in zebrafish following exposure to it.

3.5. Immunohistochemistry TH

To confirm the locomotor disruption observed after treatment with the biopesticide, animals were treated to A6 or control DMSO. Changes in the patterning of their dopaminergic neurons were examined at 6 dpf. The nomenclature used for the dopaminergic neurons was as described previously (Tay et al. 2011). Low doses of A6 resulted in appreciable changes of brain TH expression compared with that in the control (Fig. 5). The first changes in neuronal patterning were observed in the olfactory bulb (OB) at all concentrations tested, where labeling of these neurons showed a decrease in fluorescence intensity (Fig. 5B–D) in contrast to control (**Fig. 5A**). The subpallium within the telencephalon (SP) also appears not to be altered by A6. In the pre-tectal (Pr) area, the density of neuronal labeling was not affected (Fig. 5F–H) compared with that in the control DMSO group (Fig. 5E). In addition, neuronal labeling in the area postrema (AP) showed a reduction with only the 50 nM of A6 (Fig. 5L) compared with the control (Fig. 5I).

4. Discussion

Our study investigated the deleterious effects of A6 on zebrafish larvae. The results showed that this biopesticide has adverse effects on neurodevelopment after its accumulation in the lateral line (Nasri et al. 2016a) in the organs (Fig. 2). It also has disturbed the expression of the key genes related to the control of neurodevelopment. The exposure of zebrafish larvae to A6 resulted in malformations only at 50 nM. Vismara et al. (2000) showed that the herbicides (paraquat and diquat) are highly embryotoxic and cause malformations in zebrafish larvae, primarily by causing lipid peroxidation. Generally, pesticides act by affecting various visceral organs in a way that causes abnormalities in different parts of the corps (Hamm and Hinton 2000; Willey and Krone 2001). In addition to these morphological changes, exposure to pesticides at the embryonic and larval stages also results in decline

hatching success and larval death (Aydin and Köprücü 2005) or behavioral modifications (González-Doncel et al. 2003; Kienle et al. 2009). These data are alike to those in our current study in which A6-treated embryos showed abnormal behaviors. Slotkin (2004) asserted that organophosphate pesticides cause developmental neurotoxicity and long-term cognitive and behavioral effects through routes including catecholaminergic mechanisms, as well as affecting cell signaling pathways involved in neural cell differentiation. In our study, in the A6-exposed groups, the locomotor behavior was disturbed. This may have been associated with the potential neurotoxicity of biopesticides (Fig. 3), which was in agreement with a previous article by (Nasri et al. 2016a), or related to nervous system alteration.

Generally, behavior is used as an index for evaluating neurodevelopment and has been appeared to be very sensitive to environmental pollutants early in life (Rihel et al. 2010). Exposure to members of the organophosphate pesticide family has been shown to alter swimming behavior (Sledge et al. 2011) and the reply to a surprising excitation (Eddins et al. 2010). Several studies have also observed reduced locomotor movement in larval fish immediately following embryonic treatment to chlorpyrifos (Richendrfer et al. 2012; Dishaw et al. 2014). In addition, exposure to the insecticide imidacloprid during the embryonic period was found to reduce the motion activity of zebrafish larvae (Crosby et al. 2015). Numerous analyses have displayed that various neurotransmitter systems and cellular functions are affected by exposure to organophosphate pesticides during the developmental period. A significant quantity of investigation has concentrated on recognizing the mechanisms by which pesticides apply their effects on normal behavioral development. However, exposure to organophosphates during development leads to transcriptional changes in genes linked to the action of the serotonin, dopamine, and norepinephrine neurotransmitter systems (Slotkin et al. 2007). Several factors may contribute to altered behavior at low doses, such as a rise in dopamine release in synapses caused by stress (Abercrombie et al. 1989) and a decrease in the

uptake of dopamine by dopamine transporters (Araki et al, 2006). The activity change may be due to dopamine-initiated modification in other nervous system pathways that regulate locomotion (Geyer 1995). The appearance of behavioral defects after exposure to A6 could be linked to possible effects in the brain, mainly on neurotransmitters. These play an essential role in the nervous system development (Lu et al. 2018; Tufi et al. 2015). A number of studies have published that chemical pollutants might have adverse effects on zebrafish neurotransmitters (Q. Li et al. 2018; R. Li et al. 2018; Tufi et al. 2016). In particular, the dopaminergic system is considered to consist of regulator neurotransmitters and is linked to behavior and nervous disorders (Zagrean 2014).

Dopamine is a well-described neurotransmitter known for its initiating function of motor activity in the vertebrates' brain (Yamamoto et al. 2013). A variety of pesticide groups have been related with selective dopaminergic degeneration, including dithiocarbamates, and organochlorines (Hatcher et al. 2008; Jones and Miller 2008), many of which have been shown to induce a loss of dopaminergic neurons causing a parkinsonian syndrome (Bové et al. 2005; Drechsel and Patel 2008). Other studies have shown that neurobehavioral defects are associated with developmental exposure to chlorpyrifos and can change brain dopamine content (Aldridge et al. 2005; Chen et al. 2011). Sallinen et al. (2009) showed that the pesticide MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) causes specific loss of dopaminergic neurons, decreases of the dopamine levels, and impaired motility in zebrafish larvae.

Changes in dopaminergic neuron and neurotransmitter levels were also found to be correlated with a locomotor phenotype in larval zebrafish described as a deficit in global swimming parameters (Tufi et al. 2016). In our study, the biopesticide A6 appeared to induce damage to dopamine neurons by initiating a Bax-dependent apoptosis pathway and decreasing cell proliferation in the brain (Nasri et al, 2016a). Wang et al. (2017) reported that the

exposure of adult zebrafish to rotenone decreased the levels of dopamine and TH, and caused alteration in motor function and behavior, as well as olfactory dysfunction. Precedent studies indicated that dopaminergic neurons are susceptible to paraquat (McCormack et al. 2002; Prasad et al. 2009) and a diminution of motor activity can result (Shepherd et al. 2006; Litteljohn et al. 2009).

Tyrosine hydroxylase is the first enzyme involved in the synthesis of catecholamines, especially dopamine (Nagatsu et al. 1964). It is the molecular marker most commonly used in teleosts as in mammals to evaluate the rate of biosynthesis of neurotransmitters by measuring TH enzymatic activity (Chaube and Joy 2002) or TH protein (Pasqualini et al. 1991) or mRNA levels (Weltzien et al. 2006). Our results showed that treatment of zebrafish embryos with A6 significantly decreased the mRNA level of th1 at only the highest concentration of 50 nM and the level of th2 at all tested concentrations. In a preceding study, Brooks et al. (1999) showed that paraquat can diminish TH levels in the striatum in mice. In addition, Reeves et al. (2003) measured TH levels in striatal sections of rat brains treated chronically with paraquat by both HPLC and immunohistochemistry and observed a decrease in TH in both analyses. Many studies have related the exposure to chlorpyrifos to cell signaling perturbations affecting accordingly dopamine receptor signaling (Adigun et al. 2010; Meyer et al. 2003, 2004). The decreased TH expression observed in our present report is possibly attached to the potential of the biopesticide A6 to inhibit cell proliferation (Nasri et al. 2016a). Additionally, A6 has the potential to cause endocrine disruption, being known to change the mRNA expression of estrogenic hormone-related genes such as *esr1* and *esr2a* (Nasri et al. 2016a). The involvement of estrogenic hormones in the regulation of TH expression has been suggested by several groups (Thompson and Moss 1994; Zhang et al. 2011; Tobiansky et al. 2016). This regulation takes place through the control of the transcription of the sensitive genes by nuclear receptors and may explain the TH expression identified in our work.

5. Conclusions

Our current results demonstrate that the exposure of zebrafish larvae to A6 led to a significant modification of the locomotion profile associated with the observation of neurological damage and a decrease in the expression of TH. These changes recorded were generally correlate with the alteration of physiological hormonal functions (Scott and Sloman 2004; Tierney 2011; Nasri et al. 2016a). These negative effects are triggered by disruption of estrogenic signaling pathways. Our results have just approved and added additional information on the impact of pesticides of plant origin on non-target species and highlight the importance of toxicological studies on the zebrafish model species.

Figure Captions

Fig. 1 Effect of A6 on zebrafish morphology after 6 and 9 days of treatment. (A): PE, pericardial edema; YSE, yolk sac edema; SC: spinal curvature; TD, tail deformity. (B): Percentage malformation rate for all tested concentrations of A6. Asterisks indicate significantly differences from the control, symbols: * = $p < 0.05$; ** = $0.05 \leq p < 0.001$; *** = $0.001 \leq p < 0.0001$.

Fig. 2 A6 accumulation in zebrafish tissue. (A, C, E, G, I) head with Bright field and Fluorescence of confocal microscopy; (B, D, F, H, J) the rest of the body. Scale bars: 100 μm . Asterisks indicate significantly differences from the control, symbols: * = $p < 0.05$; ** = $0.05 \leq p < 0.001$; *** = $0.001 \leq p < 0.0001$.

Fig. 3 Effect of A6 on larval swimming behavior. Species are put individually in an observation chamber and their movements are continuously recorded over the next 10 min. Figure shows four representative tracks from 20 larvae as well as the duration of trips to the center or to the periphery (expressed in s) after A6 exposure or to control (DMSO). The overall activity for each exposure group was represented by symbols: +: active; -: less active;

--: very less active. Asterisks indicate significantly differences from the control (* = $p < 0.05$; ** = $0.05 \leq p < 0.001$; *** = $0.001 \leq p < 0.0001$).

Fig. 4 Relative gene expression of th1 and th2 after A6 exposure. Results are given as mean values \pm SEM (n = 9 replicates). Asterisks indicate significantly differences from the control, symbols: * = $p < 0.05$; ** = $0.05 \leq p < 0.001$; *** = $0.001 \leq p < 0.0001$.

Fig. 5 Tyrosine hydroxylase immunohistochemistry in wild type zebrafish larvae (6 dpf) exposed to A6. Olfactory bulb (OB), telencephalon (Tel), subpallium (SP), pretectum (Pr), optic tectum (TeO), area postrema (AP), Green staining is tyrosine hydroxylase-positive catecholaminergic cells. (A, E, I) represent a strong positive labeling of tyrosine hydroxylase in the forebrain in control animals. (B, C, D), (F, G, H) and (J, K, L) show the areas of immunostaining of tyrosine hydroxylase respectively in the telencephalon and the diencephalon (pretectum and postrema) in individuals treated with A6. These are dorsal views of 100 μ m sections of the brain.

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Disclosure statement

The authors have no conflict of interest.

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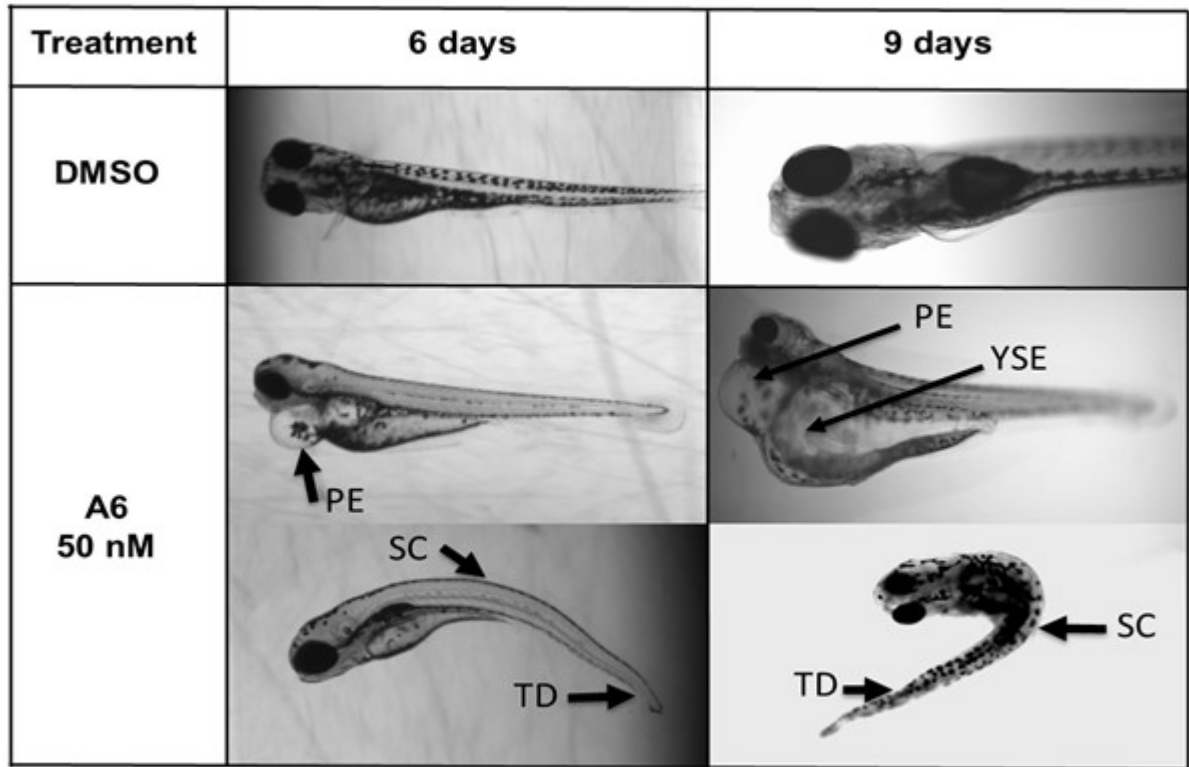
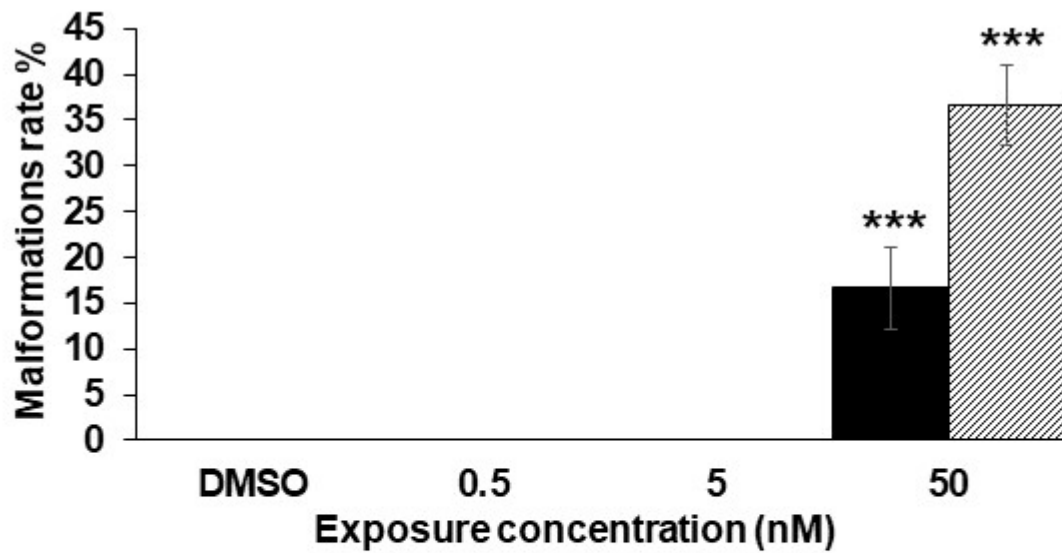
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Table 1. Primers used for qRT-PCR analyses.

Target genes	Accession numbers	Primer sequence
<i>th1</i>	XM_682702.1	(fw) 5'-GAC-GGA-AGA-TGA-TCG-GAG-ACA-3' (rev) 5'-CCG-CCA-TGT-TCC-GAT-TTC-T-3'
<i>th2</i>	NM_001001829	(Fw) 5'-CTCCAGAAGAGAATGCCACATG-3' (Rev) 5'-ACGTTCACTCTCCAGCTGAGTG-3'
<i>ef1a</i>	NM_131263.1	(fw) 5'-AGCAGCAGCTGAGGAGTGAT-3' (rev) 5'-CCGCATTTGTAGATCAGATGG-3'

A**B****Figure 1**

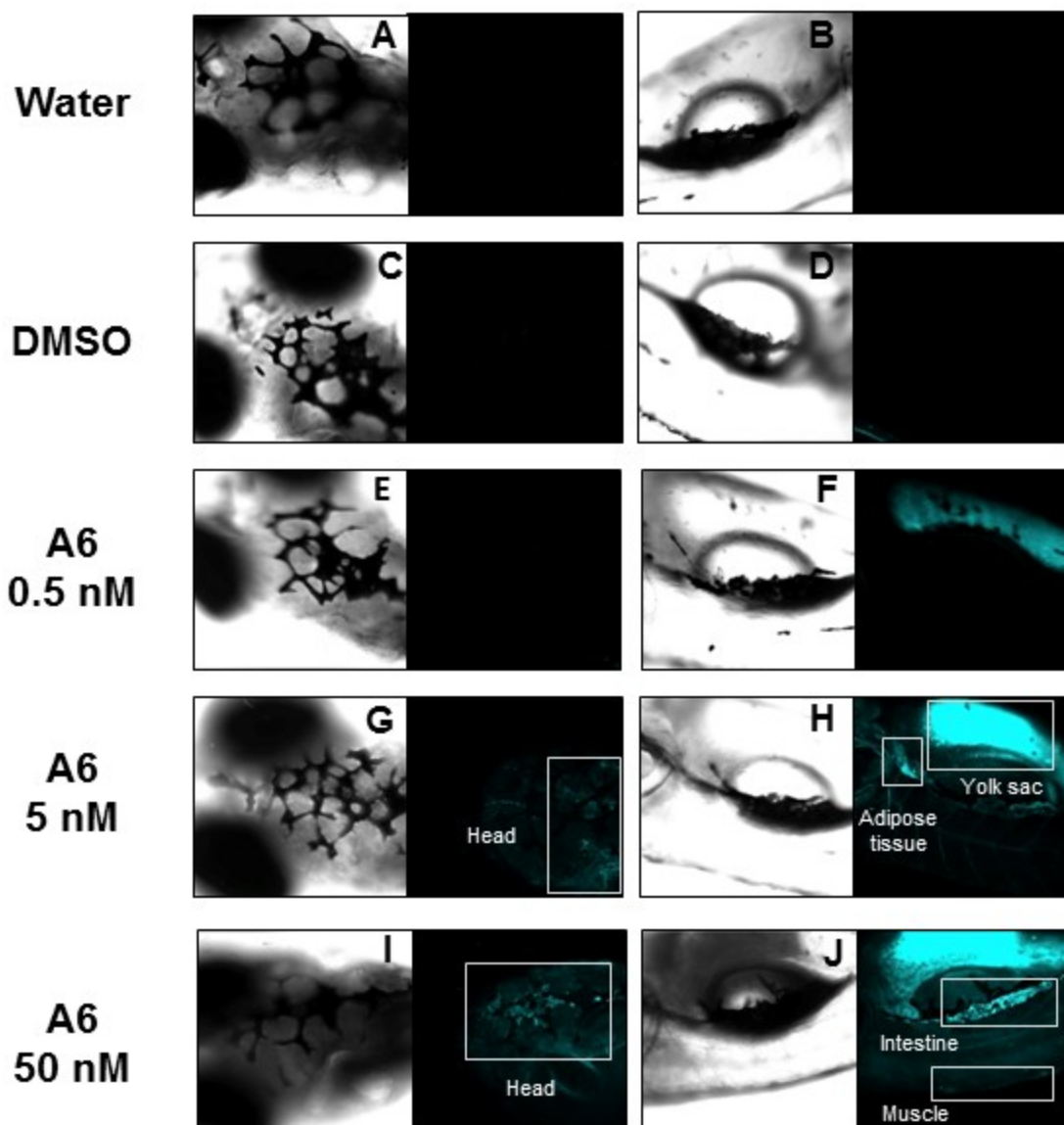
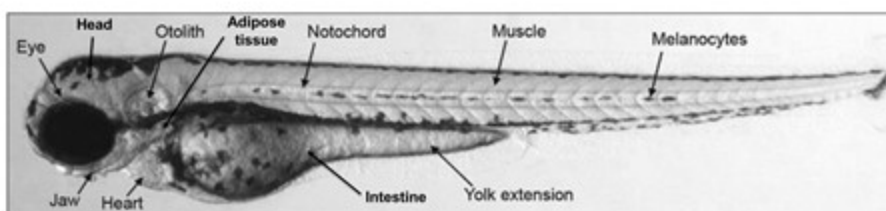
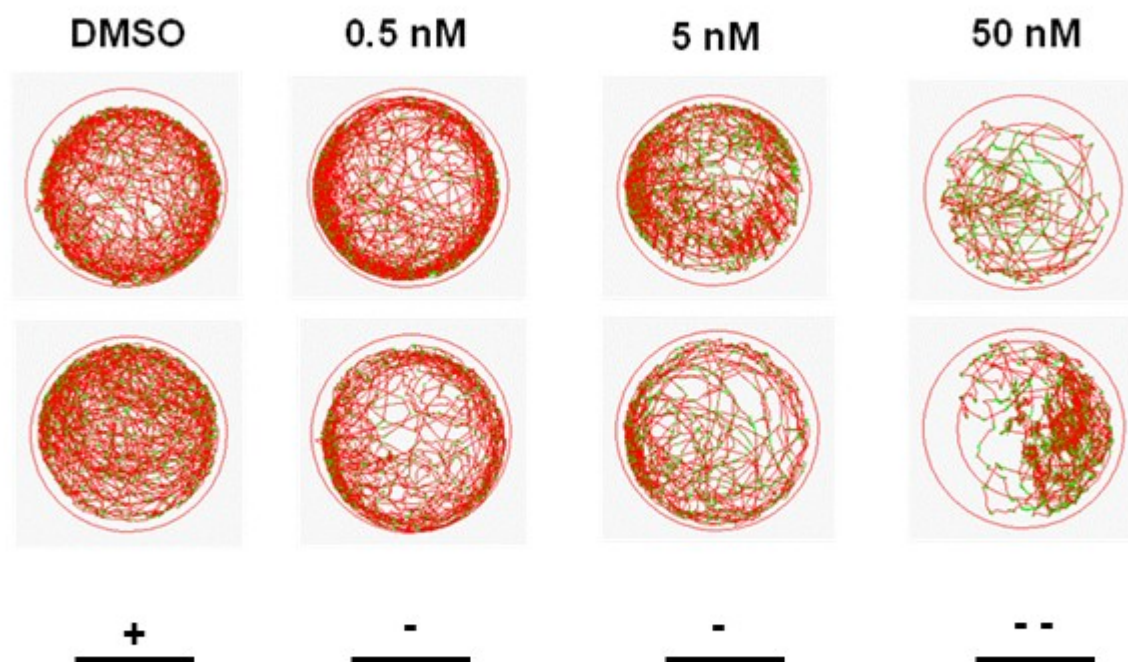
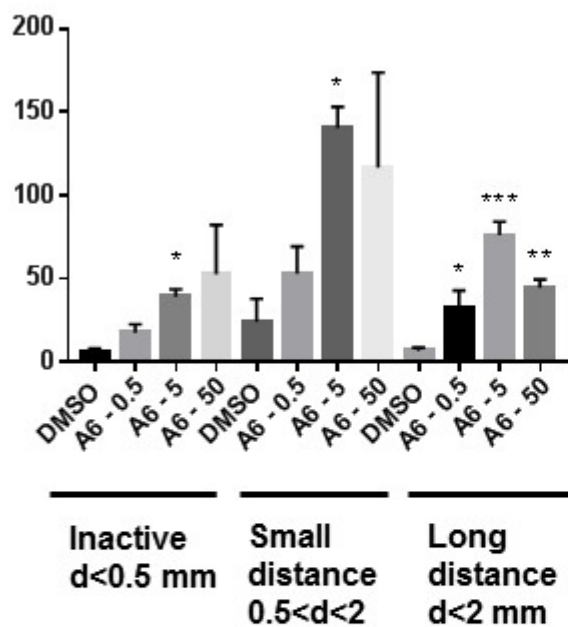


Figure 2



Duration of trips to the center
(expressed in s)



Duration of trips to the periphery
(expressed in s)

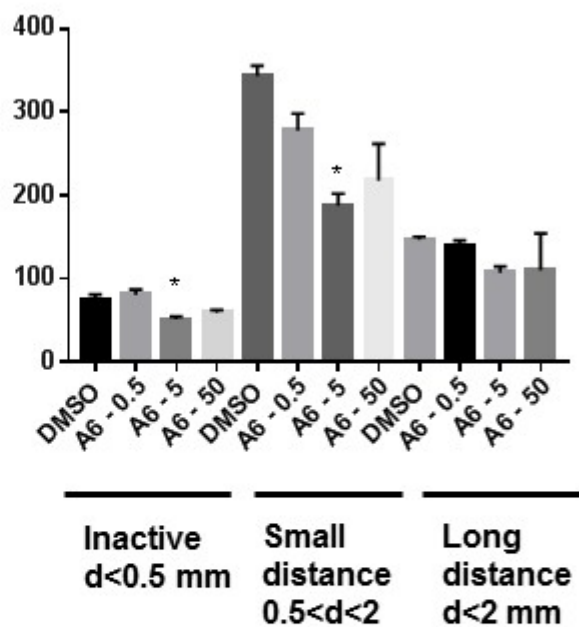


Figure 3

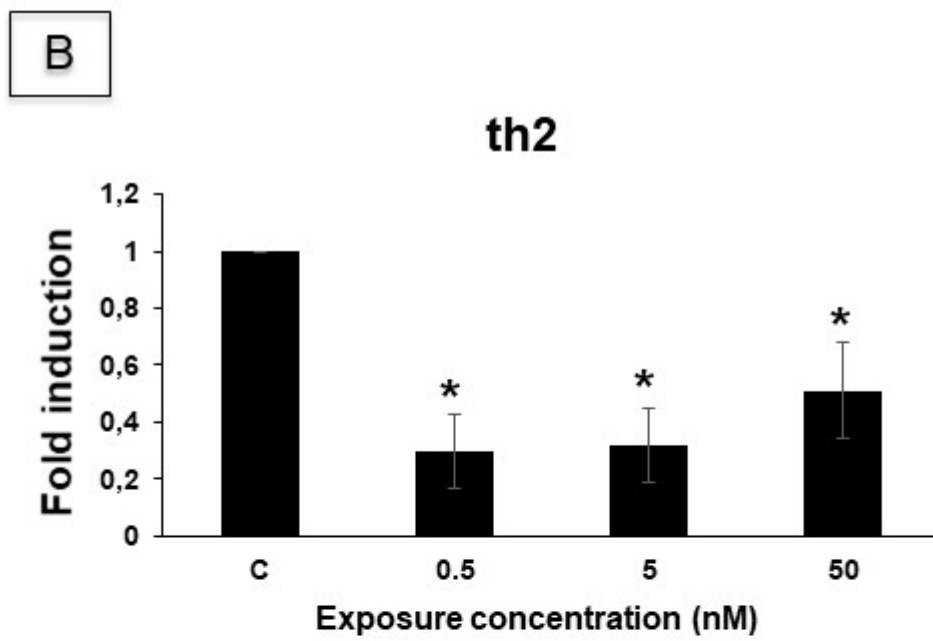
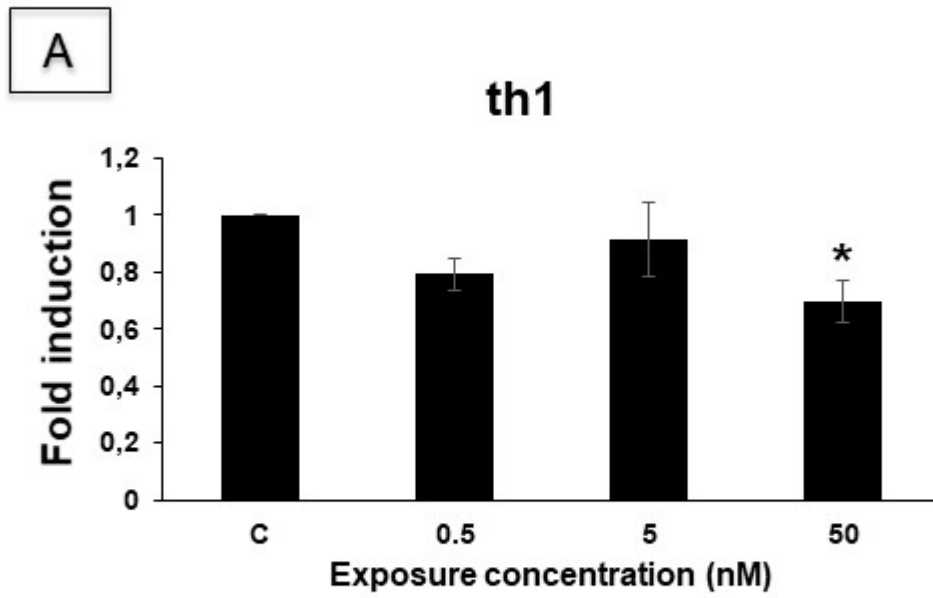
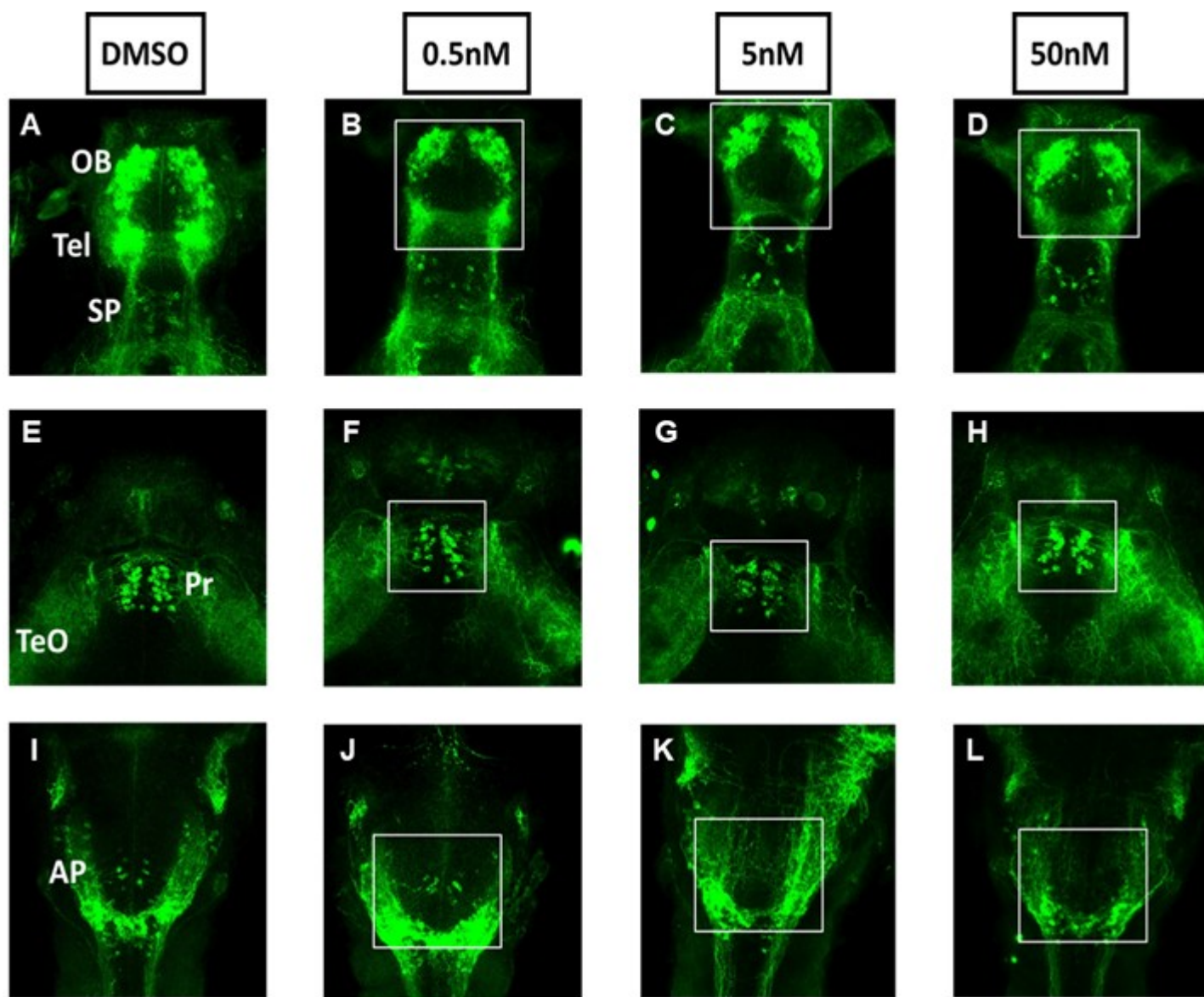


Figure 4



Fluorescence intensity
(arbitrary units)

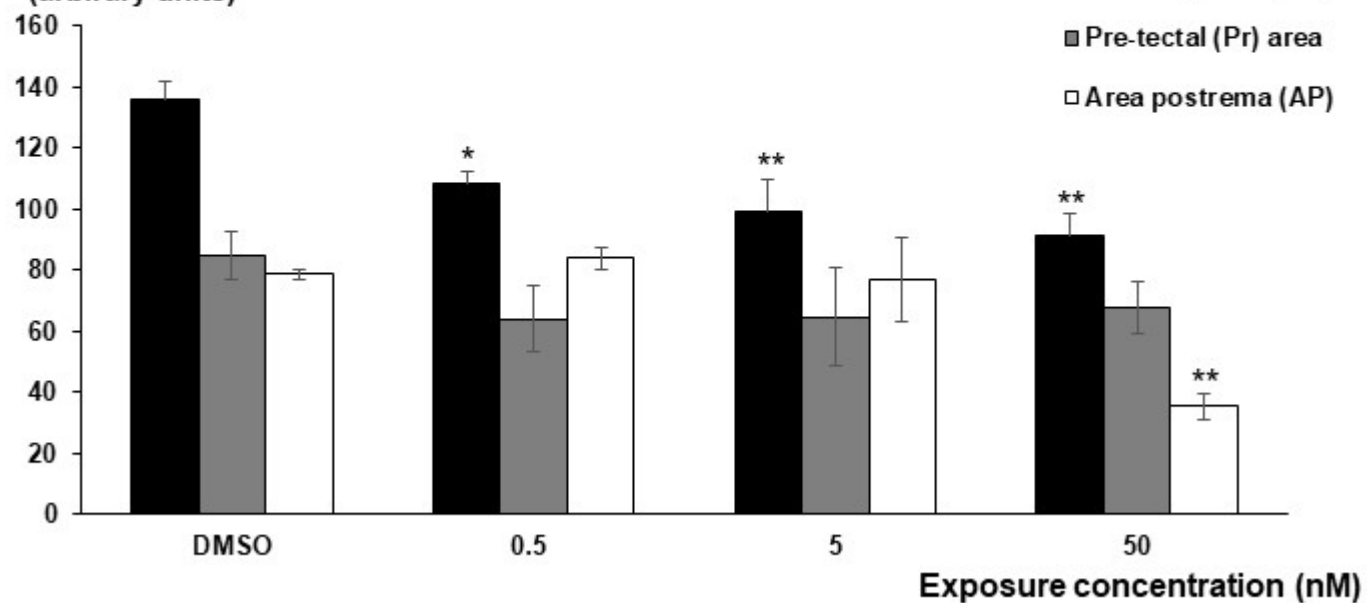


Figure 5