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The role of zebrafish larvae for studying anxiety-like behaviour

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Chapter 4

Serotonin toxicity-like phenotypes in zebrafish larvae – chronic treatment with serotonergic psychotropic drugs fails to attenuate thigmotaxis

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Abstract

Serotonin toxicity is a life-threatening disorder observed in patients consuming serotonergic drugs excessively. We report the presence of phenotypes that resemble symptoms of serotonin toxicity in 5 days post fertilized (dpf) zebrafish larvae treated with serotonergic psychotropic drugs only (amitriptyline, buspirone, and fluoxetine), but not after exposure to diazepam. We used behavioural assays that evaluated larval locomotion, startle response, and thigmotaxis, which commonly used as proxies for anxiety-like behaviour to identify the serotonin toxicity. Untreated zebrafish larvae show reduced thigmotaxis levels during a dark challenge phase. Overall, larval zebrafish retained the reduced thigmotaxis levels after acute pre-exposure to all drugs. However, chronic pre-exposure to amitriptyline and fluoxetine impaired this robust behavioural activity. To confirm our hypothesis that serotonergic drugs could cause serotonin toxicity in zebrafish larvae, we evaluated larval burst activity after the vibrational stimulus. Amitriptyline and buspirone impaired the response to the stimulus. Our results suggest that zebrafish larvae show phenotypes resembling serotonin toxicity after chronic treatment with serotonergic drugs. Moreover, only acute exposure to amitriptyline (2.5 mg/L) and diazepam (0.71 and 1.42 mg/L) attenuated thigmotaxis resembling putative pharmacological effects. In conclusion, we suggest that young larvae are at a critical time point of development that may affect the outcome of the behavioural response to environmental stimuli.

Introduction

Anxiety-related disorders are recognised as one of the great challenges of the 21st century, in terms of health, economy, and society [1]. The current major pharmaceutical for anxiety-related disorders include benzodiazepines such as diazepam and 'non-benzodiazepines' anxiolytics such as buspirone.[2] Moreover, health practitioners often prescribe antidepressants for anxiety-related disorders since depression is frequently comorbid with anxiety [3]. Some examples of antidepressants are selective serotonin reuptake inhibitors (SSRIs), serotonin norepinephrine reuptake inhibitors (SNRIs), tricyclic antidepressants (TCAs), and monoamine oxidase inhibitors (MAOIs).

The use of antidepressants is very common and is increasingly intensifying among all age groups [4-7]. Serotonin toxicity (serotonin syndrome) is one of the main health concerns often resulting from the excessive use of antidepressants [8, 9]. Excessive activation of central and peripheral serotonin receptors results in the clinical manifestation of serotonin toxicity [10]: (i) altered mental status, (ii) central nervous system (CNS) activation, and (iii) autonomic hyperactivity [11, 12]. These symptoms can range from a mild manifestation to being lethal [11]. Progression from mild to moderate conditions causes altered mental status (agitation, confusion, etc.), insomnia, and hypertension, and severe symptoms include muscular rigidity, seizures, and coma [13].

In most cases, serotonin toxicity is reported in patients who have consumed a combination of antidepressants [14]. However, this condition was also reported after an overdose of a single serotonergic agent [15]. According to the American Association of Poison Control Centers, in 2011, there were 1,757 serious outcomes due to SSRIs prescription, with 11 being mortal [16]. The number of serotonin toxicity incidents reported are likely an underrepresentation and the actual cases could exceed the number of reported cases since this condition is frequently under-diagnosed [17].

Given the above, the increasing prevalence of serotonin toxicity has become an important biomedical concern [8, 9, 18]. So far, rodent studies have been useful in resembling clinical phenotypes of serotonin toxicity [19-24]. For example, models lacking serotonin transporter (*SERT*) gene have been developed that displayed

elevated extracellular serotonin levels [19, 21, 23]. Although rodent studies have been useful in elucidating the neurochemistry of serotonin toxicity, a limitation consists in inbred strains which do not replicate genetic variations seen of humans [25]. In contrast, zebrafish species show considerable genetic polymorphism and therefore less inbreeding than in rodents [26, 27]. Moreover, larval zebrafish offer low husbandry cost, rapid development, and are useful in high throughput screenings [28-30]. These features can be very helpful in preclinical drug screenings and modeling [29, 30].

Zebrafish are increasingly used to study human brain disorders, including neurological toxidromes (a constellation of signs and symptoms associated with a particular substance or group of substances [31]), because of their strong similarity with human and non-human vertebrates on major brain structures, neurotransmitters, receptors, hormones and functionality [32-36]. Previous studies revealed larval zebrafish to be highly sensitive to a wide range of serotonergic drugs such as amitriptyline, buspirone, and fluoxetine, leading to changes in behaviour associated with anxiety-like phenotypes [37-40]. A recent study revealed serotonin toxicity like behavioural phenotype in adult zebrafish after acute exposure with the antidepressant amitriptyline [41].

In addition, zebrafish larvae have been used extensively in studying anxiety-like responses using behavioural assays such as the visual motor response (VMR), scototaxis (dark/light preference), and thigmotaxis (preference of peripheries/avoidance of open fields). Especially high thigmotactic behaviour in an open arena indicates a low degree of exploratory behaviour, which is associated with anxiety [42, 43]. This behavioural phenotype is evolutionarily conserved across various vertebrate species [43-45]. In addition to this, Thigmotactic behaviour can be reduced by the administration of different types of anxiolytics such as diazepam [38, 46] and fluoxetine [38].

The pharmaceuticals used in this study were amitriptyline (Elavil), buspirone (Buspar), diazepam (Valium), and fluoxetine (Prozac). These drugs are presumed to be causing their therapeutic effects via the following pharmacological interventions: (i) amitriptyline elevates neurotransmitter at the synaptic cleft by blocking reuptake of serotonin and norepinephrine [47, 48], (ii) benzodiazepines interact with the $GABA_A$ receptors in the central nervous system (CNS) [49, 50], (iii) buspirone acts as a full

agonist at presynaptic and partial agonist at postsynaptic serotonin receptors [51, 52], and (iv) fluoxetine increases serotonin concentration in many areas of the brain by blocking the reuptake pumps [53].

Objectives of study

The objective of the current study is to evaluate the incidence of serotonin toxicity in larval zebrafish, by exploring larval behaviour after exposure to serotonergic drugs. Our hypothesis was that chronic treatment with all serotonergic drugs used in this study, but not diazepam (negative control), would induce behavioural responses that resemble serotonin toxicity. Several behavioural parameters were used to assess the presence of serotonin toxicity like phenotypes in the larvae: (i) general locomotion patterns, (ii) thigmotaxis in response to a dark challenge and (iii) startle response induced by the vibrational stimulus.

Materials and methods

Ethics statement

Animal experimental procedures conducted in this study were all carried out in accordance with the Dutch Animals Act (http://wetten.overheid.nl/BWBR0003081/2014-12-18), the European guidelines for animal experiments (Directive 2010/63/EU; http://eur-lex.europa.eu/legal-content/NL/TXT/HTML/?uri=CELEX:32010L0063) and institutional regulations.

Zebrafish husbandry

Adult zebrafish (*Danio rerio*) of ABTL wild type strains were maintained in the facility according to the local animal welfare regulations and standard protocols (zfin.org). Zebrafish eggs were obtained by natural spawning (family crossings). Fertilization was performed by at the beginning of the light period. The eggs were harvested the following morning and transferred into 92 mm Ø Petri dishes (approximately 80 eggs per dish) containing 40 mL fresh embryo medium (EM) as a vehicle (control). This medium consists of 10% Hank's balanced salt solution at a concentration of 0.98 g/L in milli-Q water (resistivity = 18.2 MΩ cm), with the addition of sodium bicarbonate at 0.035 g/L to adjust pH to 7.46. Similar buffer medium has been used previously [54, 55]. Unfertilized, unhealthy and dead embryos

were identified under a stereomicroscope and discarded using a plastic Pasteur pipette.

At 1 dpf, the embryos were again screened and any dead or unhealthy embryos were removed before being transferred into 24 well plates. 24-well consist of wells with a diameter of 15.4 mm, which is sufficiently large enough to allow free swimming behaviour in zebrafish larvae [46], necessary to measure thigmotaxis [56]. Each well of a 24 well plate contained one embryo. Throughout all procedures, the embryos and the solutions were kept at 28 ± 0.5°C, under a 14:10 hours light: dark cycle (lights switches on at 08:00).

Pre-exposure to pharmaceuticals

Zebrafish larvae were exposed to amitriptyline (AMI, Sigma-Aldrich, PHR1384), buspirone (BUS, Sigma-Aldrich, B7148), diazepam (DZM, Duchefa Farma 5372) and fluoxetine (FLU, Sigma-Aldrich, F132) at different range of concentrations (see **Table 1**), prepared from a stock solution. The larvae were subjected to two different pre-exposure regimes prior to the initiation of behaviour analysis, i.e. acute (1 min) and chronic exposure (24 h). The larvae remained in the pharmaceutical solutions throughout the behavioural test. All behavioural tests were conducted at 5 dpf larvae. Hence, chronic exposure was initiated on 4 dpf larvae.

Table 1. Concentration ranges used in this study and their locations in the 24 well plates. N = 48 for both controls and untreated larvae.

		Location in 24 well plates (C=Column)					
		C1	**C2**	**C3**	**C4**	**C5**	**C6**
Drug/ DMSO concentration µg/ml [%]	AMI	0	0.625	1.25	2.5	5	10
	BUS	0	6.25	12.5	25	50	100
	DZM	0[0]	0[0.02]	0.71[0.02]	1.42[0.02]	2.84[0.02]	5.68[0.02]
	FLU	0	0.4	0.8	1.6	3.2	6.4

(DMSO = Dimethylsulfoxide. All drugs were dissolved in embryo medium except for DZM, which was dissolved using DZM. The final concentration of DMSO for each DZM treatment is 0.02%.)

Behavioural tests

In this study, we used (i) general locomotion patterns and (ii) thigmotaxis, as a response to a dark challenge [46] and a vibrational stimulus [57] to identify phenotypes that could resemble serotonin toxicity. Dark challenge experiments were

conducted in a ZebraBox (ViewPoint, Lyon, France) recording apparatus, equipped with a video camera (Point Grey FlyCap 2, Richmond, Canada) and recording software (ViewPoint, Lyon, France). Video footage was later analysed using Ethovision® XT 10 (Noldus Information Technology, Wageningen, Netherlands) software. Vibrational stimulus experiments were conducted using an inbuilt tapping device in the DanioVision™ DVOC-0040 set up while video analysis was simultaneously performed using Ethovision XT 11.5 (both from Noldus Information Technology, Wageningen, Netherlands).

Dark challenge

After an acclimatization period of 10 minutes during the light phase (L), the larvae were exposed to sudden darkness during the dark challenge of 4 minutes D), causing an immediate and significant increase in swimming activity (**Figure 1A**). The light intensity during L was 163.20 ± 17.25 (mean ±SD) lux, the light intensity during D was 0 lux. During L and D, (i) general locomotion and (ii) thigmotaxis were measured and compared between these two phases.

General locomotion was measured as total distance moved (in mm) over 10-minute intervals across the whole area of the well (arena). Thigmotaxis was measured as two different values. Absolute values represent the distance travelled away from the periphery of an arena (Ethovision XT 10.0 reference manual; in our study, a well of a 24 well plate is considered as an arena). Relative values represent percentage (%) of the total distance moved (TDM, mm) in the outer zone of the well ($\%TDM_{out}$), i.e. a peripheral zone alongside the walls of the well, with a width of approximately one body length (4 mm) [46]. Therefore, thigmotaxis was calculated as a ratio between TDM in the outer zone (TDM_{out}) and TDM over the whole test arena [58], consisting of the TDM in the outer (TDM_{out}) and TDM of the inner zone (TDM_{in}). This calculation is necessary to correct individual differences as recommended by Bouwknecht and Paylor [58]. ($\%\mathrm{TDM_{out}}$) was used to assess the pharmacological effects of the four drugs. A similar variable was used previously to show anxiolytic and anxiogenic effects in zebrafish larvae previously [46, 59].

$$\%TDM_{out} = \frac{TDM_{out}}{Total\ distance\ travelled\ in\ arena\ (TDM_{out} + TDM_{in})} \times 100$$

Vibrational stimulus

To test whether observed differences in the dark challenge exposure are due to the drugs reducing anxiety-like behaviour, or disruption of motor neurons, a reflexive startle response was elicited by a vibrational stimulus (DanioVision™ version DVOC-0040 reference manual). The experimental timeline (**Figure 1B**) was identical to the dark challenge, followed by an additional recovery light phase and then a vibrational stimulus (tapping) at the highest intensity level [57]. After two more seconds, the observation period ended. We chose maximum velocity (mm/s) as a response variable for this analysis to estimate the startle response to the tapping stimulus [57].

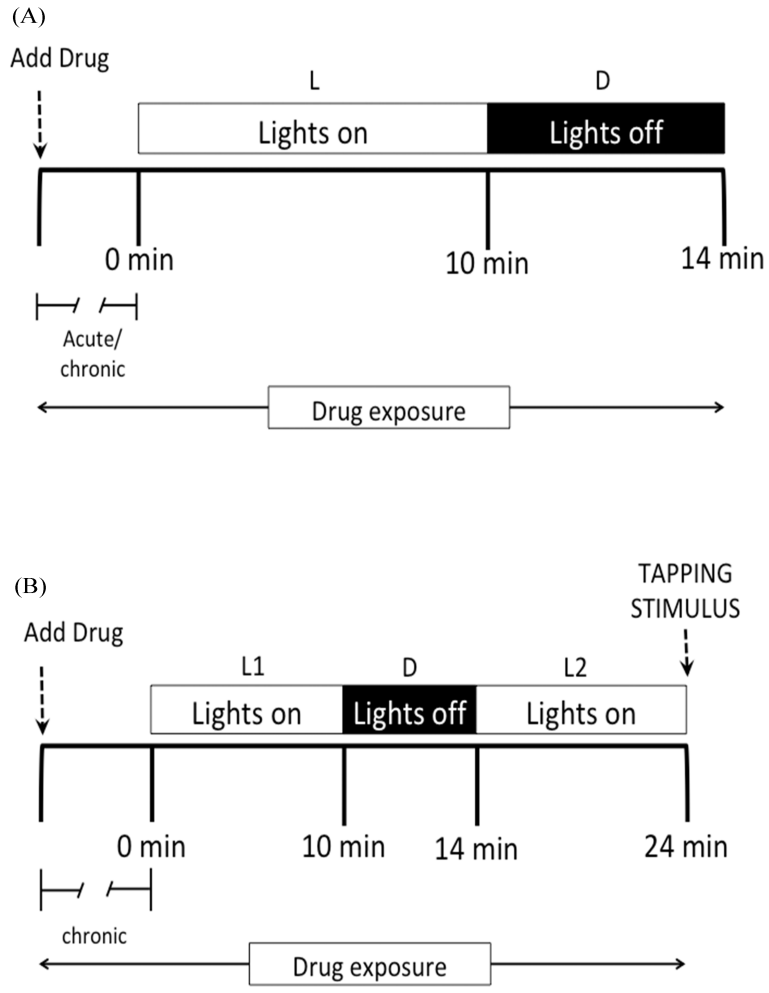

Figure 1. The experimental scheme used in the present study. (A) Thigmotaxis analysis with dark challenge stimulus. Data collected from both basal (L) and challenge (D) phase were used to assess absolute thigmotaxis levels (distance travelled away from the periphery of the wells in 24-well plate). Relative thigmotaxis levels were extracted from dark challenge phase. (B) Larval burst activity measured as maximum velocity after vibrational stimulus induced by tapping.

Statistical analyses

Behavioural data from general locomotion, relative thigmotaxis level, and larval burst activity were analysed using linear models. Residuals from the regression model were checked for normality using a Q-Q plot. One-way ANOVA with Dunnett's post hoc analysis was used to compare treatments with control larvae if the assumption of normality is not violated. When the normality test failed, Kruskal-Wallis test with Pairwise Mann-Whitney U-test as post hoc analysis was chosen. Effect sizes and degrees of freedom were always reported. Behavioural data from absolute thigmotaxis level were analysed using mixed model with repeated measures. Distance travelled away from the periphery between basal and dark challenge was compared using the estimated marginal means (emmeans) package in R studio. All statistical analyses were done using RStudio© (version 1.1.456). N was 48 for both controls and drug treatments, and significance was accepted at $p<0.05$. In order to estimate a possible plate and positional effect, we performed a Moran's I test, with full special weighing and nearest neighbours only as parameters ($n=3$, $N=24$, $p<0.05$). In all cases, there was no significant plate or positional effect.

Results

Acute drug treatment

General locomotion

AMI and BUS (**Figure 2A** and **C**, **Table 2**) showed a dose-response in locomotor activity resulting in a significant reduction starting at 2.5 mg/L for AMI and 25 mg/L for BUS. The reduction measured for both drugs at the maximum concentration was 75%. DZM (**Figure 2E**; **Table 2**) showed a plateau dose response, but with a significant reduction starting from 1.42 mg/L. Finally, FLU (**Figure 2G**; **Table 2**) showed an optimum dose response upon acute treatment with maximum locomotion levels at 0.8 and 1.6 mg/L. There was no significant effect of the DZM solvent DMSO on locomotion.

Absolute thigmotaxis level (distance travelled away from the periphery)

Untreated zebrafish larvae from the control groups show reduced thigmotaxis level (increased swimming activity/ distance travelled away from the periphery) during dark challenge phase compared to the basal phase. Acute exposure to all

concentrations of the drugs tested did not alter this robust behavioural activity whereby larvae retained the increased swimming activity during the dark challenge compared to the basal phase (**Figure 2B**, **D**, **F**, and **H**).

Table 2. AMI, BUS, DZM, and FLU concentrations that caused significant effects on general locomotion of zebrafish larvae in the basal phase after acute and chronic exposure. DMSO (dimethylsulfoxide) is used to dissolve DZM. Abbreviations: *H* = Kruskal-Wallis chi-squared values; df = degrees of freedom.

Drugs (Exposure)	Comparison (Control⇔ Drug Dose)	General locomotion (distance moved in mm) Mean ± SEM, *n*	*p*-values	Test statistics
AMI (acute)	0⇔2.5	1179.14 ± 90.09, *n = 48*	≤ 0.05	
	0⇔5.0	544.23 ± 43.74, *n = 48*	≤ 0.001	*H* = 125.47, df = 5
	0⇔10.0	424 ± 39.79, *n = 48*	≤ 0.001	
AMI (chronic)	0⇔1.25	504.51 ± 64.4, *n = 47*	≤ 0.001	
	0⇔2.5	271.35 ± 16.6, *n = 46*	≤ 0.001	*H* = 201.26, df = 5
	0⇔5.0	127.24 ± 10. 2, *n = 38*	≤ 0.001	
	0⇔10.0	2.59 ± 2.77, *n = 37*	≤ 0.001	
BUS (acute)	0⇔25	1168.67 ± 82.74, *n = 48*	≤ 0.05	
	0⇔50	698.81 ± 47.88, *n = 48*	≤ 0.001	*H* = 134.67, df = 5
	0⇔100	421 ± 25.68, *n = 48*	≤ 0.001	
BUS (chronic)	0⇔6.25	696.78 ± 96.87, *n = 48*	≤ 0.05	
	0⇔12.5	423.50 ± 53.02, *n = 48*	≤ 0.001	*H* = 60.83, df = 4
	0⇔25	219.05 ± 31.14, *n = 41*	≤ 0.001	
	0⇔50	184.44 ± 66.67, *n = 32*	≤ 0.001	
DZM (acute)	0[0]⇔1.42[0.02]	1111.03 ± 95.81, *n = 48*	≤ 0.05	
	0[0]⇔2.84[0.02]	854.13 ± 65.34, *n = 48*	≤ 0.001	*H* = 45.06, df = 5
	0[0]⇔5.68[0.02]	936.20 ± 82.57, *n = 48*	≤ 0.001	
DZM (chronic)	0[0]⇔5.68[0.02]	728.41 ± 74.89, *n = 48*	≤ 0.01	*H* = 26.80, df = 5
FLU (acute)	0⇔0.8	1833.83 ± 97.30, *n = 48*	≤ 0.001	*H* = 22.61, df = 5
	0⇔1.6	1798.22 ± 87.69, *n = 48*	≤ 0.001	
FLU (chronic)	0⇔0.8	761.82 ± 91.27, *n = 48*	≤ 0.001	
	0⇔1.6	752.38 ± 88.50, *n = 48*	≤ 0.001	*H* = 98.09, df = 5
	0⇔3.2	475.34 ± 51.61, *n = 48*	≤ 0.001	
	0⇔6.4	331.26 ± 38.56, *n = 48*	≤ 0.001	

Chronic drug treatment

General locomotion

Chronic treatment of AMI (**Figure 3A**; **Table 2**) caused a significant drop in locomotion from concentrations of 1.25 mg/L and above. Moreover, chronic exposure to amitriptyline caused lethal effects at the hree highest concentrations. BUS (**Figure 3C**; **Table 2**) led to a reduction of locomotion at 6.25 mg/L and above, and lethal effects starting at 25 mg/L, with 100% mortality at 100 mg/L. DZM led to a reduced in locomotion only at 5.68 mg/L (**Figure 3E**; **Table 2**). FLU caused a reduction in locomotion starting at 0.8 mg/L (**Figure 3G**; **Table 2**). Chronic exposure to DMSO had no measurable effect.

Absolute thigmotaxis level (distance travelled away from the periphery)

Chronic treatment with amitriptyline and fluoxetine impaired increased swimming activity in the dark challenge phase at certain concentrations. For example, 2.5 and 5 mg/L of amitriptyline reduced larval swimming activity resulting in similar levels of

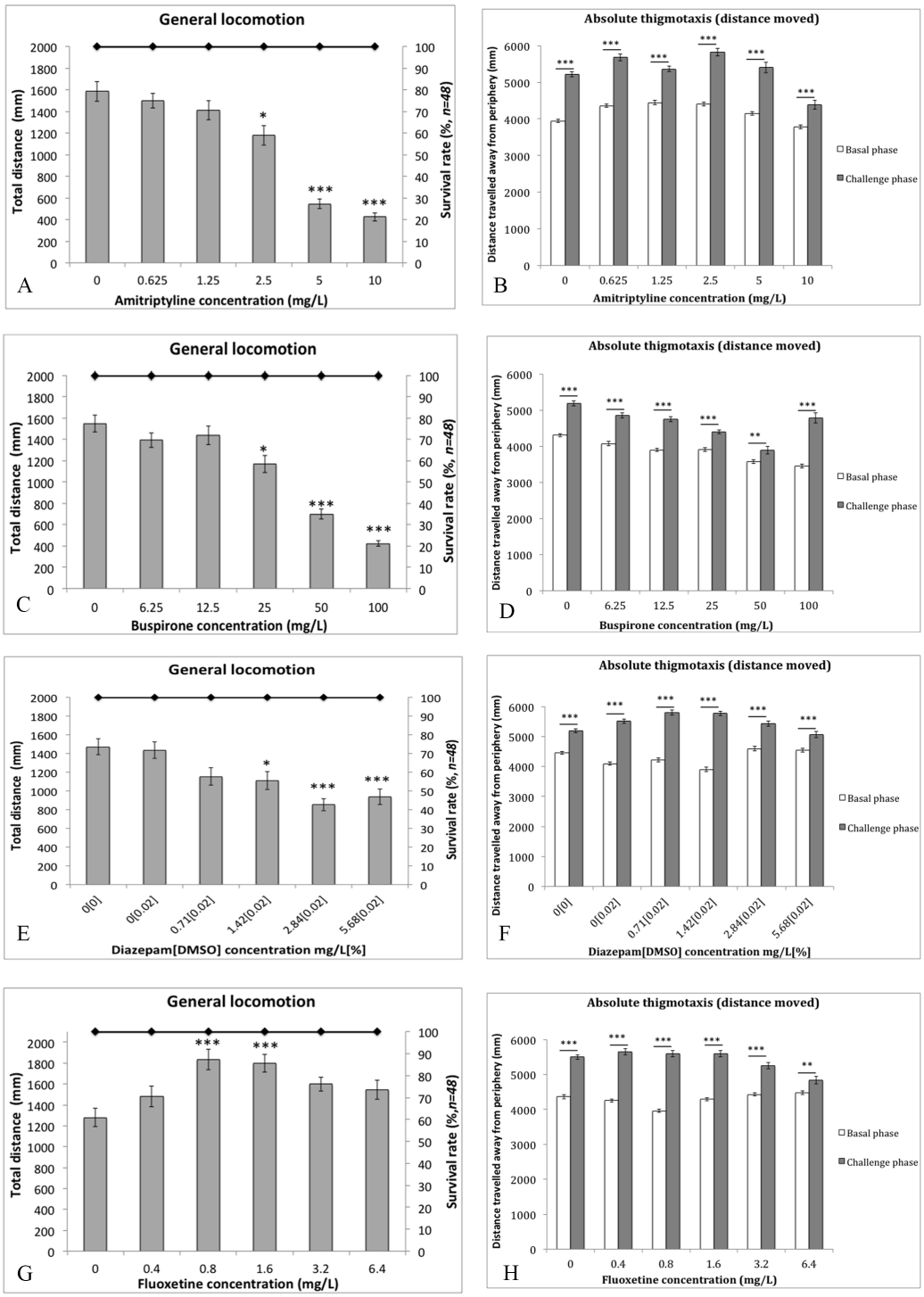

Figure 2. Impact of drugs on general locomotion (A, C, E, and G) and absolute thigmotaxis level (B, D, F, and H) after acute exposure. Absolute thigmotaxis is measured as distance traveled away from the periphery of the wells. Line represents survival rate. Bar chart represents mean ± standard errors of mean (SEM) values. Statistical icons: *p-value ≤ 0.05, ** p-value ≤ 0.01 and *** p-value ≤ 0.001. Abbreviation: DMSO = dimethylsulfoxide.

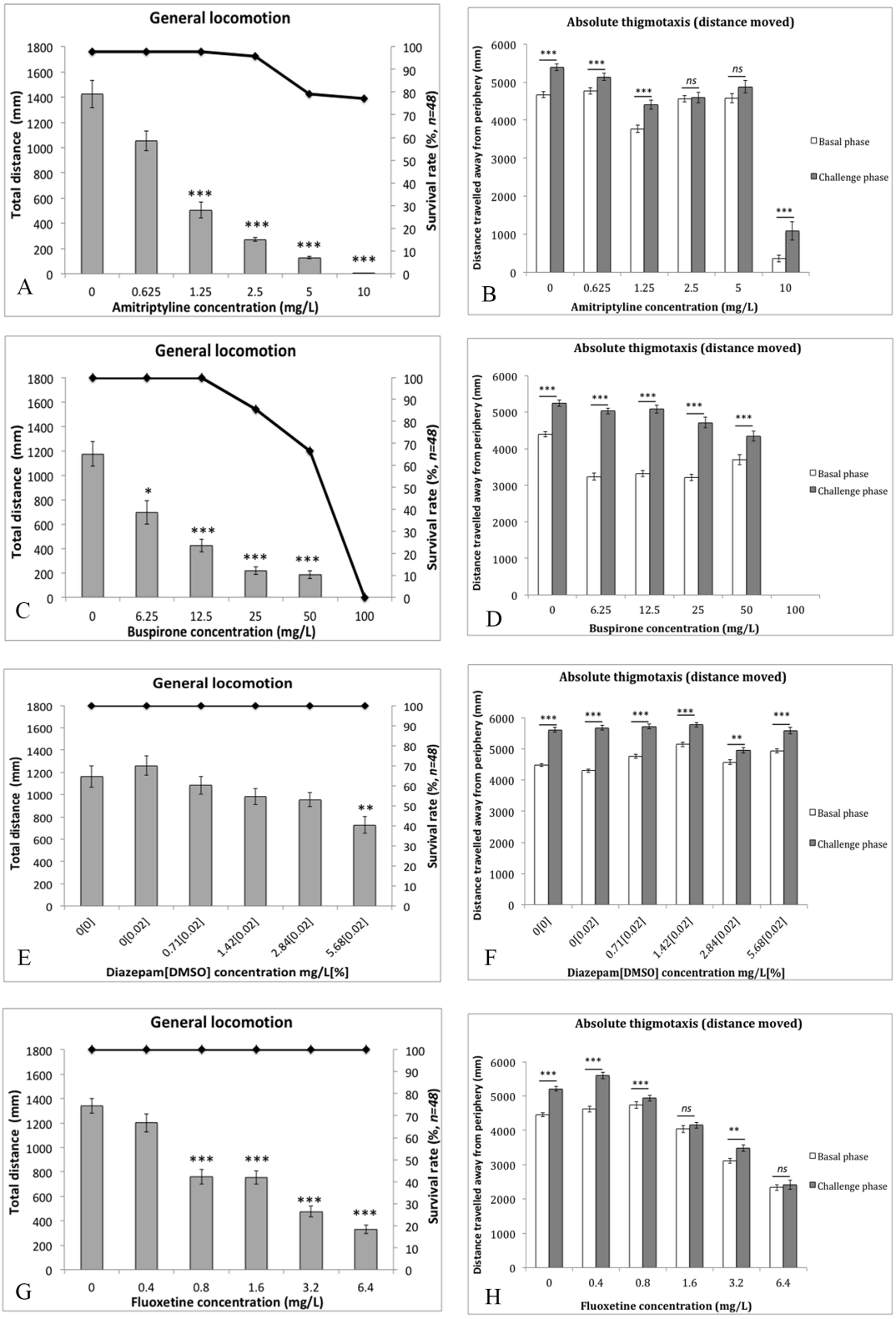

Figure 3. Impact of drugs on general locomotion (A, C, E, and G) and absolute thigmotaxis level (B, D, F, and H) after chronic exposure. Absolute thigmotaxis was measured as distance travelled away from the periphery of the wells. Line represents survival rate. Bar chart represents mean ± standard errors of mean (SEM) values. Statistical icons: *p-value ≤ 0.05, ** p-value ≤ 0.01 and *** p-value ≤ 0.001. *ns*: statistically not significant. Abbreviation: DMSO = dimethylsulfoxide.

thigmotaxis, measured as distance travelled away from the periphery, in both basal and dark challenge phase (**Figure 3B**). At a concentration of 10 mg/L, larval swimming activity in both basal and dark challenge phase dropped to less than 20% compared to the untreated larvae, resulting in significant ($p \leq 0.001$) differences between the phases. FLU at 1.6 and 6.4 mg/L also induced reduction of swimming activity resulting in equal thigmotaxis level in both basal and the dark challenge phase (**Figure 3H**). Chronic treatment with BUS and DZM did not alter thigmotaxis level in larvae after dark challenge phase (**Figure 3D** and **F**), whereby larval swimming activity significantly differed between basal and dark challenge phase overall concentrations.

Relative thigmotaxis level (% TDM in outer zone)

When measuring thigmotaxis as % TDM in outer zone, acute treatment with diazepam ($H_{(5)} = 38.538$, $p \leq 0.001$; **Figure 4A**) significantly reduced % TDM in the outer zone at 0.71 ($n = 48$, value = 60.33 ± 1.30) and 1.42 mg/L ($n = 48$, value = 62.96 ± 1.55) relative to the untreated larvae. Larval preference for the outer zone was not changed after chronic exposure to diazepam at all tested concentrations (**Figure 4B**). Acute amitriptyline (**Figure S1A**; **Table S1**) significantly reduced larval movement in the outer zone at 5 mg/L, however, at 10 mg/L % TDM in the outer zone increased compared to the untreated larvae. Larvae exposed chronically to amitriptyline showed increased % TDM in the outer zone at 1.25, 2.5 and 5 mg/L (**Figure S1B**; **Table S1**). Larval movement in the outer zone increased after both acute and chronic treatment with Buspirone (**Figure S1C** and **D**; **Table S1**) and fluoxetine (**Figure S1E** and **F**; **Table S1**).

Vibrational stimulus

One-way ANOVA test and Dunnet's post hoc analysis showed that larvae exposed chronically to amitriptyline ($F_{(2,63)} = 7.313$, $p \leq 0.05$) and buspirone ($F_{(4,87)} = 2.961$, $p < 0.05$) did not respond to the high intense vibrational stimulus at certain concentrations. For example, 10 mg/L of amitriptyline reduced the burst activity of larvae after the vibrational stimulus (**Figure 5A**; Table 3). Buspirone at 6.25 and 12.5 mg/L also caused a reduction in maximum velocity (**Figure 5B**; Table 3). All other drugs had no effect on larval burst activity after the vibrational stimulus.

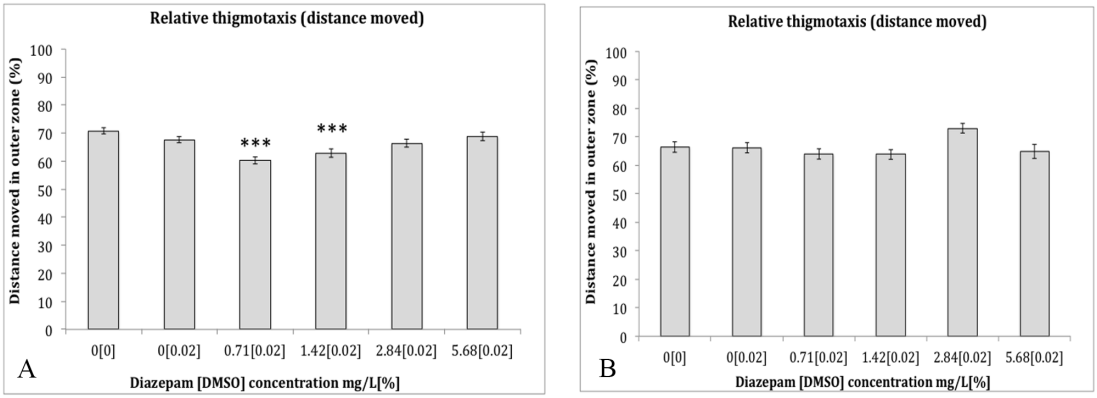

Figure 4. Impact of diazepam on relative thigmotaxis level after acute and chronic exposure. Relative thigmotaxis level was measured as *% TDM* in the outer zone as compared to the whole arena. Bar chart represents mean ± standard errors of mean (SEM) values. Statistical icon: *** p-value ≤ 0.001. Abbreviation: DMSO = dimethylsulfoxide and *% TDM* = percentage of total distance moved.

Table 3. AMI and BUS concentrations that reduced larval burst activity significantly after vibrational stimulus. Drugs were exposed chronically.

Drugs	Comparison (Control⇔ Drug Dose)	Burst activity (maximum velocity in mm/s) Mean ± SEM, *n*	*p*-values	Test statistics
AMI	0⇔10	15.07 ± 2.45, *n = 18*	≤ 0.05	$F_{(2,63)} = 7.313$
BUS	0⇔6.25	17.97 ± 1.91, *n = 23*	≤ 0.05	$F_{(4,87)} = 2.961$
	0⇔10	18.18 ± 1.77, *n = 24*	≤ 0.05	

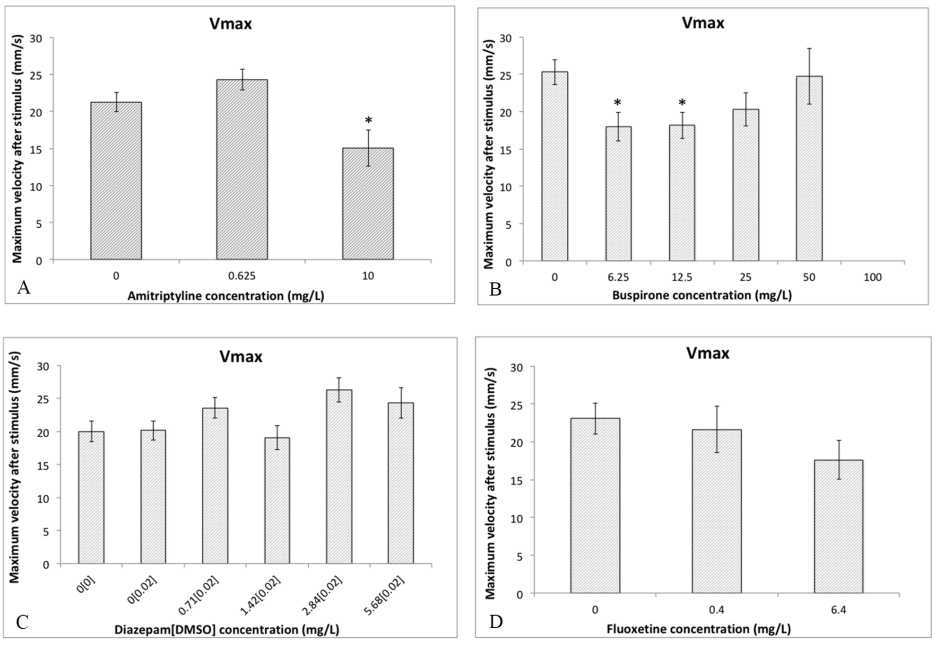

Figure 5. **Impact of tapping (vibrational) stimulus on larval zebrafish burst activity after chronic treatment with drugs.** Bar charts represent mean ± standard errors of mean (SEM) values. Statistical icons: *p-value ≤ 0.05. Abbreviation: DMSO = dimethylsulfoxide. Vmax = Maximum velocity. Chronic exposure to 10 mg/L resulted in survival rate of 78.26%. Larval survival rate after chronic treatment with buspirone at 6.25, 25 50 and 100 mg/L are 95.84%, 79.17%, 15% and 0% respectively.

Discussion

Serotonin toxicity-like phenotype

The goal of the current study was to identify the presence of phenotypes resembling serotonin toxicity in zebrafish larvae. A previous study showed that acute amitriptyline at 1 and 5 mg/L resulted in putative serotonin toxicity-like phenotypes in adult zebrafish, including hypolocomotion and ataxia (vertical swimming and falling on a side) at the bottom of the tank [41]. As of now, it is still not known if serotonin toxicity can be observed in larval zebrafish. Hence, this is the first behavioural study showing serotonin toxicity-like phenotypes in early developing larvae after chronic exposure to serotonergic psychotropic drugs.

Results from the dark challenge test indicate that acute treatment with all drugs did not alter absolute thigmotaxis level represented by increased distance travelled away from the periphery, although hypolocomotion was observed. Chronic exposure to the SNRI, amitriptyline, and SSRI, fluoxetine also caused severe hypolocomotion. In addition, these two drugs also impaired increased absolute thigmotaxis levels in the dark challenge compared to the untreated larvae.

Serotonin toxicity can result in movement and motor disturbances [17]. Larval burst activity measured after chronic treatment with amitriptyline and buspirone further corroborates the presence of impaired motor responses after. Zebrafish larvae respond to the vibrational stimulus by short latency C-bend responses (SLC) that occur within 15 milliseconds of the stimulus [60, 61]. In contrast to the dark challenge stimulus, vibrational stimulus induces a reflex behaviour, modulated by Mauthner cells, without the involvement of CNS [62]. Therefore, our results showing impaired larval burst activity after chronic treatment with amitriptyline and buspirone could indicate phenotypes of serotonin toxicity. Nonetheless, chronic fluoxetine treatment did not show impaired motor responses in behavioural test with vibrational stimulus.

We speculate that young larvae used in this present study have a plastic serotonergic system that may results in high individual variations in response to the treatment with serotonergic psychotropic drugs. Our suggestion could offer a possible explanation on larval responses to chronic fluoxetine (vibrational stimulus experiments) and buspirone (dark challenge stimulus experiments) treatments.

Previous studies show expression of 5-HT_{1A} and 5-HT_2 receptors in larval zebrafish [37, 63, 64], which are implicated in serotonin toxicity [18, 65]. Several studies with larval zebrafish reported increased levels of extracellular serotonin levels with increased heart rate, suggesting resemblance tachycardia observed in clinical serotonin toxicity [18, 66]. A very recent study recapitulated rhabdomyolysis in 4 dpf zebrafish larvae after chronic treatment (48 h) with a psychoactive designer drug that acts through serotonin-2A (5-HT_{2A}) receptor [67]. Rhabdomyolysis is a condition that induces serious muscle injury [68] and often observed in serotonin toxicity.

Pharmacological and physiological drug effects

A previous study shows that diazepam significantly attenuated thigmotaxis and therefore reduces anxiety-like behaviour in AB wild type zebrafish larvae after acute exposure with a concentration of 0.71 mg/L [46]. Our current results with the ABTL wild type zebrafish larvae are also in agreement with the previous study. In addition to this, all three serotonergic drugs used in the current study did not reduce anxiety-like behaviour in larvae since they failed to attenuate thigmotaxis.

Richendrfer *et al.* reported that acute fluoxetine (2 hours exposure) had no effect on 7 dpf larval (AB wild type) zebrafish thigmotaxis assay (retained baseline anxiety), but reduced avoidance behaviour (fear response) [38]. In our study, we did not see any pharmacological effects of fluoxetine after both acute and chronic exposure. Previous studies have shown that the choice of animal strain could influence the pharmacological effects antidepressants and this has been shown in both rodents and larval zebrafish. For example, chronic fluoxetine treatment of different mouse strains revealed that only a highly anxious strain (BALB/c) was sensitive to an SSRI [69]. Similar effects were seen in zebrafish larvae, where fluoxetine treatment attenuated startle response in gr^{s357} mutants while it had no effect in the wild type strain [70]. These studies show that fluoxetine could induce its pharmacological effects on organisms with higher baseline anxiety level. Therefore, we suggest to evaluating the drugs used in this study with highly anxious strains such as the Wild Indian Karyotype (WIK), Nadia, and Leopard strains [71].

Another concern that we want to highlight is the optimal time frame for testing larval zebrafish is relatively constricted [72]. Very young larvae (< 3 dpf) show limited behavioural repertoire [72], while larvae from 3–4 dpf are relatively on a

critical time frame of development. For example, by 5 dpf, larval brain development occurs at a slower pace and is still immature [72]. We believe that testing serotonergic drugs on very young larvae could yield individual and batch-wise variations due to possible differential expression patterns of serotonin receptors. Moreover, movement disorders seen in larvae treated chronically with serotonergic drugs could be due to development defects in addition to resembling serotonin toxicity. A previous study [63] that compared the analysis of serotonin receptors and transporters gene expression in the larval and adult zebrafish further corroborates our suggestion that larval zebrafish is developmentally naïve. According to that study, 3-dpf larval brains represent a critical stage of neural development, with similar expression domains of neurogenic genes as embryonic day 12.5/13.5 mouse embryos.

Conclusion

We report for the first time serotonin toxicity-like phenotype in zebrafish larvae treated chronically with serotonergic anxiolytic and antidepressants. This is based on the drugs capacity to impair larval swimming activity after dark challenge and vibrational stimulus. Moreover, we also want highlight that larval zebrafish used in this study is at a critical time point of neural development and this also could also be the reason for impaired locomotion after chronic treatment with serotonergic drugs. Collectively, the findings from the current study highlight the importance of serotonin neurotransmitter modulation in physiology and behaviour of early developing zebrafish larvae.

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Supplementary materials

Table S1 AMI, BUS, and FLU concentrations that caused significant effects on relative thigmotaxis level of zebrafish larvae after acute and chronic exposure. Abbreviations: *H* = Kruskal-Wallis chi squared values, df = degrees of freedom and % TDM = percentage of total distance moved.

Drugs (Exposure)	**Comparison (Control⇔ Drug Dose)**	**Relative thigmotaxis level (*% TDM* in the outer zone)**	***p*-values**	**Test statistics**
		Mean ± SEM, *n*		
AMI (acute)	0⇔2.5	59.26 ± 2.06, *n = 48*	≤ 0.001	*H* = 37.032, df = 5
	0⇔10.0	79.36 ± 3.38, *n = 48*	≤ 0.05	
AMI (chronic)	0⇔1.25	80.17 ± 2.27, *n = 47*	≤ 0.01	
	0⇔2.5	83.27 ± 2.15, *n = 46*	≤ 0.001	*H* = 45.694, df = 5
	0⇔5.0	85.93 ± 4.18, *n = 38*	≤ 0.001	
BUS (acute)	0⇔12.5	78.12 ± 1.40, *n = 48*	≤ 0.01	
	0⇔25	84.02 ± 1.10, *n = 48*	≤ 0.001	*H* = 44.084, df = 5
	0⇔50	83.30 ± 1.77, *n = 48*	≤ 0.001	
BUS (chronic)	0⇔6.25	73.73 ± 1.49, *n = 48*	≤ 0.05	
	0⇔12.5	74.10 ± 2.58, *n = 48*	≤ 0.05	*H* = 17.271, df = 4
	0⇔25	74.32 ± 1.87, *n = 41*	≤ 0.01	
FLU (acute)	0⇔1.6	75.70 ± 1.45, *n = 48*	≤ 0.01	*H* = 17.749, df = 5
FLU (chronic)	0⇔1.6	82.99 ± 1.42, *n = 48*	≤ 0.001	
	0⇔3.2	85.38 ± 1.32, *n = 48*	≤ 0.001	*H* = 122.60, df = 5
	0⇔6.4	91.57 ± 1.36, *n = 48*	≤ 0.001	

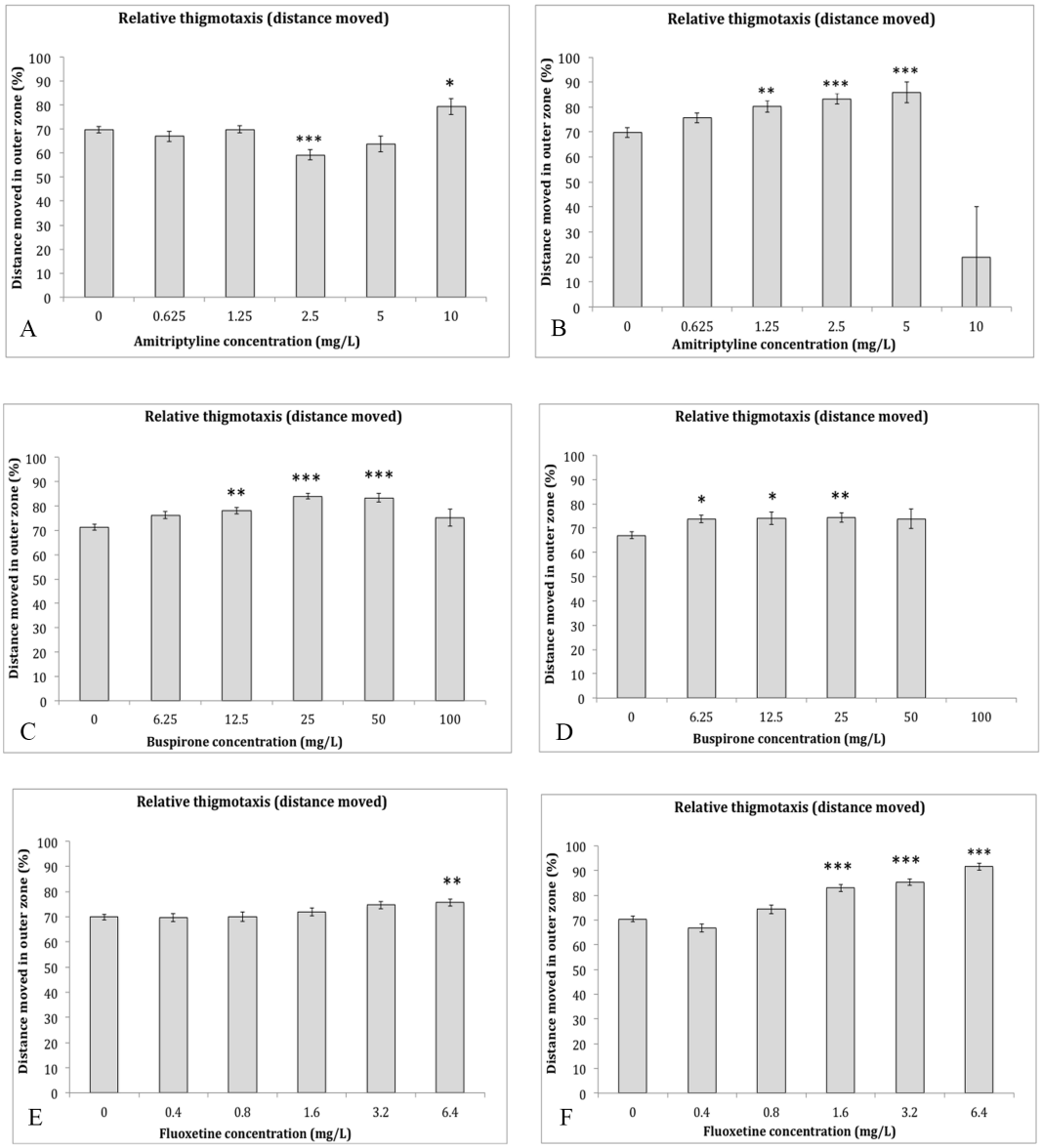

Figure S1 Impact of amitriptyline, buspirone and fluoxetine on relative thigmotaxis level after acute and chronic exposure. Relative thigmotaxis level was measured as *% TDM* in the outer zone as compared to the whole arena. Bar chart represents mean ± standard errors of mean (SEM) values. Statistical icons: *p-value ≤ 0.05, ** p-value ≤ 0.01 and *** p-value ≤ 0.001. Abbreviation: DMSO = dimethylsulfoxide and *% TDM* = percentage of total distance moved. Larval survival rate after chronic exposure to the drugs are same as reported in Fig. 3.