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Fluoxetine and WAY 100,635 dissociate increases in scototaxis and analgesia induced by conspecific alarm substance in zebrafish (*Danio rerio* Hamilton 1822)

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ABSTRACT

Alarm reactions to a substance secreted by the damaged skin of conspecifics and closely-related species are increasingly being recognized as fear-like responses in fish. The neurochemical underpinnings of these effects are so far unknown; however, given the role of the serotonergic system on defensive behavior, it is possible that the alarm reaction is mediated by this monoamine. Exposure to conspecific alarm substance (CAS) increased anxiety-like behavior in the light/dark test in zebrafish and decreased nocifensive behavior. These effects were accompanied by increases in blood glucose, hemoglobin, epinephrine and norepinephrine levels, as well as extracellular levels of serotonin in the brain. Pretreatment with fluoxetine blocked the anxiogenic effects of CAS on the light/dark test as well as all physiological parameters and the increase in extracellular brain 5-HT, but not the reduction in nocifensive behavior. Conversely, pretreatment with the 5-HT_{1A}R antagonist WAY 100635 blocked the effects on nocifensive behavior, but not the effects on anxiety-like behavior nor on physiological parameters. These results point to an important and complex role of the serotonergic system in the mediation of fear-potentiated behavior in the light/dark test and in fear-induced analgesia in zebrafish.

1. Introduction

A dual role for serotonin (5-HT) has been proposed in the control of mammalian defensive behavior, with the neurotransmitter increasing anxiety-like and decreasing fear-like behavior (Graeff et al., 1996, 1997). Although it is yet unknown if such a behavioral specialization exists in teleost fish (Kalueff et al., 2012), two of the most widely used behavioral tasks in zebrafish – the novel tank test and the light/dark test – show differential pharmacological sensitivity to anxiolytic and panicolytic drugs: dark preference is sensitive to anxiolytic, but not panicolytic drugs (Maximino et al., 2011), while bottom-dwelling is also sensitive to panicolytic drugs (Stewart et al., 2011). Besides their differential pharmacological sensitivity, both tests are also under different stimulus control; while the light/dark preference test is controlled by an approach/avoidance conflict (Blaser et al., 2010; Maximino et al., 2010; Blaser and Peñalosa, 2011; Araujo et al., 2012), the novel tank test is controlled by escape from the surface (Blaser and Goldsteinholm, 2012; Luca and Gerlai, 2012). These observations closely parallel Gray's model in which anxiety involves defensive approach, generating an approach–avoidance conflict, while fear involves defensive avoidance, generating escape/withdrawal responses (Gray and McNaughton, 2000; McNaughton and Corr, 2004). Interestingly, increasing 5-HTergic transmission by blocking uptake has been shown to reduce bottom-dwelling but increase white avoidance in adult zebrafish (Sackerman et al., 2010; Maximino et al., 2013a), while 5-HT depletion produces the opposite effect (Maximino et al., 2013a).

These behavioral observations suggest different stimulus control, and coordinate well with the "dual role" of serotonin proposed by Deakin and Graeff (1991), Graeff et al. (1997), and Guimarães et al. (2010). However, the differences observed in the effects of fluoxetine and pCPA in both tests (Maximino et al., 2013a) could also be due to other factors, including, e.g., alterations in contrast discrimination (in the case of dark preference) or effects on the swim bladder (in the case of bottom-dwelling). To better discriminate these hypotheses, a clearer fear-inducing stimulus is needed. A behavioral response to an "alarm substance" released by the damaged skin of conspecifics (conspecific alarm substance, CAS), dubbed Schrecksreaktion by Von

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Frisch (1938), has been proposed in zebrafish as a model to study innate fear (Jesuthasan and Mathuru, 2008; Gerlai, 2010). In addition to increased bottom-dwelling (Egan et al., 2009), shoal cohesion (Speedie and Gerlai, 2008) and erratic movements in the novel tank test and group behavior task (Speedie and Gerlai, 2008; Egan et al., 2009; Mathuru et al., 2012), CAS has been demonstrated to promote analgesia in zebrafish (Maximino, 2011; Lima et al., 2012) as well as in the piauçu *Leporinus macrocephalus* (Alves et al., 2013). In this latter species, acute treatment with fluoxetine (10 mg/kg) reverts the locomotory-inhibiting effect of exposure to CAS (Barbosa et al., 2012). In the crucian carp, Schrecksreaktion is in part mediated by structures which receive primary and secondary olfactory projections which are conducted by the medial bundle of the medial olfactory tract (Hamdani et al., 2000; Døving and Lastein, 2009); in zebrafish, these projections integrate into a circuit of amygdala-like structures in the telencephalon, as well as hypothalamic structures (Gayoso et al., 2011, 2012). Serotonergic innervation of such structures has been described in zebrafish (Kaslin and Panula, 2001; Lillesaar et al., 2009), as well as monoamine oxidase activity and immunoreactivity (Anichtchik et al., 2006), the presence of 5-HT_{1A}-like, 5-HT_{1B} and 5-HT_{2C} receptors (Norton et al., 2008; Schneider et al., 2012), and serotonin transporters (Norton et al., 2008).

To better understand whether the serotonergic system participates in the alarm response in zebrafish, we analyzed 5-HT content in the extracellular fluid (ECF) of zebrafish exposed to conspecific skin extract, as well as the effects of the serotonin transporter antagonist fluoxetine and the 5-HT_{1A}R antagonist WAY 100,635 on the behavioral responses of conspecific skin extract in the light/dark assay and in a nocifensive behavior assay.

2. Methods

2.1. Drugs and reagents

Fluoxetine was bought from Roche (Brazil). WAY 100,635 maleate was bought from Sigma (St. Louis, USA). HPLC standards (NE, Epi, 5-HT, 5-HIAA and DHBA) were bought from Sigma (St. Louis, USA). HPLC-grade methanol was bought from Tedia (Fairfield, USA), and biotechnology-grade sodium dodecyl sulfate was bought from Amresco (Solon, USA). Reagents for hemoglobin quantification were bought from LabTest (Brazil). All other reagents were of analytical-grade and bought from Synth (Diadema, Brazil).

2.2. Animals

Wild-type longfin (n = 140) adult zebrafish were bought from a local ornamental aquarium shop and collectively maintained in 40 L tanks for at least two weeks before onset of experiments. The tanks were kept at constant temperature (28 °C), oxygenation, and light cycle (14:10 LD photoperiod). The animals were fed daily with Alcon flake food, and twice a week with live brine shrimp.

2.3. Drug treatment

30 min before exposure to either conspecific skin extract or water, the animals were cold-anesthetized and transferred to a sponge soaked in cold water for intraperitoneal injection of 5 μl of either 2.5 mg/kg fluoxetine hydrochloride, 0.003 mg/kg WAY 100635, or Cortland's salt solution (vehicle). The animals were then transferred to a 2 L beaker to recover.

2.4. Behavioral assays

2.4.1. Alarm substance preparation and exposure

Alarm substance was produced from a conspecific skin extract as described elsewhere (Speedie and Gerlai, 2008). Excess water was removed from the skin of donor animals with a paper towel, after which the animals were cold-anesthetized and quickly sacrificed by decapitation with surgical scissors. 10–15 shallow cuts were made on each side of the trunk of 10 donor fish, after which the cuts were washed with Milli-Q water. During the collection process and until further use, the solution was kept on ice. A total of 100 ml of alarm substance solution was collected, and the aliquots were diluted to a 50% concentration. Exposure was performed in a pre-treatment beaker (3.5 ml skin extract/l) for 5 min, after which the fish were removed from the exposure beaker and subjected to one of the behavioral tests.

2.4.2. Scototaxis

The light/dark assay was performed as described elsewhere (Araujo et al., 2012). Briefly, the animals were transferred to the central compartment of a black and white tank (15 cm × 10 cm × 45 cm h × d × l) for a 3-min. acclimation period, after which the doors which delimit this compartment were removed and the animal was allowed to freely explore the apparatus for 15 min. While the whole experimental tank was illuminated from above by a homogeneous light source, due to the reflectivity of the apparatus walls and floor average illumination (measured just above the water line) above the black compartment was 225 ± 64.2 (mean ± S.D.) lx, while in the white compartment it was 307 ± 96.7 lx. The following variables were recorded:

time on the white compartment: the time spent in the white half of the tank (percentage of the trial);
squares crossed: the number of 10 cm^2 squares crossed by the animal in the white compartment; latency to white: the time to first entry in the white compartment (s);
entries in white compartment: the number of entries the animal makes in the white compartment in the whole session;
erratic swimming: the number of "erratic swimming" events, defined as a zig-zag, fast, unpredictable course of swimming of short duration;
freezing: the proportional duration of freezing events (in % of time in the white compartment), defined as complete cessation of movements with the exception of eye and operculae movements;
thigmotaxis: the proportional duration of thigmotaxis events (in % of time in the white compartment), defined as swimming in a distance of 2 cm or less from the white compartment's walls;
risk assessment: the number of "risk assessment" events, defined as a fast (<1 s) entry in the white compartment followed by re-entry in the black compartment, or as a partial entry in the white compartment (i.e., the pectoral fin does not cross the midline);

Video records of the experiments were manually registered by two observers blind to treatment (inter-observer reliability > 0.85) using X-Plo-Rat 2005 (http://scotty.ffclrp.usp.br).

2.4.3. Fear-induced analgesia

The nocifensive behavior assay was modified from methods proposed elsewhere (Correia et al., 2011; Maximino, 2011; Alves et al., 2013). The locomotor activity of individual animals (n = 8–10) was recorded for 10 min before drug treatment to produce a baseline, after which the animals were subjected to drug treatment and exposure to alarm substance as above. After that, the animals were removed from the exposure beaker and quickly injected with 5 ml of Cortland's salt solution with 1% acetic acid in the tail in a region near the adipose fin. Immediately after injection, the animals were transferred to the observation tank (a 10 cm l × 10 cm w × 20 cm h Plexiglas tank containing water from the home tank) and their behavior was video recorded for 10 min. Digitized video files were then analyzed and two categories were scored: tail-beating (the number of tail-beat movements that did not lead to propulsion in the water) and locomotion (number of 9 cm^3 squares crossed). These latter measures were normalized on a fish-by-fish basis to the means of their individual pre-treatment values and expressed as percentages, so all locomotion data in this assay refers to changes relative to pre-treatment values.

2.5. Physiological parameters

2.5.1. Sample preparation

After behavioral analyses, zebrafish were cold-euthanized and the caudal fin and head were severed with a pair of fine scissors. Each fish (n = 4 per group) was put into a homemade 0.5 ml microcentrifuge tube that was previously perforated with sharp needles. This tube was then placed into a 1.5 ml microcentrifuge tube containing 500 UI/ml heparin. The assembly was then centrifuged at 50 g for 5 min at 11 °C. After this step, another cut was made closely behind the existing wound, removing the clot that had formed therein. Centrifugation was repeated with the same parameters, yielding total blood at the bottom tube (8.4 ± 1.1 μl) (Babaei et al., 2013).

While blood samples were prepared, another experimenter extracted cerebrospinal fluid (CSF) from the severed heads. 0.5 ml of CSF was extracted by quickly removing one brain from the skull and incubating it in 2 ml of 50 mM TBS, pH 7.4, containing 90 mM NaCl, 2.5 mM $CaCl_2$, and 1 mM glutathione for 30 min at 4 °C (Pradel et al., 1999). This fluid was then mixed with 0.5 ml of eluting solution, filtered through a 0.22 μm syringe filter and frozen at −20 °C until use.

2.5.2. Plasma glucose and hemoglobin levels

4 μl from the blood samples was thawed and used for glucose quantification, using Accu-Check Advantage II blood glucose strips and the Accu-Check Advantage monitor (Roche Diagnostics, Germany). Another 4 μl was added with 1 ml of Drabkin's solution (LabTest, Brazil) and measured spectrophotometrically at 540 nm; hemoglobin levels were compared against a cyanohemoglobin standard curve. All values were corrected by protein levels, according to the method of Zor and Selinger (1996).

2.5.3. HPLC analysis of monoamines

Serotonin, 5-HIAA, norepinephrine, epinephrine and 3,4-dihydroxybenzylamine (DHBA) (50 mg) were dissolved in 100 mL of eluting solution ($HClO_4$ 70% [0.2 N], 10 mg EDTA, 9.5 mg sodium metabissulfite) and frozen at -20 °C, to later be used as standards. The HPLC system consisted of a delivery pump (LC20-AT, Shimadzu), a 20 μL sample injector (Rheodyne), a degasser (DGA-20A5), and an analytical column (Shimadzu Shim-Pack VP-ODS, 250 x 4.6 mm internal diameter). The integrating recorder was a Shimadzu CBM-20A (Shimadzu, Kyoto, Japan). An electrochemical detector (Model L-ECD-6A) with glassy carbon was used at a voltage setting of +0.72 V, with a sensitivity set at 2 nA full deflection. The mobile phase consisted of a solution of 70 mM phosphate buffer (pH 2.9), 0.2 mM EDTA, 34.6765 mM SDS, 10% HPLC-grade methanol and 20% sodium metabissulfite as a conservative. The column temperature was set at 17 °C, and the isocratic flow rate was 1.6 ml/min.

2.6. Statistical analysis

Categorical data (entries on white, squares crossed on white, erratic swimming, risk assessment, tail-beating) were represented by boxplots with Tukey's whiskers, and analyzed using Kruskal–Wallis ANOVAs. Since latency estimates do not reach criteria for parametric analyses, values were analyzed by the Mantel–Cox test and are represented as Kaplan–Meier transformations in a survival plot (Jahn-Eimermacher et al., 2011). The other data were represented as means ± standard errors and analyzed by analyses of variance. p-Values < 0.05 were considered statistically significant. In addition to p-values, replication probabilities (Killeen, 2005) were also calculated for the contrasts, using a built-in function from the R package 'psych' (v. 1.2.4).

3. Results

Alarm substance significantly reduced the time spent on the white compartment of the light/dark test (Fig. 1A), an effect which was avoided by pre-treatment with fluoxetine; fluoxetine itself decreased this variable ($F_{3, 39}$ = 5. 299, p = 0.004, p_{rep} = 0.914). Likewise, alarm substance increased the latency to enter the white compartment (Fig. 1B), an effect which once again was blocked by fluoxetine pre-treatment (χ^2_3 = 8.217, p = 0.0417, p_{rep} = 0.890). No effect was observed in the number of entries in the white compartment (Fig. 1C; H_3 = 3.315, p = 0.3455, p_{rep} = 0.611) or number of squares crossed in the white compartment (Fig. 1D; H_3 = 2.343, p = 0.5043, p_{rep} = 0.497). Fluoxetine treatment and alarm substance increased freezing ($F_{3, 33}$ = 11, p < 0.0001, p_{rep} = 0.978), while pre-treatment with fluoxetine abolished the alarm substance-induced freezing (Fig. 1E). The same was observed in relation to erratic swimming events (Fig. 1F; H_3 = 15.68, p = 0.0013, p_{rep} = 0.983); while fluoxetine itself increased thigmotaxis (Fig. 1G), alarm substance did not alter this variable ($F_{3, 33}$ = 4.093, p = 0.0151, p_{rep} = 0.876). Finally, none of the treatments altered risk assessment (H_3 = 2.866, p = 0.4128, p_{rep} = 0.562).

In the nocifensive behavior test, alarm substance significantly decreased tail-beating caused by acetic acid injection at the 4–6 min time block ($F_{6, 108}$ = 5.164, p = 0.0069, p_{rep} = 0.917 for the interaction term); pre-treatment with fluoxetine did not block this effect (Fig. 2A). Likewise, alarm substance blocked the decrease in activity at the 2–4 min and 8–10 min time blocks caused by acetic acid ($F_{8, 135}$ = 2.242, p = 0.028, p_{rep} = 0.781 for the interaction term). Again, pre-treatment with fluoxetine did not block this effect (Fig. 2B).

Treatment with WAY 100,635 increased the time spent on the white compartment (Fig. 3A), but did not block the decrease in this variable in animals exposed to the alarm substance ($F_{3, 35}$ = 19.92, p < 0.0001, p_{rep} = 0.996). Likewise, pre-treatment with WAY 100,635 did not decrease the alarm substance-elicited increase in latency to white (Fig. 3B; χ^2 = 9.048, p = 0.0278, p_{rep} = 0.912). No effects were observed on entries on white nor squares crossed in the white compartment (Fig. 3C and D; H_3 < 6.72, p > 0.082, p_{rep} < 0.837). This drug did not alter freezing duration (Fig. 3E), and did not have any effect on the increase in freezing elicited by alarm substance ($F_{3, 35}$ = 8.662, p = 0.0002, p_{rep} = 0.963). A similar pattern was observed in erratic swimming (Fig. 3F; H_3 = 20.71, p < 0.0001, p_{rep} > 0.996) and thigmotaxis (Fig. 3G; $F_{3, 35}$ = 7.12, p = 0.0009, p_{rep} = 0.946). At last, WAY 100,635 decreased risk assessment (Fig. 3H), but no other effects were observed in this variable (H_3 = 14.12, p = 0.0027, p_{rep} = 0.975).

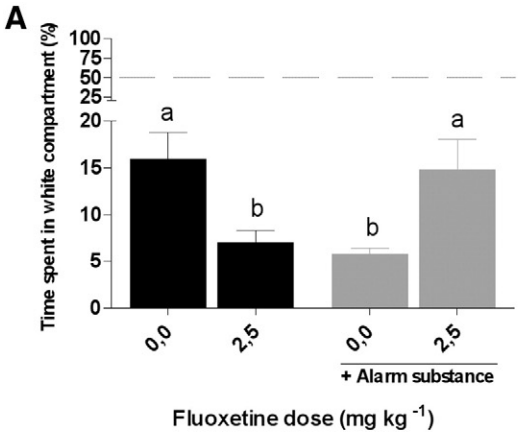
Pre-treatment with WAY 100,635 reduced the analgesic effect of alarm substance exposure on tail-beating (Fig. 4A) at the 4–6 and 6–8 min time blocks ($F_{8, 135}$ = 14.28, p < 0.001, p_{rep} = 0.993 for the interaction term) and completely blocked it on the activity decrease at the 2–4 and 8–10 min time blocks ($F_{8, 135}$ = 2.941, p = 0.0228, p_{rep} = 0.830; Fig. 4B).

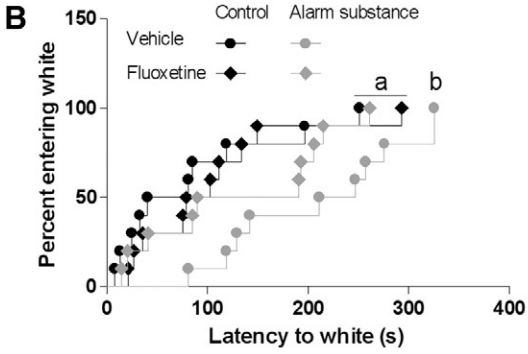
Alarm substance, but not fluoxetine, increased blood glucose, hemoglobin, epinephrine and norepinephrine levels (Table 1). Pre-treatment with fluoxetine partially blocked the effects of alarm substance on blood glucose and hemoglobin levels, and totally blocked the increases in circulating epinephrine and norepinephrine levels. WAY 100,635 did not have effects by itself, nor did it block the effects of alarm substance.

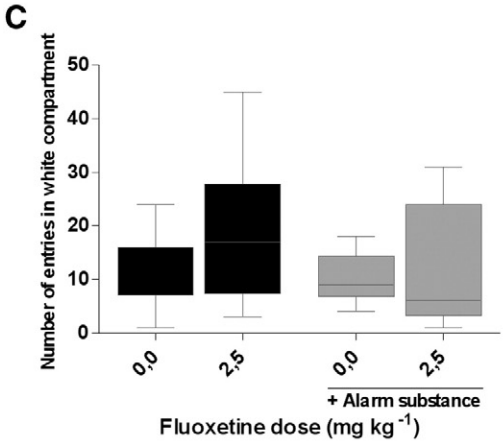
Finally, treatment with fluoxetine increased extracellular 5-HT content and blocked the alarm substance-elicited increase (Fig. 5A; $F_{3, 31}$ = 7.879, p = 0.0006, p_{rep} = 0.954). While fluoxetine did not produce an effect on ECF 5-HIAA, it blocked the alarm substance-elicited increase in this metabolite (Fig. 5B; $F_{3, 31}$ = 5.219, p = 0.0055, p_{rep} = 0.909). WAY 100,635 did not increase extracellular 5-HT nor 5-HIAA content by itself, nor did it block the CAS-elicited increases (Fig. 5C and D; $F_{3, 31}$ > 2.9, p < 0.05, prep > 0.877).

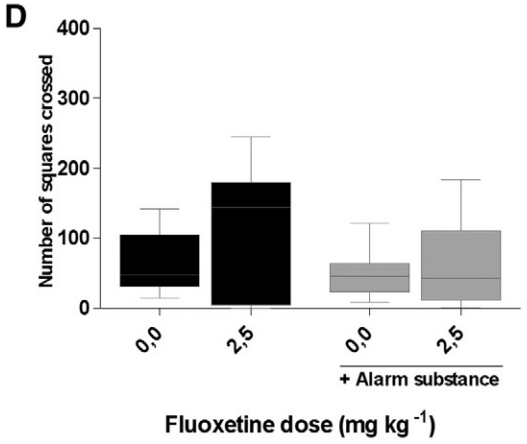
4. Discussion

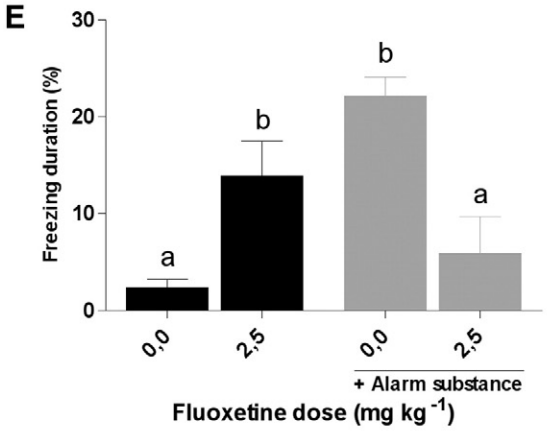
In the present work, it was demonstrated that alarm substance increases scototaxis in adult zebrafish, also increasing the latency to enter the white compartment, freezing and erratic swimming, but not risk assessment or thigmotaxis. While most papers using the light/dark test report only white avoidance (e.g., Gebauer et al., 2011; Maximino et al., 2011; Norton et al., 2011; Steenbergen et al., 2011), it

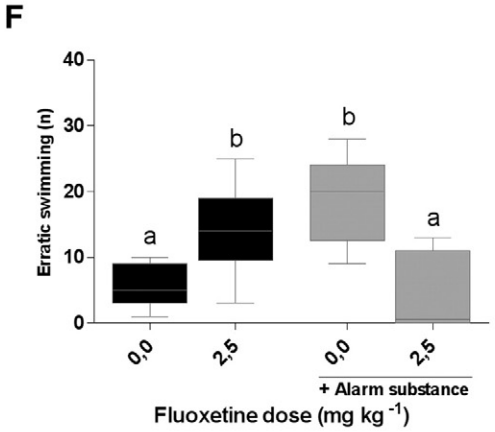

A
Time spent in white compartment (%)
100
75
50
25
20
15
10
5
0
a
b
b
a
0,0
2,5
0,0
2,5
+ Alarm substance
Fluoxetine dose (mg kg -1)

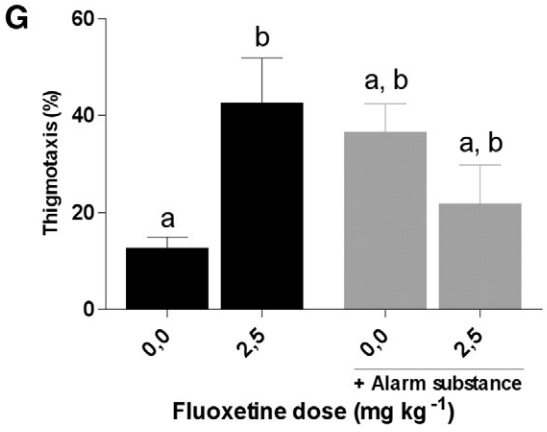

B
Control
Alarm substance
Vehicle
Fluoxetine
Percent entering white
150
100
50
0
a
b
0
100
200
300
400
Latency to white (s)

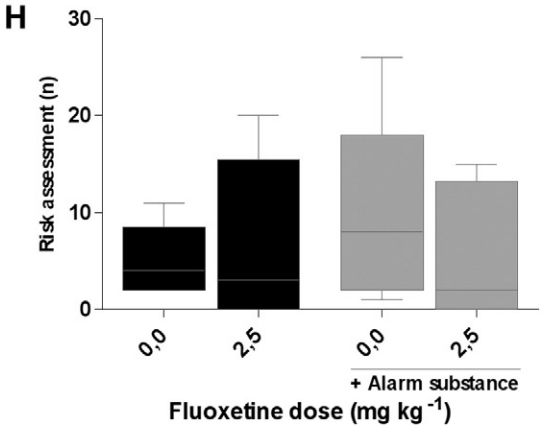

C
Number of entries in white compartment
50
40
30
20
10
0
0,0
2,5
0,0
2,5
+ Alarm substance
Fluoxetine dose (mg kg -1)


D
Number of squares crossed
400
300
200
100
0
0,0
2,5
0,0
2,5
+ Alarm substance
Fluoxetine dose (mg kg -1)


E
Freezing duration (%)
30
20
10
0
a
b
b
a
0,0
2,5
0,0
2,5
+ Alarm substance
Fluoxetine dose (mg kg -1)


F
Erratic swimming (n)
40
30
20
10
0
a
b
b
a
0,0
2,5
0,0
2,5
+ Alarm substance
Fluoxetine dose (mg kg -1)


G
Thigmotaxis (%)
60
40
20
0
a
b
a, b
a, b
0,0
2,5
0,0
2,5
+ Alarm substance
Fluoxetine dose (mg kg -1)


H
Risk assessment (n)
30
20
10
0
0,0
2,5
0,0
2,5
+ Alarm substance
Fluoxetine dose (mg kg -1)

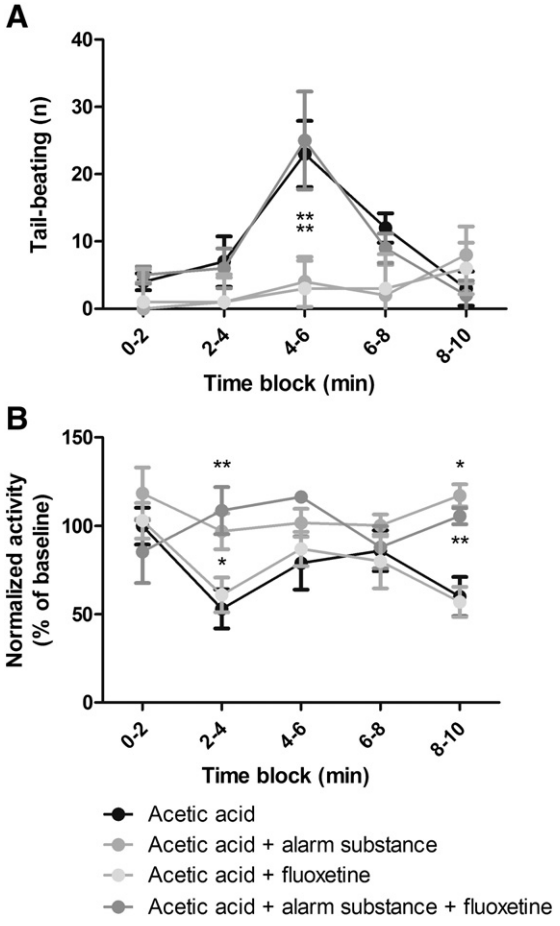


Fig. 2. Fluoxetine does not block the analgesic effect of CAS. (A) Number of tail-beating events per time bin; (B) Normalized activity. **, $p < 0.01$ vs. control; *, $p < 0.05$ vs. control.

has recently been demonstrated that risk assessment and thigmotaxis are also sensitive to serotonergic drugs (e.g., risk assessment and thigmotaxis; Maximino et al., 2013a). Moreover, zebrafish from the *leopard* phenotype, which show decreased tissue serotonin in the brain and increased serotonin metabolism (Maximino et al., 2013b) also display increased risk assessment, but not thigmotaxis. Finally, risk assessment and thigmotaxis are also decreased by anxiolytic drugs with different molecular targets, while anxiogenic drugs increase these measures (Maximino et al., in press). Overall, these results suggest that, while the light/dark test seems to model conflict-induced anxiety (Maximino et al., in press), the effects of fear-inducing stimuli such as acute alarm substance alter specific behavioral measures which are not usually affected by manipulations which increase or decrease anxiety.

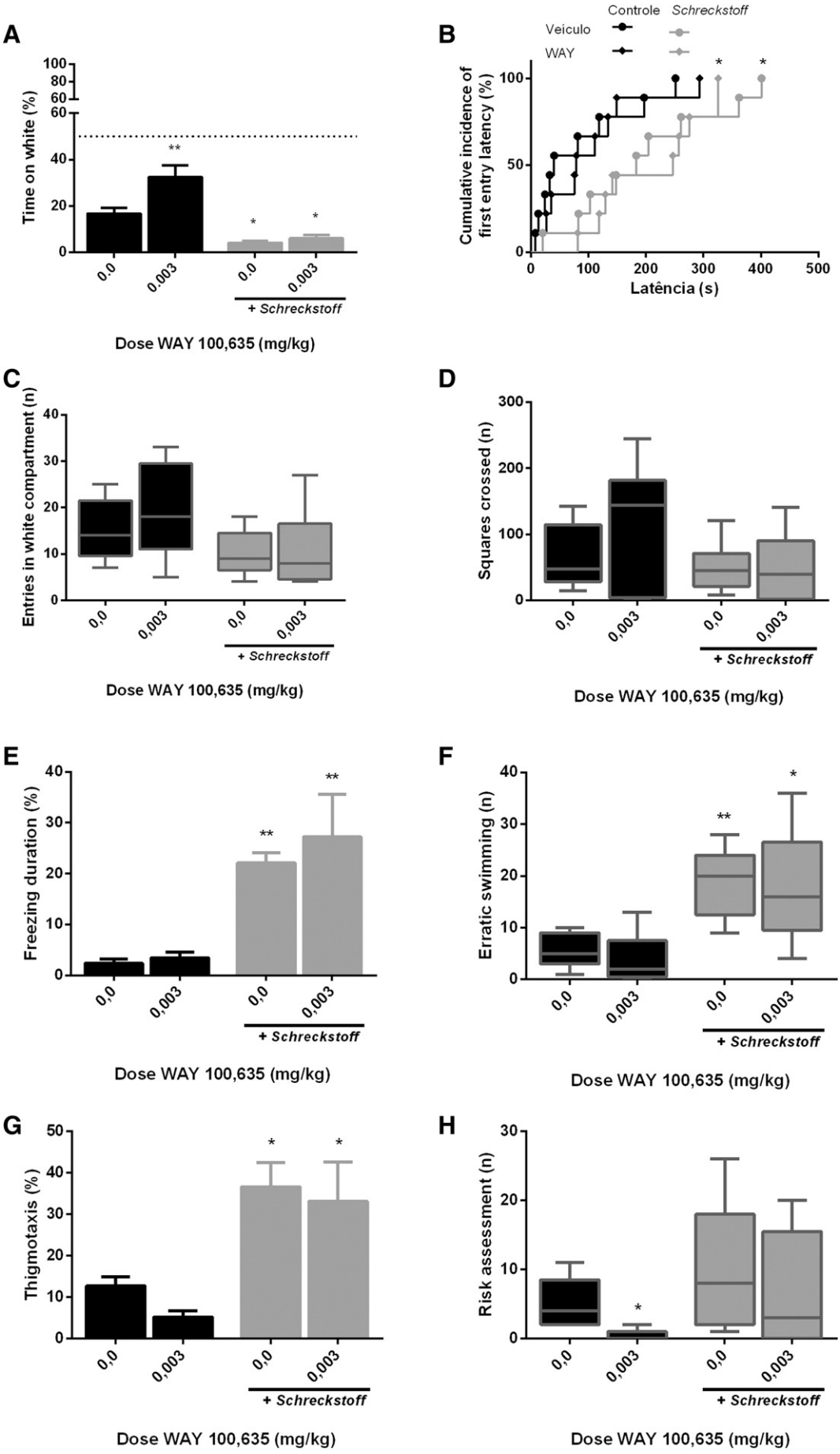
As reported elsewhere (Maximino, 2011), alarm substance also produced analgesia. Moreover, extracellular 5-HT and 5-HIAA levels in the brain were increased after exposure to *Schreckstoff* and blood glucose, hemoglobin, epinephrine and norepinephrine levels were also increased; these latter results suggest increased sympathetic activity, which can precede or parallel the increase in whole-body cortisol that is observed in these animals after CAS exposure (Schirmer et al., 2013). Fluoxetine treatment blocked the effects of alarm substance on the light/dark assay and on blood parameters, but did not block the fear-induced analgesia; conversely, WAY 100,635 did not block the effects of CAS on scototaxis or blood parameters, but blocked fear-induced analgesia. Pre-treatment with fluoxetine also blocked the alarm substance-elicited increase in 5-HT and 5-HIAA, an effect which was not observed with WAY 100,635.

The role of serotonin transporter (SERT) on the behavioral responses to fear stimuli has been investigated in rodents (Adamec et al., 2006; Wellman et al., 2007; Ravinder et al., 2011), humans (Perkins et al., 2009; Schruers et al., 2011) and fish (Beulig and Fowler, 2008; Maximino et al., 2011; Roussigné et al., 2011; Barbosa et al., 2012). In piauçu fish, pre-treatment with fluoxetine (10 mg/kg) decreases the hypoactivity induced by exposure to conspecific skin extract (Barbosa et al., 2012); the opposite effect was observed in Arabian killifish (Barry, 2013). In mice, acute fluoxetine (5 mg/kg, but not higher doses) increases escape responses in the Mouse Defense Test Battery (Blanchard et al., 1997). Interestingly, while fluoxetine by itself increased white avoidance in the scototaxis test in the present experiments, it reversed the increase caused by alarm substance. The same dose of fluoxetine was shown to decrease the exaggerated scototaxis and risk assessment seen in *leopard* zebrafish in the light/dark test (Maximino et al., 2013b). These results are consonant with the hypothesis that, in zebrafish as well as in mammals, 5-HT increases anxiety and decreases fear (Graeff et al., 1996, 1997; Zangrossi et al., 2001; Hale and Lowry, 2011; Maximino et al., in press), but suggest that the serotonergic modulation of zebrafish defensive behavior shows complexities which are not explainable solely by this 'dual role' hypothesis.

The dose of fluoxetine used in the present experiment was enough to increase extracellular 5-HT levels, but the increase induced by alarm substance was quantitatively equivalent to that caused by blocking SERT, which could indicate that this effect was mediated by an action on SERT to increase 5-HT levels. Moreover, SERT inhibition also blocked the effects of alarm substance on both 5-HT levels and scototaxis, which could indicate that this effect was mediated by an action on SERT. Whether this results from an inhibition of 5-HT transport or a reversal of uptake is yet unclear, and further studies are necessary to test this hypothesis.

While fluoxetine treatment blocked the effects of conspecific alarm substance on scototaxis, it was not able to block the antinociceptive effect of the alarm reaction. Analgesia during and after presentation of predators and partial predator stimuli has been described in rodents (Kavaliers and Douglas, 1991; Wiedenmayer and Barr, 1995) and fish (Maximino, 2011; Lima et al., 2012; Alves et al., 2013). In rodents, opioid and non-opioid components of fear-induced analgesia have been described, with the non-opioid component being mediated by GABAergic and serotonergic mechanisms (Kavaliers and Douglas, 1991). While an opioid component has been described in the piauçu fish (Alves et al., 2013), the role of serotonin has not yet been described. While fluoxetine did not block the antinociceptive effect of alarm substance, pre-treatment with the 5-HT_{1A}R antagonist WAY 100,635 had this effect. While WAY 100,635 decreased scototaxis and risk assessment by itself, it had no effect on the alarm substance-induced increase in white avoidance, latency to white, freezing, or erratic swimming. While 5-HT_{1A} partial agonists and full antagonists have been described as decreasing anxiety-like behavior in different tests and species (Zangrossi et al., 2001; Herculano and Maximino, in press), the role of these receptors in fear responses is less certain. In zebrafish, scototaxis and geotaxis are equally decreased by WAY 100,635 and buspirone (Maximino et al., 2013a). 5-HT_{1A} receptor knockout mice show normal conditional fear (Groenink et al., 2003), and 5-HT_{1A} partial agonists do not affect one-way escape in the

Fig. 1. Fluoxetine, at an anxiogenic dose, blocks the behavioral effects of alarm substance on the light/dark test. (A) Time spent on the white compartment; (B) Latency to enter the white compartment; (C) Number of entries in the white compartment; (D) Number of squares crossed in the white compartment; (E) Total duration of freezing events in the white compartment; (F) Frequency of erratic swimming events in the white compartment; (G) Proportion of time on white spent in thigmotaxis; (H) Frequency of risk assessment events. Black bars represent animals exposed to water, and gray bars animals exposed to conspecific alarm substance (CAS). Different letters indicate statistically significant differences.


A
Time on white (%)
100
80
60
60
40
20
0
**
*
*
0,0
0,003
0,0
0,003
+ Schreckstoff
Dose WAY 100,635 (mg/kg)
B
Controle
Schreckstoff
Veiculo
WAY
Cumulative incidence of first entry latency (%)
100
50
0
*
*
0
100
200
300
400
500
Latência (s)
C
Entries in white compartment (n)
40
30
20
10
0
0,0
0,003
0,0
0,003
+ Schreckstoff
Dose WAY 100,635 (mg/kg)
D
Squares crossed (n)
300
200
100
0
0,0
0,003
0,0
0,003
+ Schreckstoff
Dose WAY 100,635 (mg/kg)
E
Freezing duration (%)
40
30
20
10
0
**
**
0,0
0,003
0,0
0,003
+ Schreckstoff
Dose WAY 100,635 (mg/kg)
F
Erratic swimming (n)
40
30
20
10
0
**
*
0,0
0,003
0,0
0,003
+ Schreckstoff
Dose WAY 100,635 (mg/kg)
G
Thigmotaxis (%)
50
40
30
20
10
0
*
*
0,0
0,003
0,0
0,003
+ Schreckstoff
Dose WAY 100,635 (mg/kg)
H
Risk assessment (n)
30
20
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0,0
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0,003
+ Schreckstoff
Dose WAY 100,635 (mg/kg)

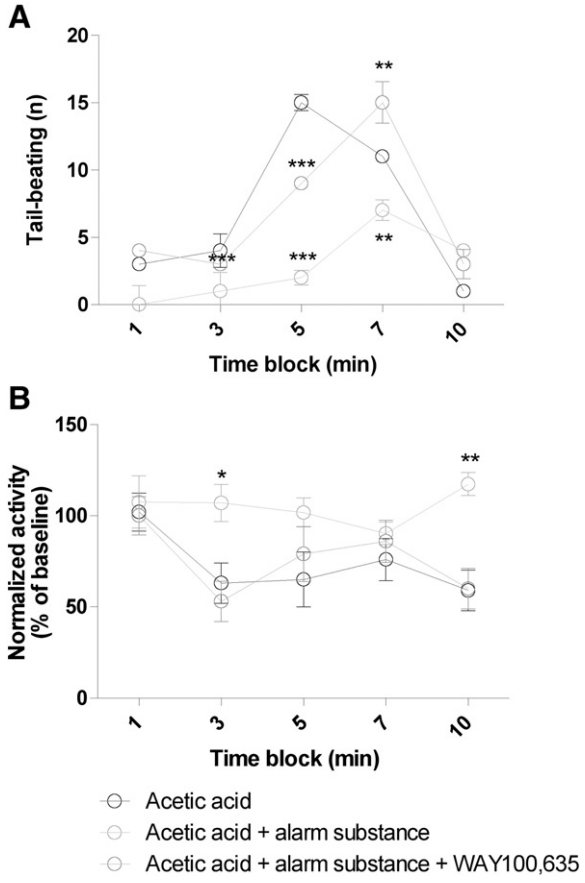

Fig. 4. WAY 100,635 blocks the analgesic effect of CAS. (A) Number of tail-beating events per time bin; (B) Normalized activity. ***, $p < 0.001$ vs. control; **, $p < 0.01$ vs. control; *, $p < 0.05$ vs. control.

rat elevated T-maze (Zangrossi et al., 2001). Nonetheless, the 5-HT_{1A} full agonist 8-OH-DPAT decreases the threshold for periaqueductal gray stimulation-induced escape responses (Jenck et al., 1989).

From a pharmacological point of view, 5-HT_{1A} partial agonists effectively act as full antagonists at postsynaptic sites and full agonists at presynaptic sites. In rats, microinjection of 8-OH-DPAT in the amygdala does not block stress-induced analgesia (Nunes-de-Souza et al., 2000), but decreases one-way escape in the elevated T-maze (Guimarães et al., 2010); reduction of one-way escape is also observed with microinjection in the periaqueductal gray, while microinjection of 8-OH-DPAT in the hippocampus and lateral septum increases inhibitory avoidance in the elevated T maze (Guimarães et al., 2010). Since 5-HT levels were not altered by WAY 100,635 in the present experiments, it is suggested that postsynaptic 5-HT_{1A} receptors mediate fear-induced analgesia, but not other behavioral effects of alarm substance.

Fluoxetine treatment also blocked the sympathetic burst elicited by CAS, while WAY 100,635 had no such effect. These results suggest that the fear-like reaction to CAS is mediated by a serotonin-induced sympathetic activation, and that this reaction is not mediated by the $5HT_{1A}$ receptor. These results are consistent with the observation that treatment with WAY 100,635 reduces conditioned ultrasonic vocalizations in rats without altering plasmatic ACTH, corticosterone, prolactin or glucose levels (Groenink et al., 1996). They are also consistent with the observation that footshock-induced analgesia is independent on sympathetic nervous system activity (Watkins et al., 1982).

Overall, these results suggest that, in zebrafish, fear-induced increases in defensive behavior and sympathetic activation are mediated by the serotonin transporter, while fear-induced analgesia is mediated by the 5-HT_{1A} receptor. The precise site of action of these drugs, however, awaits further experiments. The present work underlies the evolutionary conservation of the role of serotonin in fear-like behavior in vertebrates, opening avenues for future investigations in the genetic underpinnings of these functions.

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Table 1
Fluoxetine, but not WAY 100,635, blocks sympathetic activation elicited by CAS, as measured by blood catecholamine (epinephrine and norepinephrine), hemoglobin and glucose levels.

Parameter	Control	CAS	Drug	CAS + drug	Statistical analysis
Fluoxetine					
Blood glucose (mg/dl)	50.3 ± 16.25	121.8 ± 48.6**	48.1 ± 16.09	75.3 ± 19.21*	$F_{3,15} = 9.052$, $p = 0.0021$
Hemoglobin (mmol/l)	4.4 ± 1.4	8.3 ± 0.9**	4.32 ± 0.3	6.7 ± 0.7*	$F_{3,15} = 29.14$, $p < 0.0001$
Norepinephrine (pg/ml)	803.4 ± 82.7	1245.7 ± 128.3**	851.5 ± 87.7	769.7 ± 79.3	$F_{3,15} = 11.83$, $p = 0.0007$
Epinephrine (pg/ml)	400.1 ± 120.1	1015.1 ± 123.7***	357.4 ± 103.1	451.1 ± 58.1	$F_{3,15} = 29.53$, $p < 0.0001$
WAY 100,635					
Blood glucose (mg/dl)	51.3 ± 3.1	132.1 ± 23.1**	57.5 ± 8.3	142.1 ± 31.2**	$F_{3,15} = 17.54$, $p = 0.0001$
Hemoglobin (mmol/l)	4.8 ± 0.3	9.1 ± 1.3***	3.9 ± 1.5	10.2 ± 0.8***	$F_{3,15} = 29.9$, $p < 0.0001$
Norepinephrine (pg/ml)	798.7 ± 81.1	1232.1 ± 118.3***	815.1 ± 58.9	1313.6 ± 129.4***	$F_{3,15} = 54.4$, $p < 0.0001$
Epinephrine (pg/ml)	403.5 ± 98.7	1021.9 ± 98.1***	420.1 ± 101.7	997.1 ± 81.4***	$F_{3,15} = 42.24$, $p < 0.0001$

*** $p < 0.001$ vs. control.
** $p < 0.01$ vs. control.
* $p < 0.05$ vs. control.

Fig. 3. WAY 100,635, at an anxiolytic dose, does not block the behavioral effects of CAS on the light/dark test. (A) Time spent on the white compartment; (B) Latency to enter the white compartment; (C) Number of entries in the white compartment; (D) Number of squares crossed in the white compartment; (E) Total duration of freezing events in the white compartment; (F) Frequency of erratic swimming events in the white compartment; (G) Proportion of time on white spent in thigmotaxis; (H) Frequency of risk assessment events. Black bars represent animals exposed to water, and gray bars animals exposed to conspecific alarm substance (CAS). Different letters indicate statistically significant differences.

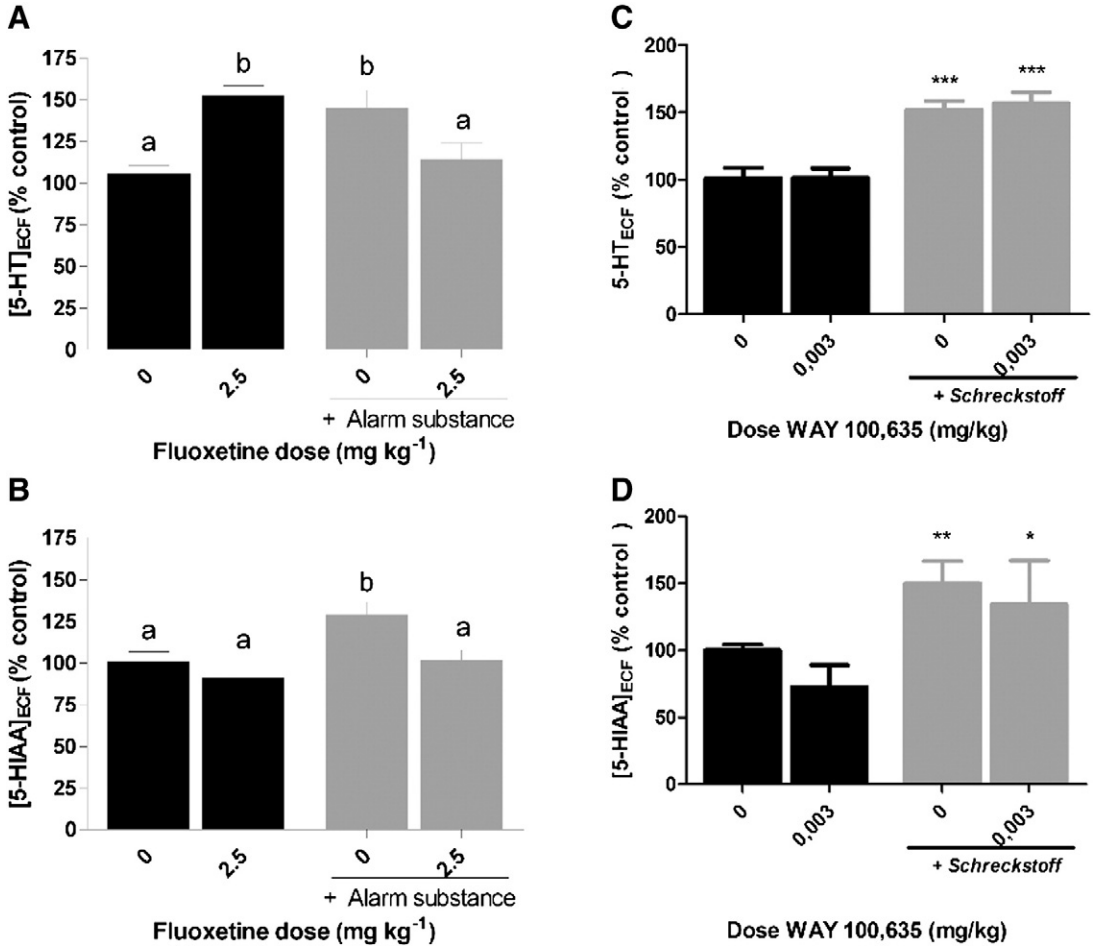

Fig. 5. Fluoxetine, but not WAY 100,635, blocks the CAS-elicited increases in extracellular serotonin in the brain. Extracellular 5-HT levels in water- (black bars) and CAS-exposed (gray bars) animals treated with (A) fluoxetine or (C) WAY 100,635; extracellular 5-HIAA levels in water- (black bars) and CAS-exposed (gray bars) animals treated with (B) fluoxetine or (D) WAY 100,635.

References

Adamec R, Burton P, Blundell J, Murphy DL, Holmes A. Vulnerability to mild predator stress in serotonin transporter knockout mice. Behav Brain Res 2006;170:126–40.

Alves FL, Barbosa Jr A, Hoffmann A. Antinociception in piauçu fish induced by exposure to the conspecific alarm substance. Physiol Behav 2013;110–111:58–62.

Anichtchik O, Sallinen V, Peitsaro N, Panula P. Distinct structure and activity of monoamine oxidase in the brain of zebrafish (*Danio rerio*). J Comp Neurol 2006;610:593–610.

Araujo J, Maximino C, de Brito TM, da Silva AWB, Batista Ede JO, Oliveira KRM, et al. Behavioral and pharmacological aspects of anxiety in the light/dark preference test. In: Kalueff AV, Stewart AM, editors. Zebrafish protocols for neurobehavioral research. New York: Humana Press; 2012.

Babaei F, Ramalingam R, Tavendale A, Liang Y, Yan LSK, Ajuh P, et al. Novel blood collection method allows plasma proteome analysis from single zebrafish. J Proteome Res 2013;12:1580–90.

Barbosa Jr A, Alves FL, Pereira Ade SF, Ide LM, Hoffmann A. Behavioral characterization of the alarm reaction and anxiolytic-like effect of acute treatment with fluoxetine in piauçu fish. Physiol Behav 2012;105:784–90.

Barry MJ. Effects of fluoxetine on the swimming and behavioural responses of the Arabian killifish. Ecotoxicology 2013;22(2):425–32.

Beulig A, Fowler J. Fish on Prozac: effect of serotonin reuptake inhibitors on cognition in goldfish. Behav Neurosci 2008;122:426–32.

Blanchard RJ, Griebel G, Henrie JA, Blanchard DC. Differentiation of anxiolytic and panicolytic drugs by effects on rat and mouse defense test batteries. Neurosci Biobehav Rev 1997;21:783–9.

Blaser RE, Goldsteinholm K. Depth preference in zebrafish, *Danio rerio*: control by surface and substrate cues. Anim Behav 2012;83:953–9.

Blaser RE, Peñalosa YM. Stimuli affecting zebrafish (*Danio rerio*) behavior in the light/dark preference test. Physiol Behav, 104. Elsevier Inc.; 2011. p. 831–7.

Blaser RE, Chadwick L, McGinnis GC. Behavioral measures of anxiety in zebrafish (*Danio rerio*). Behav Brain Res 2010;208:56–62.

Correia AD, Cunha SR, Scholze M, Stevens ED. A novel behavioral fish model of nociception for testing analgesics. Pharmaceuticals 2011;4:665–80.

Deakin JFW, Graeff FG. 5-HT and mechanisms of defense. J Psychopharmacol 1991;5:305–15.

Døving KB, Lastein S. The alarm reaction in fishes — odorants, modulation of responses, neural pathways. Ann N Y Acad Sci 2009;1170:413–23.

Egan RJ, Bergner CL, Hart PC, Cachat JM, Canavello PR, Elegante MF, et al. Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. Behav Brain Res 2009;205:38–44.

Gayoso JÁ, Castro A, Anadón R, Manso MJ. Differential bulbar and extrabulbar projections of diverse olfactory receptor neuron populations in the adult zebrafish (*Danio rerio*). J Comp Neurol 2011;519:247–76.

Gayoso JÁ, Castro A, Anadón R, Manso MJ. Crypt cells of the zebrafish *Danio rerio* mainly project to the dorsomedial glomerular field of the olfactory bulb. Chem Senses 2012; 37:357–69.

Gebauer DL, Pagnussat N, Piato ÂL, Schaefer IC, Bonan CD, Lara DR. Effects of anxiolytics in zebrafish: Similarities and differences between benzodiazepines, buspirone and ethanol. Pharmacol Biochem Behav 2011;99:480–6.

Gerlai R. Zebrafish antipredatory responses: a future for translational research? Behav Brain Res 2010;207:223–31.

Graeff FG, Guimarães FS, De Andrade TGCS, Deakin JFW. Role of 5-HT in stress, anxiety, and depression. Pharmacol Biochem Behav 1996;54:129–41.

Graeff FG, de Viana MB, Mora PO. Dual role of 5-HT in defense and anxiety. Neurosci Biobehav Rev 1997;21:791–9.

Gray JA, McNaughton N. The neuropsychology of anxiety: an enquiry into the functions of the septo-hippocampal system. 2nd ed. Oxford: Oxford University Press; 2000.

Groenink L, Mos J, van der Gugten J, Olivier B. The 5-HT_{1A} receptor is not involved in emotional stress-induced rises in stress hormones. Pharmacol Biochem Behav 1996;55:303–8.

Groenink L, Pattij T, De Jongh R, Van der Gugten J, Oosting RS, Dirks A, et al. 5-HT_{1A} receptor knockout mice and mice overexpressing corticotropin-releasing hormone in models of anxiety. Eur J Pharmacol 2003;463:185–97.

Guimarães FS, Zangrossi Jr H, Del-Ben CM, Graeff FG. Serotonin in panic and anxiety disorders. In: Müller C, Jacobs B, editors. Handb behav neurobiol serotonin. Amsterdam: Elsevier B. V.; 2010. p. 667–85.

Hale MW, Lowry CA. Functional topography of midbrain and pontine serotonergic systems: implications for synaptic regulation of serotonergic circuits. Psychopharmacology 2011; 213:243–64.

Hamdani E, Stabell OB, Alexander G, Døving KB. Alarm reaction in the crucian carp is mediated by the medial bundle of the medial olfactory tract. Chem Senses 2000;25:103–9.
Herculano AM, Maximino C. Serotonergic modulation of zebrafish behavior: towards a paradox. Prog Neuropsychopharmacol Biol Psychiatry 2014. (in press).
Jahn-Eimermacher A, Lasarzik I, Raber J. Statistical analysis outcomes in behavioral experiments. Behav Brain Res 2011;221:271–5.
Jenck F, Broekkamp CL, Van Delft AM. Opposite control mediated by central 5-HT1A and non-5HT1A (5-HT1B or 5-HT1C) receptors on periaqueductal gray aversion. Eur J Pharmacol 1989;161:219–21.
Jesuthasan SJ, Mathuru AS. The alarm response in zebrafish: innate fear in a vertebrate genetic model. J Neurogenet 2008;22:211–29.
Kalueff AV, Stewart AM, Kyzar EJ, Cachat J, Gebhardt M, Landsman S, et al. Time to recognize zebrafish "affective" behavior. Behaviour 2012;149:1019–36.
Kaslin J, Panula P. Comparative anatomy of the histaminergic and other aminergic systems in zebrafish (*Danio rerio*). J Comp Neurol 2001;440:342–77.
Kavaliers M, Douglas DC. Sex differences in opioid and non-opioid mediated predator-induced analgesia in mice. Brain Res 1991;568:173–7.
Killeen PR. An alternative to null-hypothesis significance tests. Psychol Sci 2005;16:345–53.
Lillesaar C, Stigloher C, Tannhäuser B, Wullimann MF, Bally-Cuif L. Axonal projections originating from raphe serotonergic neurons in the developing and adult zebrafish, *Danio rerio*, using transgenics to visualize raphe-specific pet1 expression. J Comp Neurol 2009;512:158–82.
Lima MG, Maximino C, Batista Ede JO, Oliveira KRM, Herculano AM. Nocifensive behavior in adult and larval zebrafish. In: Kalueff AV, Stewart AM, editors. Zebrafish protocols for neurobehavioral research. New York: Humana Press; 2012.
Luca RM, Gerlai R. In search of optimal fear inducing stimuli: differential behavioral responses to computer animated images in zebrafish. Behav Brain Res 2012;226:66–76.
Mathuru AS, Kibat C, Cheong WF, Shui G, Wenk MR, Friedrich RW, et al. Chondroitin fragments are odorants that trigger fear behavior in fish. Curr Biol 2012;22:538–44.
Maximino C. Modulation of nociceptive-like behavior in zebrafish (*Danio rerio*) by environmental stressors. Psychol Neurosci 2011;4:149–55.
Maximino C, de Brito TM, Colmanetti R, Pontes AAA, de Castro HM, de Lacerda RIT, et al. Parametric analyses of anxiety in zebrafish scototaxis. Behav Brain Res 2010;210:1–7.
Maximino C, de Silva AWB, Gouveia Jr A, Herculano AM. Pharmacological analysis of zebrafish (*Danio rerio*) scototaxis. Prog Neuro-Psychopharmacol Biol Psychiatry 2011;35(2):624–31.
Maximino C, Puty B, Benzecry R, Araújo J, Lima MG, Batista EJO, et al. Role of serotonin in zebrafish (*Danio rerio*) anxiety: relationship with serotonin levels and effect of buspirone, WAY 100635, SB 224289, fluoxetine and *para*-chlorophenylalanine (pCPA) in two behavioral models. Neuropharmacology 2013a;71:83–97.
Maximino C, Puty B, Oliveira KRM, Herculano AM. Behavioral and neurochemical changes in the zebrafish leopard strain. Genes Brain Behav 2013b;12:576–82.
Maximino C, da Silva AWB, Araújo J, Lima MG, Miranda V, Puty B, et al. Fingerprinting of psychoactive drugs in zebrafish anxiety-like behaviors. PLoS ONE 2014. (in press).
McNaughton N, Corr PJ. A two-dimensional neuropsychology of defense: fear/anxiety and defensive distance. Neurosci Biobehav Rev 2004;28:285–305.
Norton WHJ, Folchert A, Bally-Cuif L. Comparative analysis of serotonin receptor (*HTR1A*/*HTR1B* families) and transporter (*slc6a4a*/*b*) gene expression in the zebrafish brain. J Comp Neurol 2008;511:521–42.
Norton WHJ, Stumpenhorst K, Faus-Kessler T, Folchert A, Rohner N, Harris MP, et al. Modulation of Fgfr1a signaling in zebrafish reveals a genetic basis for the aggression-boldness syndrome. J Neurosci 2011;31:13796–807.
Nunes-de-Souza RL, Canto-de-Souza A, Da-Costa M, Fornari RV, Graeff FG, Pelá IR. Anxiety-induced antinociception in mice: effects of systemic and intra-amygdala administration of 8-OH-DPAT and midazolam. Psychopharmacology 2000;150:300–10.
Perkins AM, Ettinger U, Davis R, Foster R, Williams SCR, Corr PJ. Effects of lorazepam and citalopram on human defensive reactions: ethopharmacological differentiation of fear and anxiety. J Neurosci 2009;29:12617–24.
Pradel G, Schachner M, Schmidt R. Inhibition of memory consolidation by antibodies against cell adhesion molecules after active avoidance conditioning in zebrafish. J Neurobiol 1999;39:197–206.
Ravinder S, Pillai AG, Chattarji S. Cellular correlates of enhanced anxiety caused by acute treatment with the selective serotonin reuptake inhibitor fluoxetine in rats. Front Behav Neurosci 2011;5. (Article 88).
Roussigné M, Blader P, Wilson SW. Breaking symmetry: the zebrafish as a model for understanding left–right asymmetry in the developing brain. Dev Neurobiol 2011;72:269–81.
Sackerman J, Donegan JJ, Cunningham CS, Nguyen NN, Lawless K, Long A, et al. Zebrafish behavior in novel environments: effects of acute exposure to anxiolytic compounds and choice of *Danio rerio* line. Int J Comp Psychol 2010;23(1):43–61.
Schirmer A, Jesuthasan S, Mathuru AS. Tactile stimulation reduces fear in fish. Front Behav Neurosci 2013;7. (Article 167).
Schneider H, Fritzky L, Williams J, Heumann C, Yochum M, Pattar K, et al. Cloning and expression of a zebrafish 5-HT_{2C} receptor gene. Gene 2012;502:108–17.
Schruers K, Esquivel G, Van Duinen M, Wichers M, Kenis G, Colasanti A, et al. Genetic moderation of CO_2-induced fear by 5-HTTLPR genotype. J Psychopharmacol 2011;25:37–42.
Speedie N, Gerlai R. Alarm substance induced behavioral responses in zebrafish (*Danio rerio*). Behav Brain Res 2008;188:168–77.
Steenbergen PJ, Richardson MK, Champagne DL. Patterns of avoidance behaviours in the light/dark preference test in young juvenile zebrafish: a pharmacological study. Behav Brain Res 2011;222:15–25.
Stewart A, Wu N, Cachat J, Hart P, Gaikwad S, Wong K, et al. Pharmacological modulation of anxiety-like phenotypes in adult zebrafish behavioral models. Prog Neuropsychopharmacol Biol Psychiatry 2011;35:1421–31.
Watkins LR, Cobelli DA, Newsome HH, Mayer DJ. Footshock induced analgesia is dependent neither on pituitary nor sympathetic activation. Brain Res 1982;245:81–96.
Wellman CL, Izquierdo A, Garrett JE, Martin KP, Carroll J, Millstein R, et al. Impaired stress-coping and fear extinction and abnormal corticolimbic morphology in serotonin transporter knock-out mice. J Neurosci 2007;27:684–91.
Wiedenmayer CP, Barr GA. Developmental changes in responsivity to threat are stimulus-specific in rats. Dev Psychobiol 1995;39:1–7.
Zangrossi Jr H, de Viana MB, Zanoveli J, Bueno C, Nogueira RL, Graeff FG. Serotonergic regulation of inhibitory avoidance and one-way escape in the rat elevated T-maze. Neurosci Biobehav Rev 2001;25:637–45.
Zor T, Selinger Z. Linearization of the Bradford protein assay increases its sensitivity: theoretical and experimental studies. Anal Biochem 1996;236:302–8.