Scientific Research

Effect of Risperidone and Fluoxetine on the Movement and Neurochemical Changes of Zebrafish

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ABSTRACT

Brain developmental disorders in humans, including Autism Spectrum Disorders (ASD) and Down's syndrome, have been linked to increased serotonin levels. This work was designed to study changes in serotonin levels in the early stages of development with two classes of antipsychotic drugs: Risperidone, a drug that blocks serotonin and dopamine receptors, and fluoxetine, a serotonin reuptake inhibitor. The use of antipsychotic drugs is a solid choice to study the decrease and increase of these neurotransmitters and their influence on development. The study of these parameters will give an idea of the effects of serotonin in early developmental stages. To this end, we examined the effects of risperidone and fluoxetine on the locomotor activity, heart rate and brain development of zebrafish larvae. Our results showed that in larvae exposed to fluoxetine alone, swimming was significantly increased at 9 dpf (days post-fertilization). Erratic and abnormal movements were observed suggesting a toxic effect of fluoxetine. No erratic swimming was observed in larvae treated with fluoxetine plus risperidone. Both drugs presented morphological changes in dopaminergic neurons and mononeurons. Exposure to fluoxetine plus risperidone indicated possible reversal effects. Studies in zebrafish allow obtaining new insights into the side effects of these drugs as well as into the brain control of locomotor activity. Testing several drug-induced changes in behavior and serotonin levels is one of the experimental approaches for screening a new therapeutically relevant compound, and thus, merits further research.

Keywords: Zebrafish; Risperidone; Fluoxetine; Serotonin; Behavioral

1. Introduction

Serotonin has shown to play a role in regulating brain and spinal cord development before assuming its role as a neurotransmitter in the mature central nervous system (CNS) [1,2]. Serotonin plays an important role in modulating motor pathways in all vertebrates. The early appearance of serotonin neurons and their wide distribution of extra-synaptic and synaptic terminals are consistent with the notion that serotonin acts as a growth factor or a signal molecule for multiple developmental mechanisms.

Increased serotonin levels have also been linked to brain developmental disorders in humans, including Autism Spectrum Disorders (ASD) and Down's syndrome [3-7]. ASD include different neurodevelopmental disorders that become manifest mainly in the earlier years of life [8] and affect the development of language, communication, and reciprocal social interaction [9], with an incidence of 1 out of 150 individuals [10].

The main problem with the theory of serotonin as a primary cause of autism is that treatments which further increase serotonin levels seem to improve some symptoms of autism, such as obsessiveness and social relatedness [11], while the depletion of tryptophan, a serotonin precursor, seems to exacerbate autistic symptoms such as flapping, banging and self-hitting, rocking and increased anxiety [12].

On the other hand, a recent clinical study has reported that children exposed to therapeutic levels of a selective serotonin reuptake inhibitor (SSRI), such as is the case of fluoxetine, in the third trimester of pregnancy, scored lower in psychomotor and behavioral tests administered at 6 - 40 months of age compared to children who were not exposed to a SSRI during pregnancy [13]. Delays on psychomotor development and changes in movement and control at these later ages imply a more permanent problem in motor circuitry. This is why more studies are needed to understand the neurochemistry of serotonin in the early stages of development.

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Fluoxetine has many characteristics that make it a useful tool to study increases in serotonin neurotransmission. Fluoxetine is one of several SSRIs used for treatment of clinical depression, anxiety disorder, obsesssive-compulsive disorder, and bulimia. It is also one of the most heavily prescribed antidepressant drugs worldwide. It is a potent and highly selective inhibitor of the transporter enzyme for serotonin reuptake at the presynaptic membrane, causing an increase in serotonin concentrations at postsynaptic receptor sites [14].

On the other hand, atypical antipsychotic drugs, such as risperidone, 3-[2-[4-(6-fluoro-1,2-benzisoxazol-3-yl)-1-piperidinyl]ethyl]-6,7,8,9-tetrahydro-2-methyl-4H-pyrido [1,2-*a*] pyrimidin-4-one (Risp), have been increasingly used to treat anxiety-related disorders in addition to their use in the treatment of psychosis and ASD. Risperidone has been reported to act therapeutically by blocking serotonin and dopamine receptors [15]. This drug is useful to study increases in serotonin neurotransmission. Su *et al.* [16] showed that risperidone inhibits N-methyl-D-aspartate antagonist-induced glutamate release in the medial prefrontal cortex by blocking serotonin 5-HT2A receptors on glutamatergic terminals, leading to attenuation of the activity of cortico-subcortical glutamatergic neurons. This attenuation, in turn, decreases MK-801-induced hyper-locomotion [16].

In this work, we used the zebrafish (*Danio rerio*) as an ideal model for studying developmental neurobiology and other fields of biomedicine. The zebrafish is a teleost of the Cyprinid family, and has very advantageous features to be used in the laboratory: its small size (no more than 5 cm in adults) allows the easy maintenance of several individuals with relatively low costs; females lay a large number of eggs; embryos develop rapidly and are semitransparent 24 hours post-fertilization (hpf); and embryos have a sequenced genome and numerous mutant and transgenic lines.

It is known that serotonin plays an important role in modulating the motor output in vertebrates [17,18]. Serotonin neurons particularly innervate motor neurons projecting to axial muscles, which are associated with rhythmic locomotor movements. In developing zebrafish, serotonin neurons and projections appear in the hindbrain and spinal cord at about 2 days post-fertilization (dpf) [19].

Based on the above, the use of antipsychotic drugs is a solid choice to study the increase and decrease of this neurotransmitter and its influence on development. This study was designed to study changes in serotonin levels in the early stages of development induced by two classes of antipsychotic drugs: Risperidone, a drug that blocks serotonin and dopamine receptors; and fluoxetine, a serotonin reuptake inhibitor. To this end, we examined the effects of risperidone and fluoxetine on the locomotor activity, heart rate, and brain development of zebrafish

larvae. Study of these parameters will give an idea of the effects caused by serotonin in early developmental stages.

2. Materials and Methods

2.1. Animals

Adult zebrafish (*Danio rerio*) belonging to the AB line, provided by the Department of Cell Biology and Pathology, University of Salamanca, Spain, were used as breeding individuals. The animals were kept in tanks at a temperature of 28°C, with 14 h of light and 10 h of darkness, as previously established [20]. In this study, "embryos" refer to zebrafish prior to hatching (0 - 3 dpf), while "larvae" refer to post-hatch animals (over 3 dpf). Embryos were obtained from natural mating, and all embryos and larvae used in these experiments were reared at 28.5°C on a 14/10 h light/dark cycle in conditioned E3 medium (NaCl 0.29 g/l, KCl 0.012 g, $CaCl_2$ 0.036 g/l and $MgSO_4$ 0.039 g/l in deionized water), and 50 ppb methylene blue (Panreac) to inhibit fungal growth. The animals were handled following the European Union directives (86/609/EEC and 2003/65/EC) and Spanish legislation (RD 1201/2005, BOE 252/34367-91, 2005).

Embryo Collection

The evening before spawning, breeding pairs of specimens were transferred to rearing tanks. These tanks were kept at 28.5°C. The first light stimulus after the dark cycle induced egg laying. The eggs obtained were prepared in petri dishes in E3 medium. Only the eggs that were fertilized and in good condition were selected for further treatment; the others were discarded. Egg observation was followed by stereomicroscopy (Leica Zoom, 2000).

2.2. Exposure to Risperidone and Fluoxetine

Risperdal tablets (Jannsen Cilag Laboratory, 2 mg risperidone) were dissolved in E3 medium and prepared as a 5 μM solution. Prozac tablets (Lilly Laboratory, 20 mg fluoxetine) were dissolved in E3 medium and prepared as a 5 μM solution. In Airhart *et al.* 2007 [7], zebrafish embryos were exposed to seven different fluoxetine concentrations beginning at 10 pf and up to 11 dpf, to determine the lowest observable effective concentration (LOEC). In larvae exposed to 4.6 μM (LOEC) fluoxetine for 24 h intervals between 4 and 5 dpf, spontaneous swimming activity was significantly depressed compared to controls and remained depressed through 14 dpf. In addition, the core neural migration raphe to the spinal cord was observed between 3 and 6 dpf [7] and serotonin levels could affect normal development of CNS. In this respect, in this work, we selected the following exposure concentrations: 1) Risp 5 μM at 4 dpf for 24 h; 2) Risp 5 μM at 6 dpf for 24 h; 3) Prozac 5 μM at 4 dpf for 24 h; 4) Prozac 5 μM at 4 dpf for 24 h and Risp 5 μM at 6 dpf for 24

h. Controls were untreated larvae. Larvae were exposed to 5 μM risperidone or fluoxetine for periods of 24 h at 4 or 6 dpf and subsequently rescued into a preconditioned E3 medium (**Figure 1**).

2.3. Behavioral Testing: Spontaneous Swimming Activity (SSA) Measurement

At 4 dpf, larvae were exposed for 24 h in a risperidone or fluoxetine solution of 5 μM and at 6 dpf, larvae were monitored daily. Animals were placed individually in a 6-well multiplate at 28°C. After a 1-min acclimation period, the SSA was measured as the number of times the animal crossed the gridlines in 1' under a magnifying glass (**Figure 2(a)**). For treatments at 4 dpf and 6 dpf or only at 6 dpf, at 6 dpf larvae were monitored daily.

The data was analyzed with Prism GradPad. Experiments were performed with groups of 6 larvae and the data was collected three times per group.

2.4. Heart Rate Measurements

The heart rate was assessed at 8 and 10 dpf. Control and experimental zebrafish larvae were individually transferred to a depression slide with methylcellulose and placed under a binocular microscope. The heart rate was determined by counting the number of beats per 15 s and recorded as beats per minute (bpm). Experiments were performed thrice on three larvae per group for each time point.

2.5. Preparation of Histological Sections

For the fixation of samples, both treated and control animals were anesthetized with a tricaine methanesulfonate solution (MS-222, Sigma) at 0.3 g/l. They were then fixed by immersion in 4% v/v paraformaldehyde (PFA) in phosphate buffer 0.1 M (PB), pH 7.4 for 24 h at 4°C. Following fixation, the PFA was removed with five 5-minute washes in PBS.

Then the samples were embedded in a mixture 1.5% of agar and 10% of sucrose (Panreac) in PB. The mixture
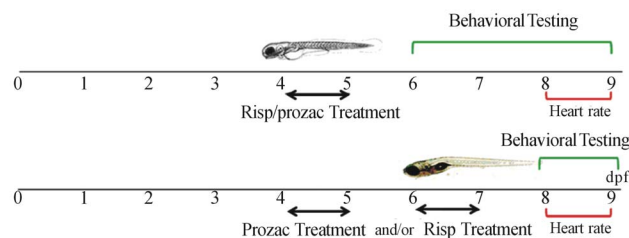

Figure 1. Timeline representing the stage specificity of the effects of fluoxetine and risperidone in the developing zebrafish. Larvae were exposed to 5 μM risperidone and/or fluoxetine for 24 h periods from 4 dpf and subsequently rescued into a conditioned E3 medium. Larvae were monitored through 9 dpf.

animals were targeted. After the mixture was solidified, the larvae were cryoprotected in a 30% w/v sucrose solution in PB for 24 h. Agar blocks containing cryoprotected larvae were frozen in a cryostat (Microm HM 560) and then cut at –28°C in 10 μm thick parasaggital serial sections, which were collected on gelatinized slides and stored at –20°C until further use.

2.6. Hematoxylin-Eosin Staining

Histological sections were obtained as mentioned above and stained with hematoxylin-eosin to observe possible morphological changes. Briefly, the technique involved immersing the sections in eosin for 1 min, then washing them with water for 30 min and incubating them for 1 min in hematoxylin. Finally, the samples were dehydrated in alcohols of increasing concentration for 5 min each, ending with three tanks of xylene, for 3 min each. The slides were mounted in Entellan (Merck) for analysis and storage.

Images of hematoxylin-eosin staining were taken in a light microscope (Olympus Provis AX70) coupled to a digital camera (DP70, Olympus).

Finally, to adjust the brightness and contrast of those observed directly under the microscope, Adobe® Photoshop CS2® version 9.0 (Adobe Systems) was used.

2.7. Immunohistochemistry in Tissue Sections

The sections were washed three times in PB for 10 min to rehydrate and remove the agar and then incubated for 1 h at room temperature (RT) in non-immune serum (Sigma) 5.0%, detergent Triton X-100 (Sigma) 0.2% and 1.0% DMSO in PB.

Then, the primary antibodies were added and incubated for 24 h at RT. After this incubation, the exceeding antibodies were removed with three washes of PB and then the sections were incubated with the corresponding secondary antibodies conjugated with the appropriate fluorochrome for 1 h at RT. The secondary antibody was removed with three 10-min washes in PB with 0.4% fish gelatin (Sigma-Aldrich).

In order to mark cell nuclei, tissue sections were incubated in 4',6-diamidino-2-phenylindole (DAPI, Sigma) at a 1:10,000 concentration for 7 min at RT, and then washed in PB three times of 10 min each.

Antibodies Used

The antibodies used were:

The polyclonal anti-calretinin (CalR) antibody 7696 (# 6B3 Swant, Bellinzona, Switzerland), at a 1:10,000 concentration. This antibody has been widely used in the study of the neuroanatomy of teleosts, both in adult animals [21-23] and in embryos, larvae and juveniles [23].

The secondary antibody was labeled with Cy3 (red).

The anti-tyrosine hydroxylase (TH) antibody (Incstar, Stillwater, MN, USA) [24] at a 1:1000 concentration. The secondary antibody was labeled with Cy2 (green).

The sections were examined under a microscope (Olympus Provis AX70) coupled to a digital camera (XM10, Olympus). The images were coded green (Cy2) and red (Cy3), giving yellow co-localization in merged images. The images were adjusted for brightness, contrast and colors using Adobe Photoshop 7.0 (Adobe Systems).

2.8. Statistical Analysis

Data was presented as a mean ± standard deviation (S.D.) and analyzed by one-way analysis of variance (ANOVA) and Tukey's Multiple Comparison Test using GraphPad Prism v. 5. Only values with $P < 0.05$ were accepted as significant.

3. Results

3.1. Heart Rate Measurements

The effect of fluoxetine and risperidone exposure on circulation was qualitatively evaluated by observing the heart rate and blood flow through the ventral aortaposterior cardinal vein channel in control versus treated larvae.

The aim of this study was to determine whether 5 μM fluoxetine and/or risperidone affected blood circulation. To this end, at 4 and 6 dpf, larvae were exposed to 5 μM fluoxetine and/or risperidone for 24 h and their heart rate monitored at 8 and 9 dpf. This data demonstrated that larvae treated with 5 μM fluoxetine and/or risperidone exhibited a normal heart rate at 8 and 9 dpf compared to controls (**Table 1**, $P > 0.05$).

Therefore, the effect of 5 μM fluoxetine or risperidone on locomotor development in larvae was most likely based on neurological grounds rather than on cardiovascular ones.

3.2. Behavioral Testing

In this specific experiment, larvae were exposed to 5 μM fluoxetine or risperidone at 4 and 6 dpf for 24 h. After fluoxetine or risperidone exposure, larvae were rescued by transfer into E4 medium. SSA in these larvae was monitored daily from 6 to 9 dpf. All SSA measurements were performed three times per larva; each data point represented the mean ± S.D. of six larvae.

Risperidone treatment at 4 dpf only transiently decreased SSA, with full recovery after 7 dpf.

SSA was significantly below that of controls (**Figure 2(b)**) in the first days after treatments (6 dpf, $P < 0.05$). After 7 dpf, SSA had recovered to control levels, these levels were maintained throughout the remaining experimental period (**Figure 2(b)**).

The same occurred in zebrafish treated with 5 μM risperidone at 6 dpf and monitored at 8 dpf (8 dpf, $P < 0.05$).

A 24-h exposure to fluoxetine at 4 dpf resulted in a significant decrease in SSA as compared to controls (6 dpf, $P < 0.01$). SSA recovered fully after 7 dpf, and increased significantly at 9 dpf (9 dpf, $P < 0.01$) (**Figure 2 (b)**); however, the movements were erratic and abnormal. It is worth mentioning that when fish were exposed to fluoxetine at 4 dpf and then to risperidone at 6 dpf, there was no significant change in SSA with respect to control (**Figure 2(b)**).

3.3. Tissue Sections

3.3.1. Morphological Changes

Both treated and control animals were fixed at 10 dpf, cut in serial sections and stained, as mentioned above. A 24-h exposure to fluoxetine at 4 dpf resulted in an observable decrease in the number of nucleated areas of the dorsalis telencephali area. The same occurred in the dorsal area of the optic tectum (**Figure 3(b)**) compared with the control (**Figure 3(a)**).

There was also a larger area in the postoptic commissure and the raphe population zone and a cellular disorganization in the latter. On the other hand, the fissure that separates the midbrain at forebrain increased in size (**Figure 3(b)**). All these changes were reflected as an increase in abnormal and erratic swimming in the behaveioral test. Undoubtedly, the administration of this drug to animals at 4 dpf caused dramatic changes that persisted overtime.

In the treatment with risperidone at 4 and 6 dpf (**Figures 3(c)** and **(d)**), an increase in the nucleated zone of

Table 1. Post-treatment heart rates in zebrafish larvae after a 24 h exposure to 5 μM Fluoxetine or risperidone. A mean number of heart beats per minute (bpm) (±S.D.) in control and 24 h fluoxetine or risperidone treated zebrafish larvae. There was no significant difference between control and fluoxetine or risperidone exposed larvae at either 8 or 9 dpf ($P > 0.05$, n = 6).

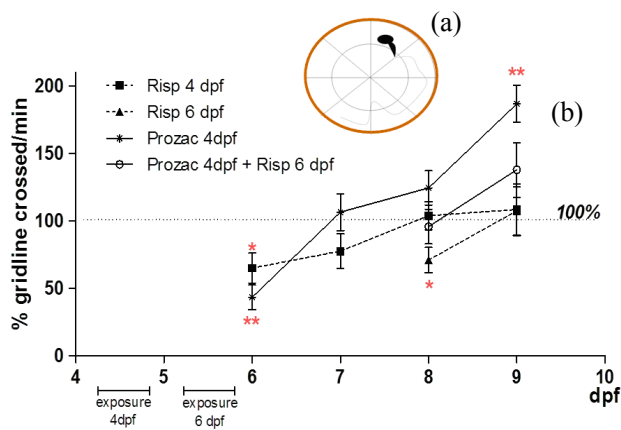|	Treatment				
Heart rate measurements	**Prozac 4 dpf**	**Risp 6 dpf**	**Risp 4 dpf**	**Prozac 4 dpf + Risp 6 dpf**	**Control**
8 dpf	121 ± 11	128 ± 7	127 ± 7	131 ± 6	131 ± 9
9 dpf	115 ± 9	125 ± 7	130 ± 3	121 ± 10	122 ± 6

Figure 2. (a) Schematic representation of a 6-well multiplate with a dividing line for monitoring the spontaneous swimming activity (SSA); (b) Spontaneous swimming activity (SSA) measurements. All SSA measurements began at 6 dpf and were performed three times; each data point represents the mean ± S.D. of six larvae.

the postoptic commissure and a cellular disorganization in the raphe populations zone were observed. However, these changes would not be sufficient to affect the SSA.

Zebrafish exposed to fluoxetine at 4 dpf and then to risperidone at 6 dpf showed no significant changes with respect to control (**Figure 3(e)**).

3.3.2. Immunohistochemistry

For the histological analysis, crop images were obtained to include the reference space area and optical angle for brain tissue. In this work, we studied two proteins to determine dopaminergic neurons, labeled with TH, and motoneurons, labeled with CalR.

When the larvae were exposed to free risperidone, an increase in the levels of TH + dopaminergic neurons was observed in the dorsal region of the optic tectum of the brain (**Figures 4(c)** and **(d)**) compared with the control (**Figure 4(a)**). On the other hand, increases in the levels of TH + cells in the midbrain region were also observed. However, no significant changes in brain tissue (**Figures 4(b)** and **(e)**) were observed with fluoxetine either alone or combined with risperidone.

In the spinal cord, we found a decrease in the levels of CalR-positive motoneurons in all treatments with risperidone alone (**Figures 5(c)** and **(d)**) compared with the control (**Figure 5(a)**). In the case of fluoxetine, an increased width in the nerve fiber formed by the axons of motoneurons (**Figure 5(b)**) was observed. In the case of fish treated with the combination of fluoxetine and risperidone, there was an increased width in the nerve fiber formed by the axons of motoneurons compared with those treated with risperidone (**Figure 5(e)**) but less than that found in those under fluoxetine alone.

F Forebrain ; T Telencephalon ; M Midbrain ;
C Cerebellum ; HY Hypothalamus ;
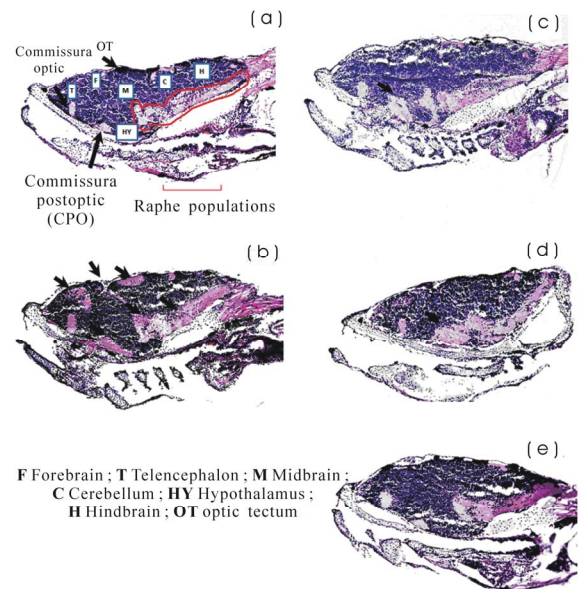H Hindbrain ; OT optic tectum

Figure 3. Images of histological sections of brain tissue stained with hematoxylin-eosin. (a) Control; (b) Fluoxetine at 4 dpf; (c) Risperidone at 6 dpf; (d) Risperidone at 4 dpf; and (e) Fluoxetine at 4 dpf and risperidone at 6 dpf. Larvae were analyzed three times (n = 3) at 10 dpf. In Figure 3(b), the arrows indicate an observable decrease in the number of nucleated areas of the dorsalis telencephali area and the optic tectum; also a larger area in the postoptic commissure and the raphe population zone and a cellular disorganization in the latter. On the other hand, the fissure that separates the midbrain at forebrain increased in size.
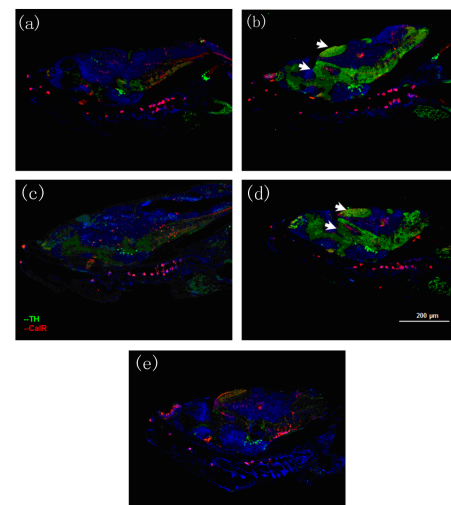

Figure 4. Immunohistochemistry images of brain tissue. Tyrosine hydroxylase, labeled with Cy2 (green) and Calretinin labeled with Cy3 (red). (a) Control; (b) Fluoxetine at 4 dpf; (c) Risperidone at 6 dpf; (d) Risperidone at 4 dpf; and (e) Fluoxetine at 4 dpf and risperidone 6 dpf. Larvae were analyzed three times (n = 3) at 10 dpf. The arrows in Figures 4(c) and (d) indicate an increase in the levels of TH + dopaminergic neurons in the dorsal region of the optic tectum of the brain and an increase in the levels of TH + cells in the midbrain region.
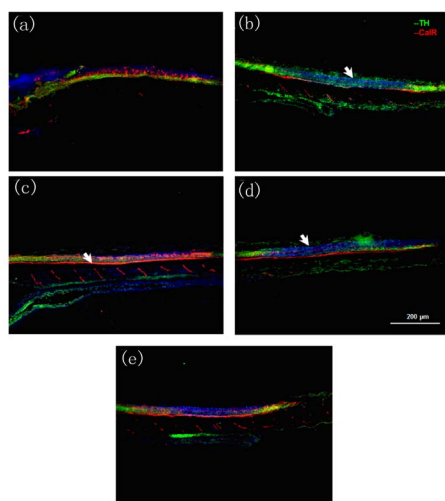
Figure 5. Immunohistochemistry images of spinal cord tissue. Tyrosine hydroxylase, labeled with Cy2 (green) and Calretinin labeled with Cy3 (red). (a) Control; (b) Fluoxetine at 4 dpf; (c) Risperidone at 6 dpf; (d) Risperidone at 4 dpf; and (e) Fluoxetine at 4 dpf and risperidone at 6 dpf. Larvae were analyzed three times (n = 3) at 10 dpf. In Figures 5(c) and (d), the arrows indicate a decrease in the levels of CalR-positive motoneurons. In Figures 5(b) and (e) the arrows indicate a change in the width of the nerve fiber formed by the axons of motoneurons.

4. Discussion

In this work, the effects of risperidone and fluoxetine on the locomotor activity, heart rate, and brain development of zebrafish larvae were studied. These parameters provide an idea of the effects caused by serotonin in early development stages and provide knowledge on the area of neuropharmacology.

The advantage of studying motoneuron diseases in zebrafish [25] is the rapid development of their spinal cord, which allows the analysis of motoneuron branching patterns as early as 24 hpf. In addition, responses to touching and swimming can be monitored after hatching around 48 hpf [26]. It is now recognized that zebrafish show great similarity to mammals and are an extremely useful model for screening compounds at several stages of the drug discovery process [27,28].

The effect of fluoxetine and risperidone exposure was evaluated by examining the heart rate. The results demonstrated that 5 μM fluoxetine and/or risperidone-treated larvae exhibit a normal heart rate at 8 and 9 dpf compared to controls. When larvae were exposed to 5 μM fluoxetine and/or risperidone at 4 and 6 dpf and for 24 h, SSA was monitored daily from 6 to 9 dpf. SSA was significantly lower than controls on the first days after treatments. After 7 dpf, SSA levels recovered up to those of control, and were maintained throughout the remaining experimental period under risperidone treatment. In larvae exposed to 5 μM fluoxetine alone, SSA was sig-

nificantly increased at 9 dpf as compared to controls. However, erratic and abnormal movements were observed with this increase, suggesting a long-term toxic effect of fluoxetine. The unusual body position exhibited in swimming during the behavioral test after exposure to fluoxetine was also observed. While control animals maintained a parallel position with respect to the water surface when swimming, the animals treated with fluoxetine were unable to maintain such a position, indicating lack of postural balance, as described in previous studies [29]. In addition, we found that fluoxetine-treated zebrafish exhibited erratic swimming patterns, manifested by bouts of vertical swimming or sideway swimming, suggesting a coordination problem. This extended effect suggests that fluoxetine effect is not pharmacological, but rather developmental. However, when fish were treated with fluoxetine and risperidone, SSA showed no significant differences with control, and no erratic swimming was observed. This result suggests a reversible process, given that fluoxetine increases the levels of serotonin and risperidone levels out this effect. So, it is important to note that changes in SSA are not pharmacological because for extended periods of time, the effects are reversed when the concentrations of the neurotransmitter are decreased.

Representatives from all major fish classes possess serotonergic neurons in the raphe nuclei, giving rise to ascending and descending pathways [18,30-47]. Due to their apparent resemblance with the mammalian raphe serotonergic neurons, these are the best studied populations within fish. Generally, the assumption for teleosts has been that the two raphe nuclei (superior and inferior raphe) innervate most brain areas in a manner similar to that described for mammals and, accordingly, that they may play equivalent functional roles. Indeed, 5-HT immunoreactive fibers have been detected throughout the fish CNS [30,33,37,39,41,42,48-51].

The raphe serotonergic innervation of the zebrafish dorsal telencephalon, in particular of the lateral zone, is very dense. Further, Lillesaar *et al.* [18] observed few serotonergic fibers in the medial zone of the dorsal telencephalon originating from the raphe fibers. Interestingly, developmental studies in zebrafish as well as axonal tracing and lesion experiments combined with behavioral assays performed in goldfish suggest that these areas are functionally equivalent to the mammalian hippocampus and amygdala, respectively [52-54]. In mammals, the hippocampus is involved in spatial, contextual, or relational memory and it is known to be supplied by a rich serotonergic innervation mainly from the median raphe populations [55,56].

Several antipsychotic drugs cause a neurotoxic mechanism resulting from an increased or decreased concentration of serotonin both in the synaptic and extracellular

spaces. In this sense, drug exposure at 4 or 5 dpf coincides with the initial appearance of inferior raphe axons distributed throughout the entire length of the spinal cord in zebrafish [7]. Growth cones of these axons at 4 dpf have been observed adjacent to reticulospinal neurons in the hindbrain and secondary motoneurons in the spinal cord. The temporal correlation between the growth of inferior raphe axons and growth cones throughout the spinal cord and the earliest morphological effects of antipsychotics drugs suggest that raphe axons are affected by the exposure to these drugs. The mechanism of excess or default of serotonin toxicity has been elusive. Considering the temporal correlation between the development of the inferior raphe pathway and risperidone exposure, it was surprising to find no change in SSA. The transient depression of SSA in larvae exposed to risperidone at 4 and 6 dpf may be due to the long half-life of the drug [57]. When the larvae were exposed to fluoxetine alone, first a decrease in swimming and then an increase with erratic movements were noted. However, in zebrafish exposed to fluoxetine at 4 dpf and then to risperidone at 6 dpf, there was no significant change with respect to control, indicating a possible reversal of the effects caused by exposure to fluoxetine observed previously.

In immunohistochemical assays, we used two proteins to determine dopaminergic neurons, labeled with tyrosine hydroxylase (TH) and motoneurons, labeled with calretinin (CalR). TH is the first enzyme in the catecholamine synthesis pathway [58]. TH has also been described to be involved in the stabilization of dopamine synapses [59]. When the larvae were exposed to free risperidone, several changes in the brain were observed. This could be because the drug is a strong blocker of dopamine receptors, which would affect dopaminergic neurons. However, despite the changes observed in the distribution pattern of dopaminergic neurons, these would not affect the SSA. On the other hand, when administered fluoxetine alone or fluoxetine and risperidone, there were no changes with respect to the control. These results indicate that risperidone alone increased the levels of TH enzymes of the dopaminergic cells when administered as a single drug.

CalR was found in several neuronal populations of the central and peripheral nervous system, primarily in motoneurons and other sensory pathways [60]. CalR is a cytosolic protein of 29-kD, belonging to the family of calcium-binding proteins "EF hand" [61], these expressed during early development of the CNS of vertebrates [62], although CalR needs some degree of cell differentiation and tissue to be expressed [63]. Calcium-binding proteins buffer intracellular calcium, thus contributing to the properties of neuronal membranes and their electrical activity. In the spinal cord, we found a decrease in the number of CalR-positive motoneurons in

all treatments with free risperidone. These changes could be due to the effect of risperidone on the core neural migration raphe (located in the hypothalamus) to the spinal cord. This migration begins to be observed between 2 and 3 dpf and extends into the caudal spinal cord after 4 days [7]. In the case of fluoxetine, an increase in the width of nerve fiber formed by the axons of motoneurons was observed. This could cause a significant increase in SSA at 9 dpf with erratic and abnormal movements, given the potential neurotoxicity of the drug. In this respect, antipsychotic drugs could alter the extracellular levels of neurotransmitters and thereby modify the development of the CNS [7,14,28,64]. These changes suggest that the neuroanatomy is affected by fluoxetine and risperidone exposure, since these changes are not sufficient to modify the SSA in the case of risperidone.

According to Seibt *et al.* [28], antipsychotic drugs show high affinity for biomembranes due to their amphiphilic property. This implies that antipsychotic drugs can also interact with membrane lipid organization. The intercalation of antipsychotic drug molecules into the plasma membrane can modify the membrane lipid dynamics, inducing a subsequent modification of the receptor response [64]. Drug interaction elicits short and long range influence on the bilayer structure, conesquently, modulating processes that range from membrane-bound enzyme activity and receptor binding to membrane permeability and transport [65]. The intercalation of antipsychotic drug molecules into the plasma membrane may thus modulate the efficacy and tolerability profile of compounds able to exert their therapeutic effect through their binding with synapse receptors. This suggests that the pharmacological activity of antipsychotic medications may result from a combination of drug-receptor and drug-membrane interactions [66]. Giacomini *et al.* [29] have suggested that the pharmacological actions of these antipsychotic drugs may be well retained in vertebrates.

Studies in zebrafish will provide an important insight into the side effects of these drugs as well as into the brain control of locomotor activity.

Testing several drug-induced changes in behavioral and serotonin levels is one of the experimental approaches for screening a new therapeutically relevant compounds, and merits further research in this animal model.

5. Acknowledgements

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Abbreviations

Hpf: Hours Post Fertilization;
Dpf: Days Post Fertilization;
SSA: Spontaneous Swim Activity;
TH: Tyrosine Hydroxylase;
CalR: Calretinin;
CNS: Central Nervous System;
ASD: Autism Spectrum Disorders;
SSRI: Selective Serotonin Reuptake Inhibitor;
Risp: Risperidone;
LOEC: Lowest Observable Effective Concentration;
bpm: Beats Per Minute;

PB: Phosphate Buffer 0.1 M;
PFA: 4% v/v Paraformaldehyde;
RT: Room Temperature;
MLF: Medial Longitudinal Fascicle.