The role of zebrafish larvae for studying anxiety-like behaviour

Muniandy, Y.

Chapter 3

Chronic treatment with serotonergic psychotropic drugs causes locomotor suppression and toxicity in 5-day zebrafish larvae

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Abstract

There is a demand for development of new psychotropic drugs to treat anxiety disorders. Although zebrafish larvae have a distinct behavioral repertoire by 5 days post fertilization (5 dpf), it is not fully known whether such young larvae respond to psychotropic drugs in the same way that adult humans and mammals do. Here we have examined the behavioral response of 5 dpf larvae after treatment with amitriptyline, buspirone, diazepam, and fluoxetine. We chose these drugs because diazepam is a gold-standard anxiolytic, while the remaining three drugs are commonly used for treating various anxiety disorders. Visual motor response (VMR) was chosen as a behavioral assay because larvae show startle response after sudden exposure to darkness. We measured larval locomotion (total distance moved) and burst activity (maximum velocity) after acute (1 min) and chronic (24 h) treatments with the four drugs. All drugs suppressed larval locomotion and burst activity in the challenge phase. However, amitriptyline and buspirone also suppressed larval locomotion in the basal phase both after acute and chronic exposure. Hence, reduction in locomotion in the challenge phase may not in itself represent anxiolytic effects; it may also indicate toxicity. This is supported by our observation that chronic exposure to two drugs (amitriptyline and buspirone) caused high mortality at the highest concentrations. Moreover, unlike diazepam that produced a monotonic suppression after acute and chronic exposures, the three serotonergic drugs (amitriptyline, buspirone, and fluoxetine) produced nuanced dose responses in larval locomotion. We suggest that 5 dpf larval serotonergic systems are still too immature to be fully responsive to the complex pharmacodynamics of the serotonergic drugs. Future studies could include older larvae and various biochemical analyses (neuroanatomical imaging, gene expression, and toxicity) and behavioral analyses (thigmotaxis, scototaxis, and swimming plus maze test) to yield a comprehensive understanding of how psychotropic drugs work in zebrafish larvae.

Introduction

Rodent models have been used widely to study the pathogenesis of affective disorders such as anxiety through various behavioral, genetic, and pharmacological assays [1-5]. Another vertebrate model organism that gained much popularity for studying anxiety and other mood disorders is the zebrafish [6-12]. Traditionally, rodent behavioral assays such as the open field-test, light dark box test, social behavior test, and novelty-based tests were used to assess anxiolytic effects of drugs [13-16]. These were later adapted to the zebrafish model [17, 18]. Some examples of the resulting assays in zebrafish include the novel tank test [19-21], light-dark preference test [19, 22, 23], open field tank test [24-26], shoaling test [27-29], and novel object approaching test [30]. Zebrafish are good candidates for studying anxiety because they have physiological and functional similarities with mammals (including humans and rodents) in brain neurotransmitters and their receptors [31-35]. Moreover, there is much evidence pointing to the fact that environmental factors (exposure to novel environments and aversive stimuli) that cause anxiety are similar in zebrafish and rodents [11, 36].

Zebrafish embryos and larvae have advantages over adults for large-scale drug screening. They are small enough to be easily plated out into multiwell plates and this feature can be adapted in high-throughput screens (HTS). HTS has been used in the past to screen different types of drugs [37-41]. In one example, Kokel *et al*. used zebrafish embryos to screen thousands of small molecules to identify neuroactive compounds [41]. In another study, a behavioral profile for zebrafish embryos was established using 60 water-soluble compounds [42].

In addition to their suitability for use in HTS, larvae offer other features that can be helpful to study behavioral, genetic, and pharmacological factors related to anxiety. These features include low husbandry cost, high fecundity, and optical transparency [43-47]. The optical transparency of zebrafish larvae facilitates imaging techniques to study the internal development of organs and tissue systems [48, 49]. Moreover, larval zebrafish are robust for preclinical studies to understand the biodistribution, toxicity, and efficacy of the test compounds [50-52].

There are many behavioral phenotypes that can be used to assess anxiety in zebrafish larvae and screen drugs with anti-anxiety effects. One of the most important behavioral phenotypes identified in larval zebrafish is the startle response [53, 54].

The visual motor response (VMR) is a type of startle response seen in zebrafish larvae at around 3 dpf (days post fertilization), which becomes robust at 5 dpf [55]. The VMR in zebrafish larvae is initiated by sudden exposure darkness [42, 55-57]. A recent study examined 3-dimensional swimming patterns including a downward (diving) response in zebrafish larvae (between 6-12 dpf). Two different experimental setups were used [58]. A cubical tank in the first experiment was used to characterize 3-D swimming patterns after visual and auditory stimuli. In another experiment, tubular tanks were used to record vertical swimming with a visual stimulus only.

In a typical VMR assay, zebrafish larvae are arrayed in a multiwell plate (normally a 96 well plate) to screen either a single or multiple drugs at different concentrations at a time point. VMR is usually measured as distance swum by a larva following the stimulus (lights off). This response variable can be measured using commercially available apparatus such as the ZebraBox (ViewPoint, Lyon, France), DanioVision™ (Noldus, Wageningen, The Netherlands), and Zantiks MWP (Zantiks UK) or with in-house systems [59, 60]. In our opinion, larval zebrafish VMR assays can serve as the first line of a battery of behavioral tests to screen new candidate drugs for anxiety. Moreover, the compatibility of VMR assays with high-throughput drug screening makes zebrafish larvae an excellent choice in preclinical anxiety model research.

In the current study, we have analysed the effects of selected drugs in the VMR assay. The drugs examined were amitriptyline (Elavil), buspirone (Buspar), diazepam (Valium), and fluoxetine (Prozac®). These drugs are used to treat anxiety and anxiety disorders in humans. Moreover, all four drugs have been shown to cause anxiolytic-like effects in adult zebrafish models using different behavioral assays [20, 61-64]. Animal experiments in the drug discovery pipelines are used to determine the efficacy, pharmacokinetics, pharmacodynamics, and toxicity of candidate drugs [51, 52]. In this context, zebrafish larvae are suitable for pre-clinical studies since they can be adapted to high throughput screening assays.

According to Dutch animal laws, larvae are considered to be experimental animals when they become free feeding. This is approximately 5dpf when the yolk sac is consumed, and 5dpf is, therefore, the limit that we have respected in this study. Larvae of this age already show a wide range of behavioral repertoires such as evoked

swimming[65], photomotor response[41], optokinetic response[66], and also VMR [67].

The goal of the current study was to develop a 96-well plate based assay, potentially adaptable for HTS screening of candidate anxiolytic drugs, and based on the 5dpf zebrafish larvae in the VMR assay. We chose four psychotropic drugs commonly prescribed for anxiety and anxiety disorders to validate our assay. Two behavioral parameters were recorded: they are locomotion (measured as total distance moved in mm) and burst activity (measured as maximum velocity in mm/s).

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; https://eur-lex.europa.eu/legal-content/NL/TXT/HTML/?uri=CELEX:32010L0063&qid=1531309204564&from=N) and institutional regulations.

Zebrafish husbandry

Male and female adult zebrafish (*Danio rerio*) of ABTL wild type strains were maintained in our facility according to standard protocols (zfin.org). Zebrafish eggs were obtained by random pairwise mating of zebrafish. Approximately 10 adult zebrafish (equal male to female ratio) were placed together in small breeding tanks the evening before eggs were required. The breeding tanks have mesh traps to prevent the eggs from being eaten by the adult fish. The eggs were harvested the following morning and transferred into 92 mm plastic Petri dishes (approximately 80 eggs per dish) containing 40 mL fresh embryo medium (EM). Unfertilized, unhealthy and dead embryos were identified under a stereomicroscope and discarded using a plastic Pasteur pipette immediately after plating into Petri dishes. The procedure for the preparation of EM is based on a previously published protocol [42, 68-70].

At 1 dpf, the embryos were again screened and any dead or unhealthy embryos were removed before the healthy embryos were transferred into 96 well plates (one

embryo per well). The transfer was done on 1 dpf to minimize potential damage when transferring at later stages. Chances of damaging the larvae after post-hatching are greater relative to the pre-hatching during transfer [71]. Throughout all procedures, the embryos and the solutions were kept in an acclimatized room at 28 ± 0.5 °C, under a light-dark cycle of 14 hours light and 10 hours dark (lights switch on at 08:00).

Exposure to psychotropic drugs

Zebrafish larvae were exposed to amitriptyline (Sigma-Aldrich, catalogue number PHR1384), buspirone (Sigma-Aldrich, catalogue number B7148), diazepam (Duchefa Farma, catalogue number 5372) and fluoxetine (Sigma-Aldrich, catalogue number F132). These drugs are referred to hereafter as AMI, BUS, DZM, and FLU respectively. **Table 1** shows the concentrations ranges used for each pharmaceutical and the spatial distribution across the 96 well plates. These concentration ranges were chosen based on previously published works [65, 72-74]. The desired final concentrations were prepared from a stock solution. Prior to the VMR behavioral assay, the larvae were pre-exposed with the pharmaceuticals either for 1 minute (acute exposure) or for 24 hours (chronic exposure). Larvae remained in the test solutions throughout the behavioral analysis. All behavior analyses were conducted with 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 96 well plates. N = 48 for both controls and untreated larvae.

		Location in 96 well plates (C=Column)					
		C1 & C7	**C2 & C8**	**C3 & C9**	**C4 & C10**	**C5 & C11**	**C6 & C12**
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

(AMI = amitriptyline, BUS = buspirone, DZM = diazepam, DMSO = dimethylsulfoxide, and FLU = fluoxetine. All drugs were dissolved in embryo medium except for DZM, which was dissolved using DMSO. The final concentration of DMSO in each DZM treatment is 0.02%.)

Experimental procedure

All behavioral experiments were done in a ZebraBox (ViewPoint, Lyon, France) recording apparatus, equipped with video camera (Point Grey FlyCap 2, Richmond, Canada) and recording software (ViewPoint, Lyon, France). Video analysis was later

done using Ethovision® XT 10 (Noldus Information Technology, Wageningen, Netherlands). Larvae were allowed to acclimatize in the test apparatus for 10 minutes in chronic exposure before recording. Plates were immediately transferred into the testing apparatus in acute exposure experiments.

Visual motor response (VMR) assay

The experimental design for the VMR assay (shown in **Figure 1**) is adapted from a previous study published in our laboratory [42]. The VMR assay consists of 10 min basal phase (light switched ON, L1), 4 minutes of challenge phase (light switched OFF, D) and 10 minutes of recovery phase (light switched ON, L2). Forty-eight larvae were used for each treatment in this study. We were interested in analyzing larval locomotion from the basal and challenge phases. Locomotion was measured as total distance moved (mm). Data from the basal phase represent the activity of larvae at rest, while the challenge phase represents the response to stimulus and it is, therefore, interesting to see whether drugs can modulate the challenge phase. The recovery phase is present to allow zebrafish larvae to recover from the shock of light stimulus; this is mainly useful in studying habituation. Therefore we do not include it in our data because it is not relevant. In addition to general locomotion, we were also interested in assessing larval burst activity (best captured by maximum velocity) in the challenge phase. This behavioral repertoire appears in larvae at 2 dpf and often associated with escape response [75].

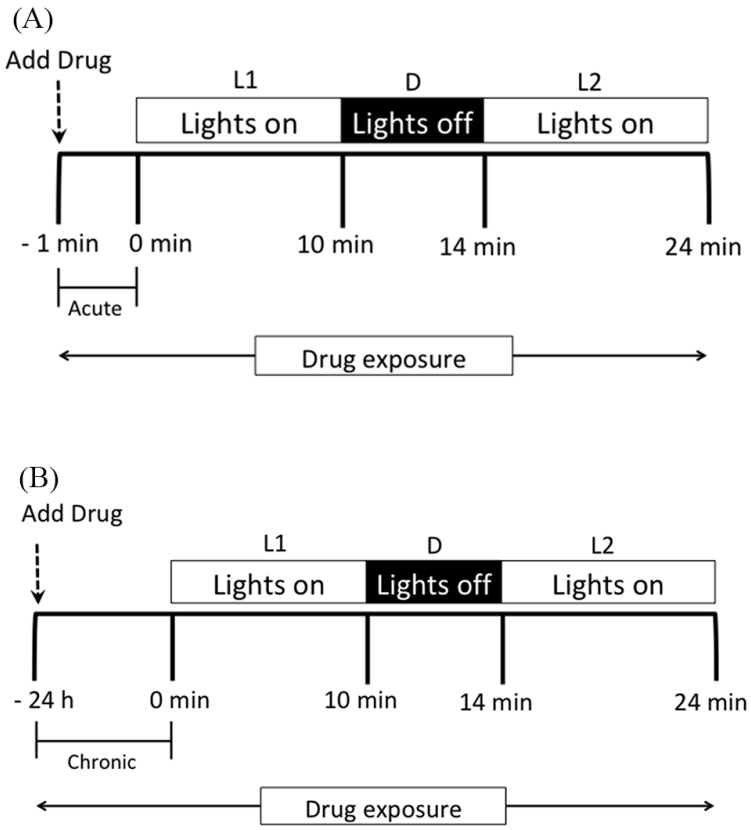

Figure 1. **Experimental design for visual-motor response assay.** Drugs were continuously present during the assay period as indicated by the box/arrow 'Drug Exposure' (Pre-exposure: 1 min is acute (A) while 24 h is chronic (B). Time points in the schematic diagram are not shown to scale. Larvae were allowed to acclimatize in the test apparatus for 10 min in chronic exposure before recording. Plates were immediately transferred into the testing apparatus in acute pre-exposure experiments. Key: L1, basal phase; D, challenge phase; L2, recovery phase

Statistical analyses

Behavioral data from locomotion was analyzed using a mixed model with repeated measures. Data from larval burst activity were analysed using a linear model. Residuals from the regression models were checked for normality using a Q-Q plot. When the normality test failed, Kruskal-Wallis tests with a Pairwise Mann-Whitney U-test as post hoc analysis were chosen to compare controls with treatments. Effect sizes and degrees of freedom were always reported. Each bar in the bar chart represents mean ± SEM (standard error of the mean). All statistical analyses were done using RStudio© (version 1.1.456). N was 48 for both controls and drug-treated larvae and results from these statistical analyses were considered significant when $p < 0.05$.

Results

Locomotion after acute treatment with AMI, BUS, DZM, and FLU

DZM (**Figure 2A** and **Table 2**) treatment also reduced zebrafish larvae locomotion in the basal phase at all concentrations. Controls alone for the solvent (0.02% DMSO) showed no effect on larval locomotion in the basal phase. Larval locomotion in the challenge phase decreased at all concentrations of DZM, including the larvae treated with 0.02% DMSO only. Larvae treated with acute DZM also had 100% survival rate.

AMI (**Figure 2B** and **Table 2**) reduced locomotion in the basal phase at all concentrations. Locomotion was also reduced after dark challenge at all concentrations compared to the untreated larvae. The survival rate after acute exposure to AMI was 100% for all concentrations.

BUS (**Figure 2C** and **Table 2**) caused a reduction in locomotion in the basal phase from 12.5 μg/ml onwards. Larvae exposed to acute BUS treatment showed reduced distance moved only at 6.25, 50, and 100 μg/ml after the dark stimulus. The survival rate for this pharmaceutical compound was 100% at all concentrations.

FLU (**Figure 2D** and **Table 2**) produced a different response, compared to the other compounds described above, after acute exposure in the basal phase. It increased larval locomotion only at concentrations of 0.8 and 1.6 μg/ml. By contrast, this drug decreased larval movement in the challenge phase at all concentrations. Similar to the other drugs, acute FLU treatment was also not toxic to the larvae at all concentrations.

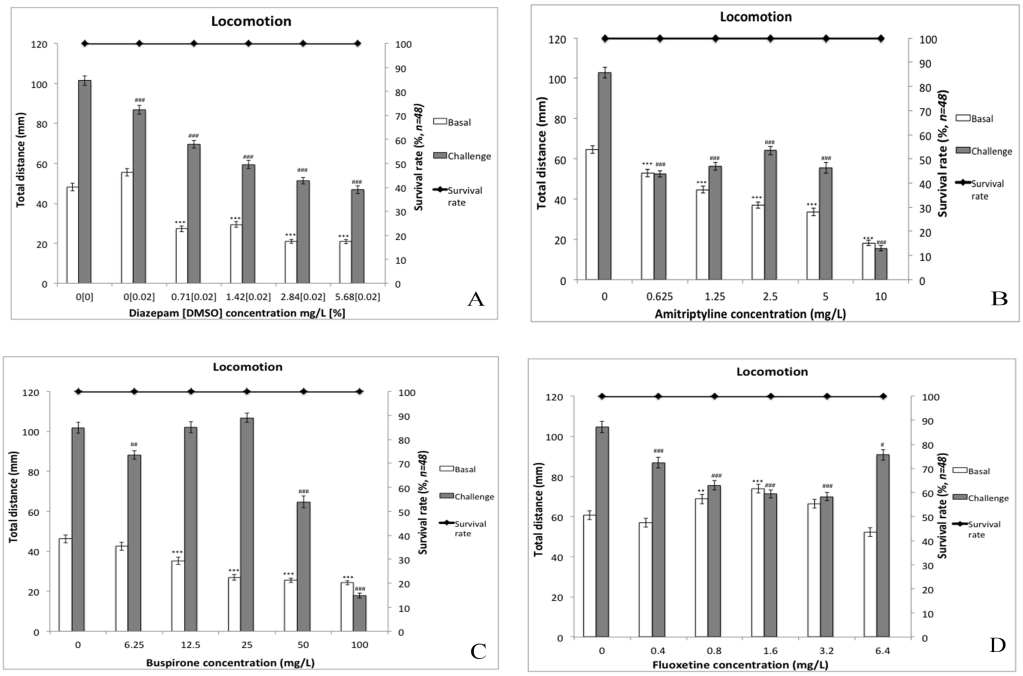

Figure 2. Locomotion (mean total distance moved, mm) of larval zebrafish after acute exposure psychotropic drugs. A, DZM; B, AMI; C, BUS and D, FLU. Error bars = ± standard errors of mean (SEM) values. Statistical symbols: (*) = statistical significance comparing control and treatment, basal phase; (#) = statistical significance comparing control and treatment, challenge phase. ** p-value < 0.01, *** p-value < 0.001, # p-value < 0.05, ## p -value <0.01 and ### p-value <0.001. Secondary (line) plot at top of chart = survival rate. *Key*: [ ], final concentration DMSO. Abbreviations: AMI, amitriptyline; BUS, buspirone; DZM, diazepam; DMSO, dimethylsulfoxide; FLU, fluoxetine.

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

Drugs (Phase)	Comparison (Control⇔Drug concentration)	Locomotion (distance moved in mm)	*p*-values	Test statistics
		Mean ± SEM, *n*		
AMI (basal)	0⇔0.625	52.89 ± 1.77, *n* = 48	<0.001	
	0⇔1.25	44.71 ± 1.70, *n* = 48	<0.001	
	0⇔2.5	37.03 ± 1.68, *n* = 48	<0.001	*H* = 469.81, df = 5
	0⇔5.0	33.57 ± 1.81, *n* = 48	<0.001	
	0⇔10.0	18.14 ± 1.32, *n* = 48	<0.001	
AMI (challenge)	0⇔0.625	52.51 ± 1.55, *n* = 48	<0.001	
	0⇔1.25	56.27 ± 1.96, *n* = 48	<0.001	
	0⇔2.5	64.08 ± 2.02, *n* = 48	<0.001	*H* = 515.44, df = 5
	0⇔5.0	55.53 ± 2.77, *n* = 48	<0.001	
	0⇔10.0	15.60 ± 1.37, *n* = 48	<0.001	
BUS (basal)	0⇔12.5	35.26 ± 1.79, *n* = 48	<0.001	
	0⇔25	26.97 ± 1.35, *n* = 48	<0.001	*H* = 56.019, df = 5
	0⇔50	25.55 ± 1.14, *n* = 48	<0.001	
	0⇔100	24.31 ± 1.04, *n* = 48	<0.001	
BUS (challenge)	0⇔6.25	88.29 ± 2.15, *n* = 48	<0.01	
	0⇔50	64.76 ± 3.00, *n* = 48	<0.001	*H* = 540.98, df = 5
	0⇔100	17.86 ± 1.25, *n* = 48	<0.001	
DZM (basal)	0[0]⇔0.71[0.02]	27.28 ± 1.48, *n* = 48	<0.001	
	0[0]⇔1.42[0.02]	29.38 ± 1.47, *n* = 48	<0.001	*H* = 288.17, df = 5
	0[0]⇔2.84[0.02]	20.92 ± 1.02, *n* = 48	<0.001	

	0[0]⇔5.68[0.02]	20.94 ± 1.13, *n* = 48	<0.001	
DZM (challenge)	0[0]⇔0[0.02]	86.85 ± 2.35, *n* = 48	<0.001	
	0[0]⇔0.71[0.02]	69.64 ± 1.96, *n* = 48	<0.001	
	0[0]⇔1.42[0.02]	59.38 ± 2.07, *n* = 48	<0.001	*H* = 376.91, df = 5
	0[0]⇔2.84[0.02]	51.38 ± 1.71, *n* = 48	<0.001	
	0[0]⇔5.68[0.02]	46.75 ± 1.90, *n* = 48	<0.001	
FLU (basal)	0⇔0.8	68.81 ± 2.41, *n* = 48	<0.01	*H* = 87.861, df = 5
	0⇔1.6	73.95 ± 2.13, *n* = 48	<0.001	
FLU (challenge)	0⇔0.4	86.81 ± 2.69, *n* = 48	<0.001	
	0⇔0.8	75.61 ± 2.30, *n* = 48	<0.001	
	0⇔1.6	71.27 ± 1.99, *n* = 48	<0.001	*H* = 147.84, df = 5
	0⇔3.2	69.92 ± 2.04, *n* = 48	<0.001	
	0⇔6.4	90.70 ± 2.57, *n* = 48	<0.05	

(AMI = amitriptyline, BUS = buspirone, DZM = diazepam, DMSO = dimethylsulfoxide, and FLU = fluoxetine)

Locomotion after chronic treatment with AMI, BUS, DZM, and FLU

Diazepam exposure (**Figure 3A** and **Table 3**) did not affect larval locomotion in the basal phase at any concentration used. However, in the solvent controls (0.02% DMSO), larvae showed increased locomotion in the basal phase. After the dark challenge, the larval movement was decreased at all concentrations of DZM. Furthermore, 0.02% of DMSO solvent alone also reduced locomotion.

Amitriptyline (**Figure 3B** and **Table 3**) significantly reduced locomotion at all concentrations where there was no mortality, both in the basal and challenge phase. At the two highest concentrations all larvae died. After 24 hours of exposure to buspirone (**Figure 3C** and **Table 3**), larvae showed reduced locomotion in the basal phase from 12.5 µg/ml onwards. However, at 6.25 µg/ml BUS increased locomotion in the challenge phase. After the dark stimulus, only the two highest concentrations of BUS were associated with reduced larval locomotion. At the two highest concentrations of BUS (50 and 100 µg/ml) survival rate was 85.42 and 4.17%, respectively. Larval locomotion in the basal phase was reduced after 24h treatment with fluoxetine treatment (**Figure 3D** and **Table 3**) only at 0.8 – 3.2 µg/ml. Larval locomotion after the dark stimulus was reduced at all concentrations of FLU.

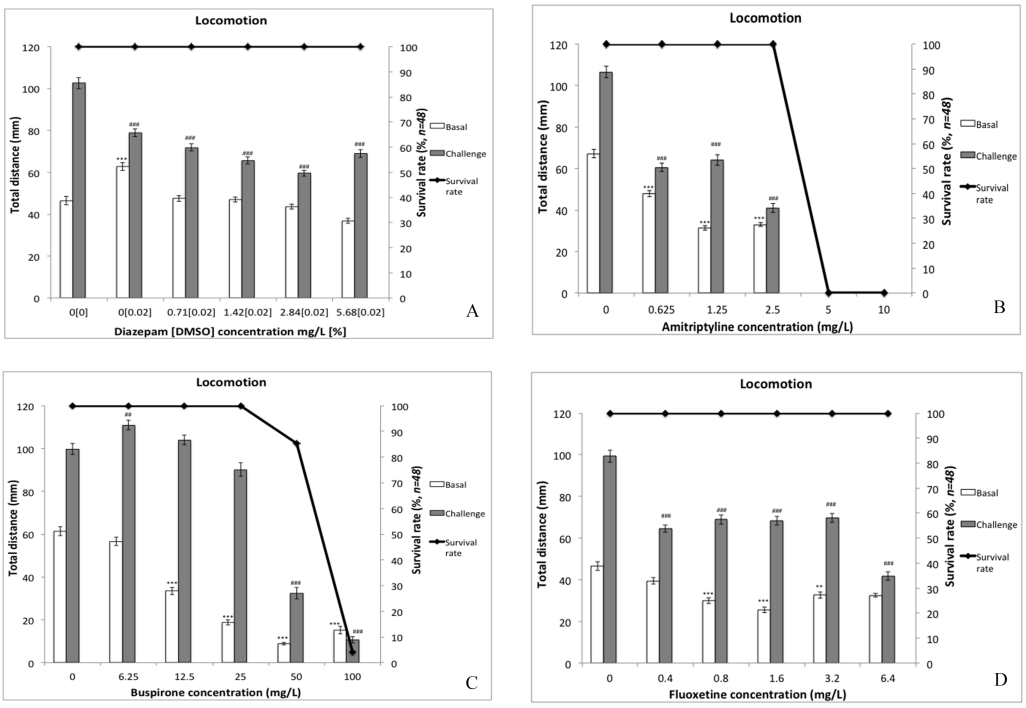

Figure 3. Locomotion (mean total distance moved, mm) of larval zebrafish after chronic exposure to psychotropic drugs. A, AMI; B, BUS; C, DZM and D, FLU. Error bars = ± standard errors of mean (SEM). Statistical symbols: (*) = statistical significance comparing control and treatment, basal phase; (#) = statistical significance comparing control and treatment, challenge phase. ** p-value <0.01, *** p-value <0.001, ## p-value <0.01 and ### p-value <0.001. Secondary (line) plot at top of chart = survival rate. *Key*: [ ], final concentration DMSO. Abbreviations: AMI, amitriptyline; BUS, buspirone; DZM, diazepam; DMSO, dimethylsulfoxide; FLU, fluoxetine.

Table 3. AMI, BUS, DZM, and FLU concentrations that caused significant effects on larval locomotion in the basal (light switch on) and challenge phase (light switch off) after chronic exposure. DMSO (Dimethylsulfoxide) is used to dissolve DZM. Abbreviations: H = Kruskal-Wallis chi-squared values; df = degrees of freedom.

Drugs (Phase)	Comparison (Control⇔Drug concentration)	Locomotion (distance moved in mm)	*p*-values	Test statistics
		Mean ± SEM, *n*		
AMI (basal)	0⇔0.625	47.83 ± 1.50, *n*= 48	<0.001	
	0⇔1.25	31.48 ± 1.13, *n*= 48	<0.001	*H* = 219.06, df = 3
	0⇔2.5	33.03 ± 0.86, *n*= 48	<0.001	
AMI (challenge)	0⇔0.625	60.61 ± 1.96, *n*= 48	<0.001	
	0⇔1.25	64.20 ± 2.59, *n*= 48	<0.001	*H* = 282.18, df = 3
	0⇔2.5	40.95 ± 2.05, *n*= 48	<0.001	
BUS (basal)	0⇔12.5	33.49 ± 1.72, *n*= 48	<0.001	
	0⇔25	18.76 ± 1.17, *n*= 48	<0.001	*H* = 644.36, df = 5
	0⇔50	8.96 ± 0.63, *n*= 47	<0.001	
	0⇔100	15.26 ± 1.82, *n*= 2	<0.001	
BUS (challenge)	0⇔6.25	110.99 ± 2.39, *n*= 48	<0.01	
	0⇔50	32.48 ± 2.68, *n*= 47	<0.001	*H* = 308.34, df = 5
	0⇔100	10.66 ± 1.82, *n*= 2	<0.001	
DZM (basal)	0[0]⇔0[0.02]	62.71 ± 1.80, *n*= 48	<0.001	*H* = 130.77, df = 5
DZM (challenge)	0[0]⇔0[0.02]	78.87 ± 1.87, *n*= 48	<0.001	
	0[0]⇔0.71[0.02]	71.78 ± 1.65, *n*= 48	<0.001	
	0[0]⇔1.42[0.02]	65.67 ± 1.69, *n*= 48	<0.001	*H* = 205.51, df = 5
	0[0]⇔2.84[0.02]	59.52 ± 1.50, *n*= 48	<0.001	
	0[0]⇔5.68[0.02]	68.95 ± 1.98, *n*= 48	<0.001	
FLU (basal)	0⇔0.8	29.87 ± 1.41, *n*= 48	<0.001	*H* = 105.26, df = 5

	0⇔1.6	25.49 ± 1.25, *n*= 48	<0.001	
	0⇔3.2	32.61 ± 1.42, *n*= 48	<0.01	
FLU (challenge)	0⇔0.4	64.54 ± 1.84, *n*= 48	<0.001	
	0⇔0.8	68.89 ± 2.17, *n*= 48	<0.001	
	0⇔1.6	68.32 ± 2.19, *n*= 48	<0.001	H = 262.84, df = 5
	0⇔3.2	69.62 ± 2.05, *n*= 48	<0.001	
	0⇔6.4	41.85 ± 2.05, *n*= 48	<0.001	

(AMI = amitriptyline, BUS = buspirone, DZM = diazepam, DMSO = dimethylsulfoxide, and FLU = fluoxetine)

Burst activity

Acute and chronic treatments with AMI (**Figure 4A** and **B; Table 4**) resulted in reduced burst activity at all concentrations tested. With chronic exposure, the two highest concentrations tested (50 and 100 mg/L) data are not shown because both concentrations showed 100% mortality. Acute BUS resulted in a decreased burst activity only at 100 mg/L only, while chronic exposure resulted in reduced burst activity at concentrations that were toxic: 50 and 100 mg/L (**Figure 4C** and **D; Table 4**). DMSO, which was used, as a carrier solvent for DZM, had no impact on burst activity in either acute or chronic treatment (**Figure 4E and F; Table 4**). With acute exposures, all concentrations of DZM tested caused a reduction in burst activity. By contrast, chronic exposure only resulted in a reduction in burst activity at 0.71, 1.42, and 2.84 mg/L. Acute exposure to FLU significantly lowered burst activity at 0.4, 0.8, 1.6, and 6.4 mg/L. However, chronic exposure to FLU reduced burst activity in larvae only at 0.8, 1.6, and 3.2 mg/L (**Figure 4G and H; Table 4**).

Discussion

In this study, we used the VMR assay adapted for zebrafish larvae to assess the effects on the locomotion of four important, widely prescribed psychotropic drugs. Based on recordings of larval locomotion and burst activity in the VMR assay performed in 96 well plates, we find that the assay holds promise for the evaluation of psychotropic drugs. Of the four drugs tested, diazepam gave classic linear (monotonic) dose-response on total distance moved both in acute (1 min) and chronic (24 h) exposure. The other three drugs gave a more heterogeneous response that was sometimes more difficult to interpret.

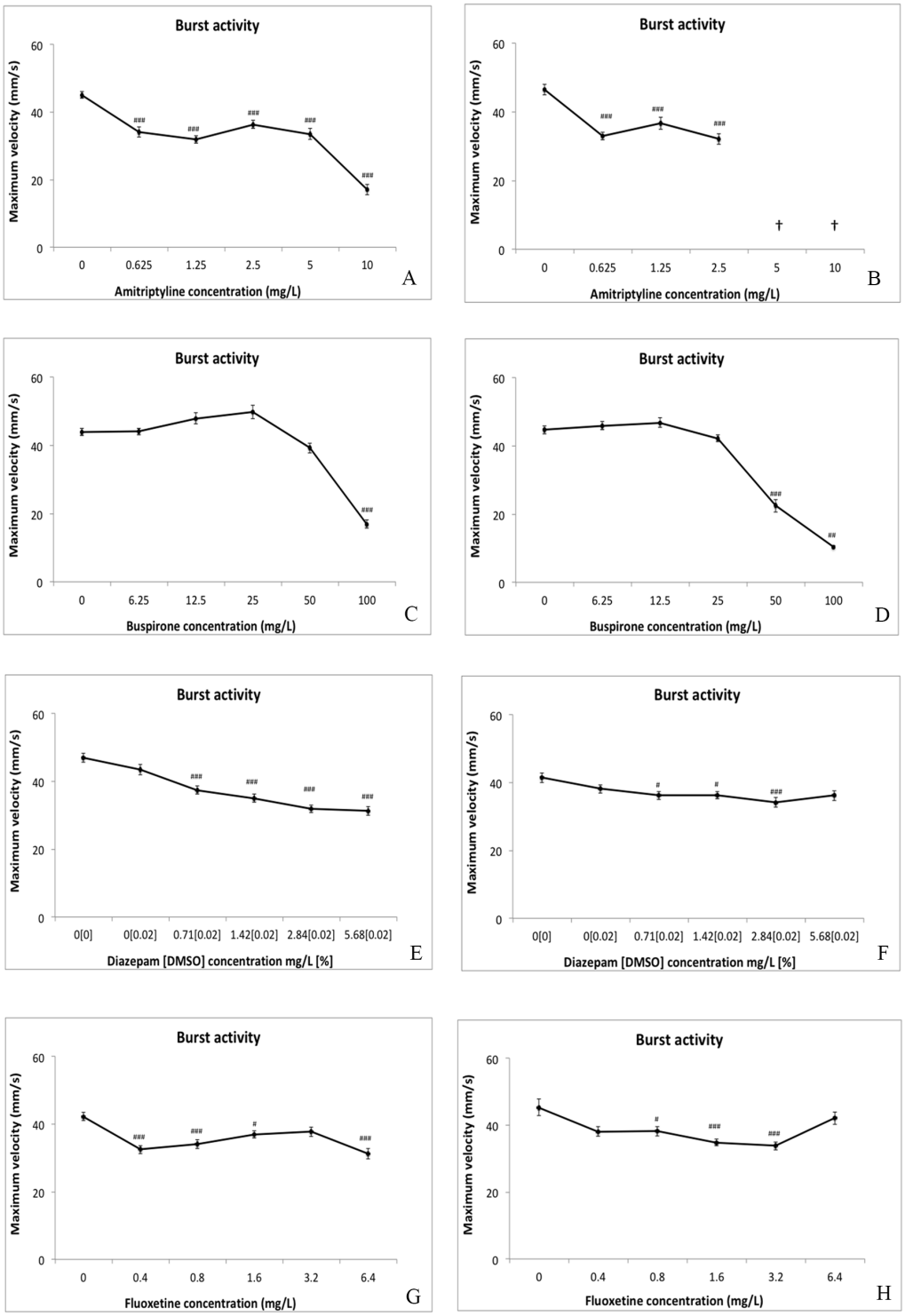

Figure 4. Impact of AMI, BUS, DZM, and FLU on larval burst activity (mean maximum velocity, mm/s) after exposure to psychotropic drugs. A, C, E, and G = acute exposure. B, D, F, and H = chronic exposure. Note that the survival rate of larvae is similar to that reported in FIG 2 and 3. Error bars = ± standard error of mean (SEM). Statistical symbols: #, *p*-value <0.05; ##, *p*-value <0.01; ###, *p*-value <0.001. *Key*: [ ], final concentration DMSO; †: larvae with 100% mortality. Abbreviations: AMI, amitriptyline; BUS, buspirone; DZM, diazepam; DMSO, dimethylsulfoxide; FLU, fluoxetine.

Table 4. AMI, BUS, DZM, and FLU concentrations that caused significant effects on larval burst activity in the basal (light switch on) and challenge phase (light switch off) after chronic exposure. DMSO (Dimethylsulfoxide) is used to dissolve DZM. Abbreviations: *H* = Kruskal-Wallis-chi squared values; df = degrees of freedom; AMI = amitriptyline, BUS = buspirone, DZM = diazepam, DMSO = dimethylsulfoxide, and FLU = fluoxetine.

Drugs (Exposure)	Comparison (Control⇔Drug concentration)	Burst activity (maximum velocity in mm/s)	*p*-values	Test statistics
		Mean ± SEM, *n*		
AMI (acute)	0⇔0.625	34.06 ± 1.45, *n*= 48	<0.001	
	0⇔1.25	31.82 ± 1.10, *n*= 48	<0.001	
	0⇔2.5	36.33 ± 1.18, *n*= 48	<0.001	*H* = 124.59, df = 5
	0⇔5	33.48 ± 1.69, *n*= 48	<0.001	
	0⇔10	17.01 ± 1.51, *n*= 48	<0.001	
AMI (chronic)	0⇔0.625	32.95 ± 1.15, *n*= 48	<0.001	
	0⇔1.25	36.63 ± 1.74, *n*= 48	<0.001	*H* = 49.887, df = 3
	0⇔2.5	32.17 ± 1.57, *n*= 48	<0.001	
BUS (acute)	0⇔100	16.91 ± 1.23, *n*= 48	<0.001	*H* = 128.07, df = 5
BUS (chronic)	0⇔50	22.45 ± 1.84, *n*= 47	<0.001	*H* = 81.401, df = 5
	0⇔100	10.40 ± 0.26, *n*= 2	<0.05	
DZM (acute)	0[0]⇔0.71[0.02]	37.45 ± 1.19, *n*= 48	<0.001	
	0[0]⇔1.42[0.02]	35.00 ± 1.25, *n*= 48	<0.001	*H* = 92.785, df = 5
	0[0]⇔2.84[0.02]	31.88 ± 1.14, *n*= 48	<0.001	
	0[0]⇔5.68[0.02]	31.31 ± 1.32, *n*= 48	<0.001	
DZM (chronic)	0[0]⇔0.71[0.02]	36.18 ± 1.16, *n*= 48	<0.05	
	0[0]⇔1.42[0.02]	36.32 ± 1.09, *n*= 48	<0.05	*H* = 25.862, df = 5
	0[0]⇔2.84[0.02]	34.17 ± 1.48, *n*= 48	<0.001	
FLU (acute)	0⇔0.4	32.44 ± 1.18, *n*= 48	<0.001	
	0⇔0.8	34.07 ± 1.30, *n*= 48	<0.001	*H* = 40.135, df = 5
	0⇔1.6	36.93 ± 1.03, *n*= 48	<0.05	
	0⇔3.2	31.22 ± 1.53, *n*= 48	<0.001	
FLU (chronic)	0⇔0.8	38.12 ± 1.36, *n*= 48	<0.05	
	0⇔1.6	34.82 ± 1.02, *n*= 48	<0.001	*H* = 37.996, df = 5
	0⇔3.2	33.78 ± 1.84, *n*= 48	<0.001	

(AMI = amitriptyline, BUS = buspirone, DZM = diazepam, DMSO = dimethylsulfoxide, and FLU = fluoxetine)

Previous behavioral studies used larval zebrafish to analyze anxiolytic drugs only in a single exposure regime [42, 72, 76]. For example, Richendrfer *et al*. exposed larvae to diazepam and fluoxetine for 2 h in behavioral assays that assessed escape responses [72]. In another study, zebrafish larvae were exposed to diazepam for 45 min to evaluate anxiolytic properties [76]. The effects of acute (short term) and chronic (long term) exposures to these compounds were not reported together in these studies. Little or no work before the current study has been done using a 96-well plate format to assess larval behavior in response to different types of anxiolytic drugs both after acute and chronic treatments.

Interestingly, we found that BUS and AMI are toxic in chronic but not in acute exposure at the same concentrations. For example, AMI at 5 and 10 mg/L and buspirone at 100 mg/L were not toxic to the larvae in acute exposure but were lethal in chronic exposure. Hence, a side-by-side comparison of acute and chronic

treatments is helpful for identifying toxic concentrations that can mimic the desired therapeutic effect. Thus, locomotor suppression could be an unwanted toxic effect or a valuable anxiolytic effect. In addition, the larval VMR assay (acute and chronic) might be useful as a first line of testing when performing HTS or developing new anxiolytic drugs.

A previous study from our laboratory identified four types of dose responses in larval locomotion after 96 h exposure to a panel of 60 water-soluble compounds [42]. Those responses were: (i) monotonic suppression, (ii) monotonic stimulation, (iii) biphasic response [stimulation followed by suppression], and (iv) no effect. In the current study, we also found heterogeneous dose responses.

In contrast to the classic monotonic dose response that we found for diazepam (see above) all serotonergic drugs tested here produced complex dose response effects in the VMR assay. For example, acute and chronic BUS exposure caused a complex dose-response during the challenge phase that deviated from a simple, monotonic suppression of locomotion. Acute FLU produced an optimum curve (inverted 'U') in the basal phase and pessimum curve (U-shaped) dose response in the challenge phase. We assume that the heterogeneity or complexity of effects produced by serotonergic drugs (AMI, BUS, FLU) in the study might be explained based on the pharmacodynamics of those drugs and also on the ontogeny of the serotonergic system of developing larvae. We shall now consider this assumption in more detail.

In humans, the serotonergic drugs used in the current study are presumed to cause their pharmacological activity by adapting, over an extended period of time, the serotonin neurotransmitter system in the brain. According to that presumption, those drugs do not act rapidly at the site of the receptors itself (for example diazepam) [77]. Buspirone acts as a full agonist at 5-HT1_A autoreceptors and a partial agonist of postsynaptic 5-HT1_A receptors [78]. The autoreceptors functions as a brake system that inhibits further release of serotonin after the initial neurotransmission event. Hence, chronic treatment is necessary for humans to desensitize the autoreceptors and increase postsynaptic activation, which is responsible for the therapeutic lag [79].

On the contrary, amitriptyline and fluoxetine are reuptake inhibitors of serotonin that actually decrease serotonin levels initially in the synapse but require chronic treatment before elevating serotonin levels to maximum concentrations where the

pharmacological effects are seen [80]. These findings from humans would explain the fact that we do not see a simple, monotonic response to serotoninergic drugs in our study. We should note at this point that our usage in this study of the terms 'acute' and 'chronic' are arbitrary, and therefore we cannot be sure whether our 'chronic' exposures equate to human 'chronic' exposure.

Another issue that might affect the outcome of our experiments is that we are using rapidly developing larvae and not an adult stage as is true of the human studies. Therefore we cannot necessarily assume that the ontogeny of the serotonergic system is complete at 5 dpf in zebrafish larvae. This immaturity might explain the complex pharmacodynamics elicited by serotonergic drugs in our system. By complex or heterogeneous we mean the non-monotonic suppression of locomotion in larvae treated with serotonergic drugs.

Further explanations for the non-monotonic responses that we observed with serotonergic drugs might come from recently published studies. For example, Tufi *et al*. proposed that changes in neurotransmitter levels during early larval zebrafish development might lead to abnormal development of the CNS (central nervous system) [81]. The authors studied different neurotransmitter profiles in early larvae (≤6 dpf) with and without pesticide treatment. Two main developmental periods or age ranges of zebrafish larvae were studied, namely: the first two days of development and 3-6 dpf. Based on hydrophilic interaction liquid chromatography (HILIC) coupled to tandem mass spectrometry (MS/MS), there were significant changes in the concentrations of many neurotransmitters and their precursors within the two tested periods. However, serotonin concentrations, by contrast, were relatively stable throughout each developmental period tested. The authors suggested that there might be some essential function of serotonin in development. If this is the case, then it could explain the toxicity of AMI and BUS observed in our study. Other studies have also suggested an important role in development for neurotransmitters in the vertebrates.

During early development, neurotransmitters are important in regulating the normal development of the CNS [81]. For example, serotonin was shown to be important for early developmental neurogenesis in Sprague-Dawley rats [82]. Similar importance for this neurotransmitter was also observed in zebrafish, in which it promotes the embryonic development of motor neurons via 5-HT1_A receptors [83]. In

another study, manipulation of serotonin levels in early zebrafish larvae could alter the expression of genes involved in diverse physiological functions including behavior, development, reproduction, and neuroendocrine systems [84]. Global gene expression analysis in early zebrafish larvae has shown that fluoxetine influences the expression of multiple genes involved in processes such as stress response, and DNA binding, replication, and repair [84].

Further examples of the developmental roles of serotonin in zebrafish larvae include the finding that intraspinal serotonergic neurons exhibit great developmental changes between 3 and 4 dpf but stabilize at 5 dpf [85]. Hence, it is possible that the targeting of serotonergic signaling with drugs at these ages could produce unwanted effects on locomotor activity. In support of this possibility, Airhart *et al*. showed that chronic exposure of zebrafish larvae to fluoxetine at 3 – 4 dpf caused a sustained reduction in larval locomotion that lasted until 14 dpf [65]. According to the authors, reduction in movement could be due to neurotoxicity to intraspinal ventromedial neurons. This suggestion was based on the observation of decreased levels of expression of SERT (serotonin transporter) and 5-HT_{1A} receptor transcripts, in the spinal cord. Another recent study also reported that exposure to the SSRI fluoxetine during the early development of zebrafish (1000-cell stage to 3 dpf) had a profoundly negative impact on the expression of several serotonin receptors [86].

Finally, we would like to discuss our choice of organic solvent (dimethylsulfoxide, DMSO) that we used to dissolve diazepam. According to our results, acute exposure to DMSO had no significant effect on locomotion. However, our experiments with chronic exposure to diazepam experiments did, in fact, show slightly increased larval locomotion in the basal phase in 0% DMSO treated larvae compared with 0.02% DMSO treated. Hallare *et al*. reported that DMSO increased hsp70 protein (marker for stress response) levels in zebrafish embryos and larvae even at very low concentrations [87]. The DMSO concentration in the present study is within the range of concentrations used in the Hallare *et al*. study, which showed elevated hsp70 levels. It is clear, therefore, that DMSO alone can have effects on locomotion in certain exposure regimes.

Conclusions and future perspectives

Our study shows that the behavior of 5-day zebrafish larvae is sensitive to four commonly used psychotropic drugs. All four drugs tested had an effect on general locomotion and maximum velocity, but only diazepam gave a classic, monotonic dose-response. Therefore, we argue that the VMR assay alone cannot be relied on for the assessment of candidate anxiolytic drugs. Additional assays for anxiolytic assessment might usefully include those based on thigmotaxis. Thus, previous work from our laboratory analyzed thigmotactic responses in zebrafish larvae. This study showed anxiolytic-like and anxiogenic-like response to diazepam and caffeine, respectively, in zebrafish larvae.[73] Other assays such as the light-dark preference test (scototaxis) could also be added to the battery of behavioral assays.

Most drugs used in the current study induced a monotonic suppression in locomotion even in the basal phase both after acute and chronic exposure, which warrants further investigations. Diazepam was the only drug that did not affect larval locomotion in the basal phase after chronic exposure, except at the highest concentration. The monotonic suppression observed in larvae treated acutely with diazepam could be due to sedative effects. In addition to this, the behavioral and mortality effects were seen in larvae treated with serotonergic drugs could be due to either developmental response or serotonin toxicity (serotonin syndrome). Presence of serotonin toxicity in adult zebrafish was shown earlier after treatment with amitriptyline [88]. Therefore, the high throughput nature of zebrafish larvae could be easily used to assess serotonin toxicity of drugs that target the serotonergic system.

In summary, our findings show that a behavioral analysis based on VMR assay using zebrafish larvae is not only sensitive for the identification of potential anxiolytic effects but also valuable in providing a measure of toxic effects of drugs. However, incorporating older larvae than 5 dpf and various physiological analyses (neuroanatomical imaging, gene expression profiling, and toxicity profiling, etc.) will provide a comprehensive understanding on the pharmacology of the anxiolytic drugs in zebrafish larvae.

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