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Serotonergic modulation of zebrafish behavior: Towards a paradox

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ABSTRACT

Due to the fish-specific genome duplication event (~320–350 mya), some genes which code for serotonin proteins were duplicated in teleosts; this duplication event was preceded by a reorganization of the serotonergic system, with the appearance of the raphe nuclei (dependent on the isthmus organizer) and prosencephalic nuclei, including the paraventricular and pretectal complexes. With the appearance of amniotes, duplicated genes were lost, and the serotonergic system was reduced to a more complex raphe system. From a comparative point of view, then, the serotonergic system of zebrafish and that of mammals shows many important differences. However, many different behavioral functions of serotonin, as well as the effects of drugs which affect the serotonergic system, seem to be conserved among species. For example, in both zebrafish and rodents acute serotonin reuptake inhibitors (SSRIs) seem to increase anxiety-like behavior, while chronic SSRIs decrease it; drugs which act at the 5-HT_{1A} receptor seem to decrease anxiety-like behavior in both zebrafish and rodents. In this article, we will expose this paradox, reviewing the chemical neuroanatomy of the zebrafish serotonergic system, followed by an analysis of the role of serotonin in zebrafish fear/anxiety, stress, aggression and the effects of psychedelic drugs.

1. Introduction

Serotonin (5-hydroxytryptamine, 5-HT) has been proposed to have a plethora of functions in vertebrates, including the control of defensive behavior (Maximino, 2012), the control of sympathetic outflow and the hypothalamus–pituitary–adrenal axis (Lowry, 2002), immunomodulation (Baganz and Blakely, 2013; Khan and Deschaux, 1997), and aggression (Carrillo et al., 2009; Takahashi et al., 2011). These functions have usually been studied largely in mammalian species. With the advent of teleost species, including zebrafish, as important model organisms in the neurosciences (Rinkwitz et al., 2011), however, a paradox begun to shape: while

Abbreviations: 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, Serotonin, 5-hydroxytryptamine; 5-HTP, 5-hydroxytryptophan; 8-OH-DPAT, 7-(Dipropylamino)-5,6,7,8-tetrahydronaphtalen-1-ol; AADC, Aromatic l-amino-acid decarboxylase (EC 4.1.1.28); ACTH, Adrenocorticotropic hormone, corticotropin; AP, Area postrema; *bdnf*, Brain-derived neurotrophic factor; BSF, Blue shortfin wild-type zebrafish; cAMP, 3′,5′-Cyclic adenosine monophosphate; *crh*, *crf*, Corticotropin-releasing hormone; CUS, Chronic unpredictable stress; dpf, Days post-fertilization; DRN, Dorsal raphe nucleus; *etv5b*, ETS variant 5b, erm; fezf2, FEZ family zinc finger 2; *tof*, *fezl*,, Forebrain embryonic zinc finger-like protein 2; GBT, Group behavior task; GC, Griseum centrale, central gray; GR, Glucocorticoid receptor; GR 125,487, 5-Fluoro-2-methoxy-[1-[2-[(methylsulfonyl)amino]ethyl]-4-piperidinyl]-1*H*-indole-3-methylcarboxylate sulfamate; Ha, Anterior paraventricular hypothalamus; Hc, Caudal paraventricular hypothalamus; HEK293, Human Embryonic Kidney 293 cells; HEK293-MSR, HEK293 cells expressing the human macrophage scavenger receptor; Hi, Intermediate paraventricular hypothalamus; hpf, Hours post-fertilization; HPI, Hypothalamus–pituitary–interrenal; HSB, High Stationary Behavior zebrafish line; IC_{50}, Half maximal inhibitory concentration; IR, Inferior raphe; K_D, Dissociation constant at equilibrium; K_m, Michaelis–Menten constant; LDT, Light/dark test; LFS, Longfin stripped wild-type zebrafish; *lmx1b*, LIM homeobox transcription factor 1β; LSD, Lysergic acid diethylamide, (6aR,9R)- *N,N*- diethyl- 7-methyl-4,6,6a,7,8,9- hexahydroindolo–[4,3-fg] quinoline- 9-carboxamide; MAO, l-Monoamine oxidase (EC 1.4.3.4); MC-LR, Microcystin-LR; MDMA, 3,4-Methylenedioxy-*N*-methylamphetamine, (*RS*)-1-(benzo[*d*][1,3]dioxol-5-yl)-*N*-methylpropan-2-amine; MiD3cm, Mauthner cell homologue MiD3cm; MK-801, Dizocilpine, [5R,10S]-[+]-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine; MPTP, 1-Methyl-4-phenyl-1,2,5,6-tetrahydropyridine; *mr*, Mineralocorticoid receptor; NAN-190, 1-(2-Methoxyphenyl)-4-(4-phthalimidobutyl)piperazine; NE, Norepinephrine; NMDA, *N*-methyl-d-aspartic acid; NOS-1, Nitric oxide synthase isoform 1; *npy*, Neuropeptide Y; NTT, novel tank test, Novel tank diving test; OCT-3, Organic cation transporter 3, extraneuronal monoamine transporter, solute carrier family 22, member 3; OFT, Open-field test; *oxtl*, Oxytocin-like; p.o, *Per os*; PCP, Phenylcyclidine, 1-(1-phenylcyclohexyl)piperidine; pCPA, *para*-Chlorophenylalanine; *pet1*, ETS domain-containing transcription factor 1, FEV; PMAT, Plasma membrane monoamine transporter, equilibrative nucleoside transporter 4, ENT4, solute carrier family 29, member 4; *pomca*, Pro-opiomelanocortin isoform A; *prl2*, Prolactin isoform 2; Rd, Dorsal raphe nucleus; Rm, Medial raphe nucleus; SB 224,289, 1′-Methyl-5-[[2′-methyl-4′-(5-methyl-1,2,4-oxadiazol-3-yl)biphenyl-4-yl]carbonyl]-2,3,6,7-tetrahydrospiro[furo[2,3-*f*]indole-3,4'-piperidinehydrochloride; SERT, Serotonin transporter, 5-HTT, solute carrier family 6 (neurotransmitter transporter), member 4; SIN-1, 3-Morpholinosydnonimine, 5-imino-3-morpholin-4-yl-5*H*-1,2,3-oxadiazol-3-ium-2-ide; SR, Superior raphe; SSRI, Selective serotonin reuptake inhibitor; TH, Tyrosine hydroxylase, tyrosine 3-monooxygenase (EC 1.14.16.2); TPH, Tryptophan hydroxylase, tryptophan 5-monooxygenase (EC 1.14.16.4); *ucn3l*, Urocortin-like isoform 3; UH-301, (S)-5-Fluoro-8-hydroxy-2-(dipropylamino)tetralin; VMAT2, Vesicular monoamine transporter 2, solute carrier family 18 (vesicular monoamine), member 2; WAY 100,635, *N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-(2-pyridyl)cyclohexanecarboxamide; ZBC, Zebrafish Behavior Catalog.

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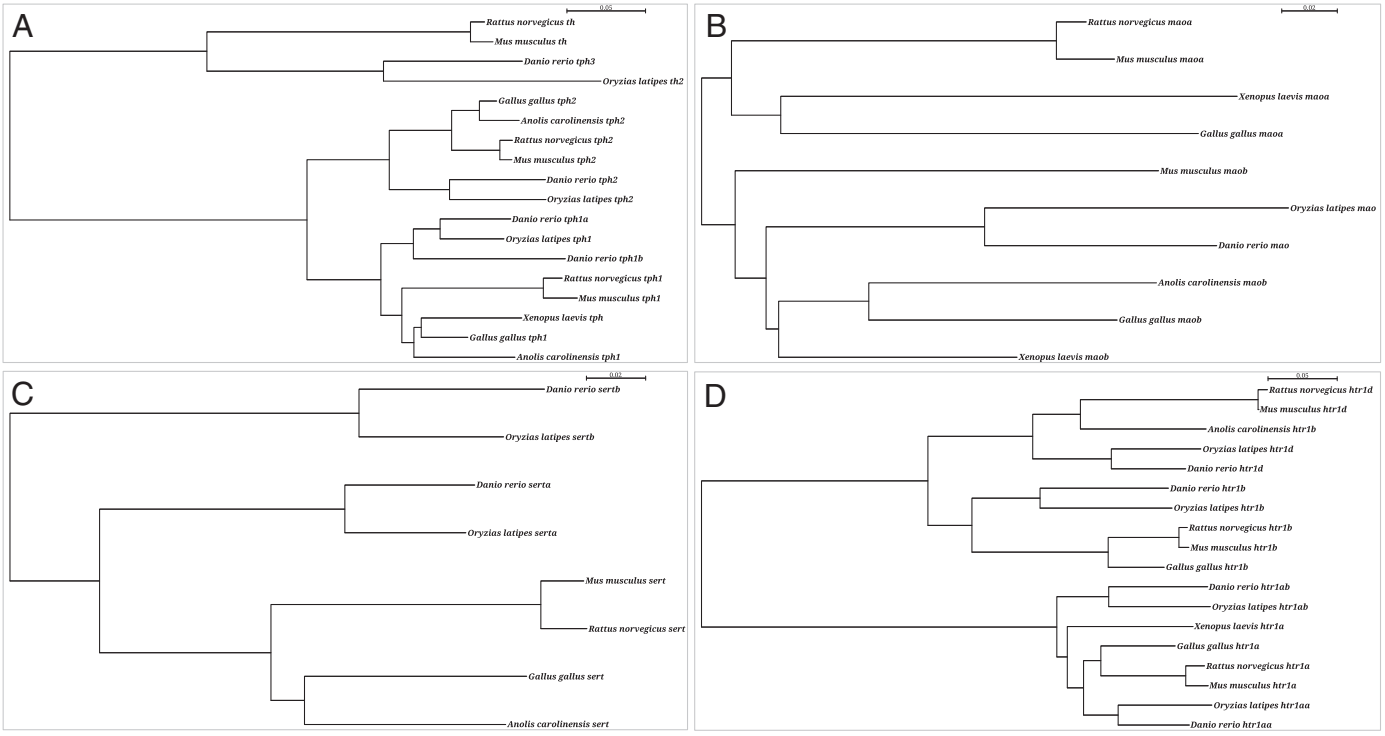

Fig. 1. Phylogenetic trees of selected genes from the serotonergic system in model organisms. (A) Tryptophan hydroxylase; (B) monoamine oxidase; (C) serotonin transporter; (D) 5-HT_{1A} and 5-HT_{1B} receptors. Trees were generated with the Neighbor-Joining with Poisson distances and 100 bootstrapped replicates.

it seems that most of the behavioral functions of serotonin, as well as the effects of drugs which act on that system, seem to be very well conserved, the degree of evolutionary conservation at the genomic and neuroanatomic level is much smaller. In this article, we will expose this paradox, reviewing the chemical neuroanatomy of the zebrafish serotonergic system, followed by an analysis of the role of serotonin in zebrafish fear/anxiety, stress, aggression and the effects of psychedelic drugs.

In order to increase comparability between studies, two strategies were used. First, to facilitate comparison between studies, behavioral variables were accompanied by their code in the Zebrafish Behavior Catalog, v. 1.0 (Kalueff et al., 2013). Second, since in waterborne treatments the unit used to report exposure concentrations varies from molarity to weight per volume, spoiling the comparison of results, all concentrations reported in this article are on the molarity scale.

2. Chemical neuroanatomy of the zebrafish serotonergic system

Early in the ray-finned fish radiation (~320–350 million years ago), prior to or coinciding with the appearance of teleost fishes, a whole-genome duplication event took place, the so-called fish-specific genome duplication (FSGD) or 3R event (Christoffels et al., 2004). Many of the duplicated genes were kept in zebrafish and closely-related teleosts, sometimes termed 'ohnologues' in deference to Ohno (1970), the first proposer of the FSGD. The significance of this event for the evolutionary history of teleosts remains elusive, with some authors proposing the possibility of neofunctionalization (Rastogi and Liberles, 2005), while others propose that the FSGD and subsequent gene loss or differential paralogue evolution in divergent populations can increase speciation (Semon and Wolfe, 2007). Naturally, some genes in the serotonin pathway are duplicated (Fig. 1). In zebrafish, the serotonin transporter and the 5-HT_{1A} receptor present ohnologues (Norton et al., 2008; Wang et al., 2006), while monoamine oxidase has only one isoform (Setini et al., 2005). Tryptophan hydroxylase 1 is duplicated, while tryptophan hydroxylase 2 exists in a single form (Bellipanni et al., 2002; Teraoka et al., 2009). Interestingly, the gene which was previously identified as coding an ohnologue of tyrosine hydroxylase actually encodes for a third tryptophan hydroxylase isoform, albeit its sequence is more similar to that of tyrosine hydroxylase than that of any tryptophan hydroxylase isoform (Ren et al., 2013). That might represent an important example of neofunctionalization of an ohnologue. This lability of the serotonergic system is not exclusive to fishes; an analysis of serotonin genes demonstrated that, while there are no signs of positive or negative selection in rodents and primates (suggesting a functional constraint as the main driving force of the evolution of these genes), considerable heterogeneity in the rate of protein evolution was observed within and between these clades (Andrés et al., 2007).

This duplication event was preceded by a reorganization of the serotonergic system. In the ascidian tunicate tadpole, serotonergic neurons are found only in the hindbrain, while in amphioxus larvae they are found in the forebrain and hindbrain (Candiani et al., 2012); while this situation may resemble that found in Actinopterygii, the existence of forebrain serotonergic nuclei in the amphioxus is probably an apomorphism due to the absence of a midbrain–hindbrain organizer in protochordates (Butler and Hodos, 2005). In the sea lamprey, serotonin-like immunoreactivity is found in the pretectal area, zona limitans intrathalamica, tuberal and mammillary hypothalamus, isthmus and vagal group, as well as in the spinal cord (Barreiro-Iglesias et al., 2009; Cornide-Petronio et al., 2013); these populations are roughly equivalent to the nuclei found in basal actinopterygian fish (López and González, 2014) and teleosts (Lillesaar, 2011; Maximino et al., 2013a). Thus, the ancestral state of the vertebrate serotonergic system is characterized by well-defined nuclei in the raphe nuclei, the preoptic area and the basal hypothalamus (Lillesaar, 2011; López and González, 2014; Maximino et al., 2013a); in amniotes, this system is reduced, as *bona fide* 5-HTergic cells are found only in the retina, pineal and raphe nuclei of these species (Hale and Lowry, 2011).

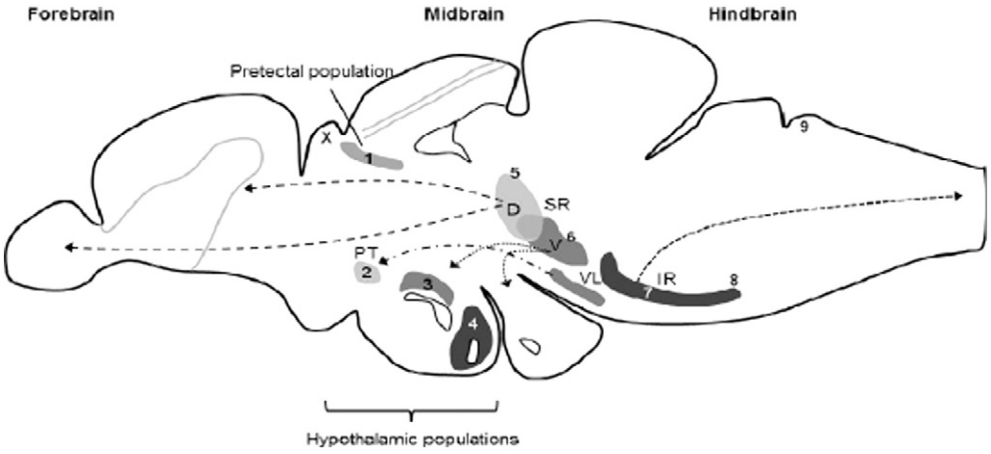

Fig. 2. Serotonergic populations in the adult zebrafish brain, with selected projection patterns from the raphe subpopulations. X — pineal population; 1 — pretectal population; 2–4 — hypothalamic periventricular population; 5–7 — rostral raphe populations; 8 — caudal raphe population; 9 — area postrema population.
Adapted from Maximino et al. (2013a) and Panula et al. (2010).

In larval zebrafish, 5-HT-like-immunoreactivity is found in the habenula and posterior tuberculum from 24 hpf onwards; immunoreactivity in the spinal cord appears at 32 hpf, in the superior raphe (SR) at 40 hpf, in the inferior raphe (IR), pretectum, posterior tuberculum, cerebellum and hypothalamus at 48 hpf, and in the area postrema it appears at 3 dpf (McLean and Fetcho, 2004a). These nuclei will develop into 9 separate *bona fide* serotonergic nuclei in the adult (Fig. 2) — the pretectal complex (serotonergic cluster 1, as per Panula et al., 2010); the anterior (2), intermediate (3) and posterior (4) paraventricular organ nuclei, which comprise the paraventricular complex; the dorsal (5), median (6) and ventrolateral (7) raphe, comprising the rostral raphe complex; and the inferior raphe (8) and area postrema (9), comprising the caudal raphe complex (Gaspar and Lillesaar, 2012; Lillesaar, 2011; Maximino and Herculano, 2010; Maximino et al., 2013a; Panula et al., 2010). These centers can be discriminated by the presence of transcription factors and mature markers, including enzymes (tryptophan hydroxylase [TPH]; aromatic acid decarboxylase [AADC]; monoamine oxidase [MAO]), isoform of the serotonin transporter (SERT) and of serotonin receptors (Lillesaar, 2011). Thus, the transcription factor *pet1* is expressed in nuclei 5–9, with non-confirmed expression in the spinal cord (Lillesaar et al., 2007, 2009), while *etv5b* is expressed in the basal forebrain and hypothalamus, where it will lead to the development of serotonergic neurons (Bosco et al., 2013). In the zebrafish raphe clusters, serotonin is synthetized by isoform 2 of TPH — which is also expressed in the epiphysis and in the pretectal area (Bellipanni et al., 2002; Lillesaar et al., 2007; Teraoka et al., 2009), while isoforms 1A and 1B are expressed in the hypothalamic cluster, as well as in the epiphysis and retina (Bellipanni et al., 2002; Lillesaar et al., 2007; Teraoka et al., 2009). Moreover, *tph3* is also expressed in serotonergic nuclei 3 and 4 (Ren et al., 2013). AADC has been described in the pretectal area, posterior tuberculum, and raphe (Filippi et al., 2010; Kaslin and Panula, 2001; Yamamoto et al., 2011).

So far, the most widely studied serotonergic cluster in teleost fishes is those comprised of the raphe nuclei. The superior raphe is located in the dorsorostral tegmentum, and is comprised of population 5, a dorsally located portion with large, elongated ovoid 5-HT-immunoreactive cells with unipolar dendrites which encircle the medial longitudinal fascicle and extend ventrolaterally (Kaslin and Panula, 2001); and population 6, a ventrally located portion with larger 5-HT-immunoreactive cells forming two parallel columns near the midline, with dendrites extending ventrally or ventrolaterally (Kaslin and Panula, 2001). Population 5, described as the dorsal raphe nucleus (Rd), is localized below the griseum centrale (GC), extending bilaterally on that portion, and projecting to the telencephalon (especially the homologue of the basolateral amygdala and hippocampus) and olfactory bulb (Lillesaar et al., 2007, 2009); population 6 is the median raphe nucleus (Rm), and projects to the hypothalamus (Lillesaar et al., 2009). A division of mRNA expression of 5-HT_{1A} receptor isoforms is also observed: isoform 1AA is expressed in the median raphe, while isoform 1AB is expressed in the dorsal raphe (Norton et al., 2008). A dorsal habenula–dorsal interpeduncular nucleus pathway projects to the GC, and the authors suggest that axon collaterals from this pathway probably synapse on Rd neurons (Agetsuma et al., 2010; Okamoto et al., 2011); the dorsal habenula has been implicated in the control of zebrafish fear/anxiety responses (Mathuru and Jesuthasan, 2013), behavioral flexibility after fear conditioning (Agetsuma et al., 2010), and behavioral control after uncontrollable stress (Lee et al., 2010). Finally, a third rostral raphe nucleus (population 7) has been described in zebrafish (Gaspar and Lillesaar, 2012; Lillesaar, 2011; Lillesaar et al., 2009) and sticklebacks (Ekström and van Veen, 1984). In the latter species, these cells have been described as homologous to mammalian group B9, the supralemniscal raphe (Ekström and van Veen, 1984), while in zebrafish it has been described as homologous to mammalian group B3, nucleus raphe magnus (Panula et al., 2010). However, given that these cells seem to project exclusively to the migrated nuclei of the posterior tuberculum, it seems unlikely that they correspond to any mammalian nucleus (Gaspar and Lillesaar, 2012; Lillesaar, 2011).

Neurons from the inferior raphe (population 8) have also been fairly well characterized, since they project extensively to the spinal cord and cerebellum (Gaspar and Lillesaar, 2012; McLean and Fetcho, 2004a,b). In larvae, this population comprises small cells (~37 μm^2 cross-sectional area) which cluster around the midline in a segmental manner, with gaps between subsequent segments (McLean and Fetcho, 2004a). They project to the brainstem escape network, which mediates escape responses and arousal (Eaton et al., 2001), where they terminate in close apposition to the ventral dendrite of the Mauthner cell and the axon collaterals of a large reticulospinal neurons, MiD3cm, as well as the dendrites of primary and secondary motoneurons in the spinal cord (McLean and Fetcho, 2004b).

Apart from population 8, serotonin-immunoreactive neurons are also found in the area postrema (AP) (Kaslin and Panula, 2001); however, since no *tph* isoform has been detected in the AP (Lillesaar et al., 2007, 2009), and since this region expresses both VMAT2 (Wen et al., 2008) and SERTA (Norton et al., 2008; Wang et al., 2006), these cells are likely to take up and use 5-HT as a transmitter, but do not synthetize it. This region is a circumventricular organ that relays baroreceptor and chemoreceptor stimuli to the hypothalamus (de Wardener, 2001); however, the role of this region in fish, or the participation of serotonin in these processes, is unknown.

Moving rostrally, the next populations after the raphe are hypothalamic. Populations 2–4 (anterior [Ha], intermediate [Hi] and caudal [Hc] parts of the paraventricular organ) represent hypothalamic paraventricular clusters (Panula et al., 2010) which do not express nor depend on *pet1* to develop (Lillesaar et al., 2007). Differently from mammalian 5-HT-accumulating cells, paraventricular neurons in the zebrafish are *bona fide* serotonergic cells (Gaspar and Lillesaar, 2012; Lillesaar, 2011), as they express *tph1a* and *tph3* (Bellipanni et al., 2002; Ren et al., 2013; Teraoka et al., 2009) and *aadc* (Filippi et al., 2010; Kaslin and Panula, 2001; Yamamoto et al., 2011); these cells also express the serotonin transporters *sertb* and *vmat2* (Norton et al., 2008; Wang et al., 2006; Wen et al., 2008; Yamamoto et al., 2011), the metabolizing enzyme *zmao* (Anichtchik et al., 2006; Sallinen et al., 2009), and the 5-HT_{1AB} receptor (Norton et al., 2008). Moreover, the 5-HT_{2C} receptor is also expressed in the larval posterior tuberculum (Schneider et al., 2012).

This peculiar expression pattern in the hypothalamic serotonergic nuclei is due to a different control mechanism: while raphe cells depend on *pet1*, hypothalamic serotonergic nuclei depend on *etv5b* (Bosco et al., 2013) and *fezf2* (Rink and Guo, 2004); both control parameters of cell cycle in progenitor cells (Berberoglu et al., 2009; Bosco et al., 2013). Interestingly, *fezf2* is expressed in radial glial progenitors, and 5-HT seems to promote neurogenesis of serotonergic neurons by promoting proliferation and migration of radial glial cells in the hypothalamus (Pérez et al., 2013). In spite of these observations, very little is known regarding the function of paraventricular serotonergic neurons in zebrafish.

Finally, the last *bona fide* serotonergic cluster is found in the pretectum of zebrafish (Lillesaar, 2011; Maximino and Herculano, 2010; Maximino et al., 2013a). Kaslin and Panula (2001) demonstrated that most of the serotonergic innervation of the tectum comes from this cluster, although it has been observed that 5-HT cells from the raphe also innervate the optic tectum (Yokogawa et al., 2012); projections from the paraventricular pretectal nucleus terminate mainly in the stratum fibrosum et griseum superficiale and stratum griseum centrale (Kaslin and Panula, 2001), while raphe neurons project to the stratum fibrosum et griseum superficiale, the stratum opticum and the stratum album centrale (Yokogawa et al., 2012).

2.1. Serotonin synthesis, uptake and metabolism

5-HT is synthesized in a two-step reaction from tryptophan to 5-hydryxytryptophan (5-HTP) and there to serotonin. The rate-limiting enzyme in the synthesis is tryptophan hydroxylase, which presents two isoforms in humans (Walther and Bader, 2003) and four in zebrafish (Bellipanni et al., 2002; Ren et al., 2013; Teraoka et al., 2009). *tph1a* is expressed in the retina, pineal, hypothalamus, and spinal cord, while *tph1b* is expressed in the pineal and, transiently, in the preoptic area (Bellipanni et al., 2002). *tph2* is expressed in the pineal, pretectal area, raphe and reticular formation (Teraoka et al., 2009). *tph3* (formerly *th2*) is expressed in the anterior, intermediate and caudal hypothalamic neural clusters (Ren et al., 2013). Among those, only the kinetics of *tph3* is known, with the purified protein synthesizing 5-HTP at a rate of ~14 nM/min/mg protein (Ren et al., 2013). Interestingly, *tph3* appears to have risen from the duplication of the *tyrosine hydroxylase* gene, but does not seem to have a participation in the synthesis of catecholamines. Once synthesized, 5-HT is transported into vesicles by isoform 2 of the vesicular monoamine transporter (VMAT), which is expressed in the pretectal area, preoptic region, posterior tuberculum, hypothalamus, raphe, reticular formation, area postrema and spinal cord (Ren et al., 2013; Wen et al., 2008).

5-HT is transported from the extracellular environment by two transport systems, $uptake_1$ and $uptake_2$. The first system represents a high-affinity, low-capacity mechanism that is dependent on sodium and chloride, and is subsumed in the serotonin transporter (SERT), which has been cloned and studied in zebrafish (Norton et al., 2008; Severinsen et al., 2008; Wang et al., 2006). The second system is a low-affinity, high-capacity mechanism that is independent on sodium and has been identified as the three isoforms of the organic cation transporter (OCT1–3) and the plasma membrane monoamine transporter (PMAT) (Duan and Wang, 2010); in anamniotes, OCT1 and OCT2 are present (Popović, 2014), and, while present in zebrafish (NCBI Reference Sequence: NP_001074041.1), PMAT has not yet been characterized.

So far, the most well characterized system in zebrafish is that mediated by SERT. Zebrafish present two isoforms of SERT, A and B, which are expressed in a complementary fashion in the brain (Norton et al., 2008; Wang et al., 2006). In 96 hpf larvae from the TL strain, SERTA mRNA is expressed in the raphe nuclei, ventral posterior tuberculum and pineal organ, while SERTB is expressed in the medulla oblongata and in the inner nuclear layer of the retina (Wang et al., 2006). In the adult brain, SERTA mRNA is expressed in the dorsal and ventral parts of the periventricular pretectal nucleus (PPd and PPv) and superior and inferior raphe nuclei, while SERTB mRNA is expressed in the paraventricular organ (PVO) and caudal zone of the periventricular hypothalamus (Hc) (Norton et al., 2008). 5-HT uptake in HEK293-MSR cells transiently transfected with SERTA is characterized by a saturating function of 5-HT concentration, with a K_m of 2.13 μM (Severinsen et al., 2008); in HEK293 cells, K_m was reported as 4.2 μM (Wang et al., 2006), which may reflect differences in transfection protocols. In whole-brain homogenates, citalopram binds with a K_D of ~16 nM (Sackerman et al., 2010), a value which is very similar to the K_D of escitalopram (~13 nM) at HEK293-MSR cells transiently transfected with SERTA (Severinsen et al., 2008).

After uptake, serotonin is metabolized in a two-step reaction to 5-hydroxyindoleacetic acid (5-HIAA); the rate-limiting step is catalyzed by monoamine oxidase (Cotzias and Dole, 1951), a mitochondrial flavoprotein that, in mammals, exists in two isoforms, MAO-A and MAO-B. In zebrafish, however, a single monoamine oxidase isoform (zMAO) has been identified (Aldeco et al., 2011; Anichtchik et al., 2006; Arslan and Edmondson, 2010; Fierro et al., 2013; Setini et al., 2005). This protein displays ~70% sequence identity with both human isoforms, and its predicted secondary structure indicates that the most important domains – the flavin-binding, substrate-binding, and membrane-binding domains – are probably conserved in the fish enzyme, without an appreciable similarity between zMAO and any of the human isoforms (Arslan and Edmondson, 2010; Fierro et al., 2013; Setini et al., 2005). In whole-brain homogenates, tyramine is the best substrate for zMAO, followed by 5-HT, phenylethylamine, 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP) and dopamine (Fierro et al., 2013). When expressed in *Pichia pastoris*, the best substrate is tyramine, followed by kynuramine, serotonin, *p*-carboxybenzylamine, benzylamine, phenylethylamine, dopamine and 4-phenylbutylamine (Anichtchik et al., 2006). In the assay conditions used in that experiment, deprenyl (a MAO-B inhibitor) is less efficacious than clorgyline, a MAO-A inhibitor (Aldeco et al., 2011; Arslan and Edmondson, 2010), with clorgyline inhibiting 5-HT metabolism with an IC_{50} of ~0.47 μM, while deprenyl inhibits 5-HT metabolism with an IC_{50} of 0.8 μM (Anichtchik et al., 2006). In vivo, deprenyl (100 μM) decreases zMAO activity by ~35% at 3 dpf and ~75% at 7 dpf (Setini et al., 2005). When zMAO is expressed in *P. pastoris*, clorgyline inhibits kynuramine metabolism with an IC_{50} of ~65 μM and deprenyl inhibits kynuramine metabolism with an IC_{50} of 6.5 μM, while the oxidation of benzylamine (a MAO-B substrate) is faster than phenylethylamine (a MAO-A substrate) (Arslan and Edmondson, 2010). Structure–activity correlations show that zMAO catalytic activity on benzylamine analogues depends on the electron withdrawing capacity of the substituent, a characteristic that is shared with MAO-A but not MAO-B (Aldeco et al., 2011). Thus, in vitro, zMAO's catalytic behavior is closer to MAO-B, while its affinity for inhibitors closely resembles that of MAO-A; moreover, some characteristics of zMAO are not present in either MAO-A or MAO-B (Aldeco et al.,

2011; Arslan and Edmondson, 2010; Fierro et al., 2013; Setini et al., 2005).

zmao mRNA is detected at 24 hpf in the locus coeruleus and diencephalon, while enzyme activity is detected using histochemistry at 42 and 48 hpf, respectively, in Türku zebrafish; at 2 dpf, *zmao* is found in the telencephalon, diencephalon, rostral raphe and internal reticular formation, and enzymatic activity is detected in these regions at 3 dpf (Sallinen et al., 2009). In adult Türku zebrafish, MAO histochemistry is low to moderate in dopaminergic cell clusters, while the highest levels are found in the noradrenergic and serotonergic cell groups, as well as in the habenulointerpeduncular pathway (Anichtchik et al., 2006). From 5 dpf onwards, *zmao* mRNA is found in the ventral telencephalon, preoptic region, habenula, thalamus, in Ha, Hi and Hc, and in the inferior raphe (Anichtchik et al., 2006; Sallinen et al., 2009).

3. Serotonergic modulation of larval and adult behavior

3.1. Serotonin and arousal

One of the first observations regarding the role of the serotonergic system in mammalian behavior regards arousal. At the end of the 1950s and beginning of the 1960s, a series of pharmacological and lesion studies led Jouvet to suggest that neurons in the dorsal raphe nucleus and posterior central nucleus were responsible for slow wave sleep (Jouvet, 1969, 1972). Soon, however, this role was inverted, and serotonin was proposed to *increase* arousal (Jouvet, 1999). In zebrafish, arousal states are usually characterized by changes in locomotor activity or sensory responsiveness triggered by intense stimuli (Chiu and Prober, 2013). For example, larvae exposed to a sudden change in water flow rate become hyperactive and sensitized to the flow stimulus, and show heightened visual sensitivity to perceived motion (Yokogawa et al., 2012). Using calcium imaging, these authors were able to demonstrate that the flow stimulus leads to increased activity in *tph2*-expressing cells in the superior raphe; moreover, genetic ablation of these cells abolishes the increase in visual sensitivity during arousal (Yokogawa et al., 2012). These authors suggested that a DR-tectal projection, which terminates at the retinorecipient upper layers of the optic tectum, is responsible for this effect. A sudden light flash enhances the Mauthner cell-mediated escape response (ZBC 1.52) in larvae, an effect which is abolished by laser ablation of monoaminergic cells in the caudal hypothalamus; while treatment with dopamine antagonists also abolishes this response, there is evidence for a modulation by serotonin as well, since knockdown of *tph3* in larvae decreases this effect (Mu et al., 2012). In larvae, the sudden onset of a high-intensity light stimulus elicits a robust motor excitation response (lasting 5–7 s), the photomotor response (ZBC 1.116). MAO inhibitors increase the magnitude and duration of the excitatory phase, and a series of coumarins which produce a similar behavioral phenotype at concentrations of 10 and 100 μM were shown to present MAO inhibiting activity (Kokel et al., 2010). The MAO inhibitors phenelzine and tranylcypromine, as well as the 5-HT_{1A} receptor antagonist UH-301 (all drugs at 10 and 30 μM), however, seem to promote sleep-like behavior, decreasing waking activity and increasing rest bout length (ZBC 1.128) (Rihel et al., 2010), while in rodents MAO inhibitors decrease slow wave sleep (Real et al., 2007). These results suggest that, in zebrafish, serotonin may act to promote sleep in the absence of stimulation, while increasing arousal in response to intense stimuli or sudden stimulus change.

These observations also have important consequences towards the assessment of emotional states which involve arousal. Generalized arousal is defined as a mechanism to provide alertness to sensory stimuli, drive voluntary motor activity, and fuel emotional reactivity (Quinkert et al., 2011), and thus alterations in this "background" may lead to misinterpretations regarding emotional states. Mice selectively bred for high arousal show elevated anxiety-like behavior and reduced exploratory behavior in the elevated plus maze and light/dark box tasks (Weil et al., 2010). In female zebrafish from a wild-type strain, locomotor activity is inversely correlated with geotaxis/bottom-dwelling (ZBC 1.46) in the novel tank test (NTT) (Tran and Gerlai, 2013), a putative measure of fear/anxiety in adult zebrafish (Iturriaga-Vásquez et al., 2012; Stewart et al., 2011a,c). Interestingly, female zebrafish show higher forebrain DA turnover and lower forebrain 5-HT turnover in relation to males (Dahlbom et al., 2012). Male fish from the AB strain show decreased PMR but increased bottom-dwelling in the NTT in relation to animals from the TU strain (Vignet et al., 2013). Thus, increased or decreased arousal can both be intervening variables in the assessment of the effects of a given manipulation on anxiety-like behavior. While decreased arousal usually manifests as hypolocomotion (ZBC 1.81), and thus it is easily factored out of the analysis, increased arousal does not necessarily imply hyperlocomotion (ZBC 1.79) (Quinkert et al., 2011; Weil et al., 2010). Two possible solutions to this conundrum arise: assessing the effects of a given manipulation on arousal measures, such as the photomotor response or the enhancement of auditory C-starts by non-auditory stimuli; and using multivariate statistical analyses to isolate the most generalized, least specific factor (Quinkert et al., 2011). Of course, this latter recourse requires a suitable dataset with a wide array of behavioral endpoints, such as those proposed in the novel tank test (NTT) by Cachat et al. (2011) and in the light/dark test (LDT) by Maximino et al. (2011a, 2013b,c), which can be labor-intensive if variables are recorded manually.

3.2. Serotonin and anxiety-like and fear-like states

3.2.1. Brain serotonin levels in zebrafish defensive behavior

Serotonin has long been implicated in the control of fear, anxiety and stress. There are several lines of evidence regarding this role in mammals: *(i)* situations which evoke approach–avoidance conflict, as well as treatment with anxiogenic peptides, increase serotonin release in prosencephalic structures associated with defensive behavior (Graeff et al., 1996; Guimarães et al., 2008, 2010; Lowry et al., 2008; Maximino, 2012); *(ii)* benzodiazepines decrease prosencephalic serotonin turnover (Lowry and Moore, 2006; Matsuo et al., 1996; Rex et al., 2005; Steckler, 2008), while anxiolytic peptides decrease serotonin release in the forebrain (Wise et al., 1972); *(iii)* microinjection of serotonergic agonists and antagonists in structures such as the septum, hippocampus, amygdala and periaqueductal gray area alters defensive behavior in different paradigms, including the elevated plus-maze and the elevated T-maze (Steckler, 2008); *(iv)* knockout of some serotonergic genes (e.g., the transcription factors Pet-1 and Lmx1b, the 5-HT_{1A} and 5-HT_{2C} receptors, the serotonin transporter) also alters anxiety- and fear-like behavior (Maximino, 2012; Pinheiro et al., 2007).

In longfin stripped (LFS) zebrafish, exposure to the LDT increases extracellular 5-HT levels, while exposure to the NTT does not (Lesch et al., 2003); moreover, tissue 5-HT levels are increased in the hindbrain and forebrain after exposure to the LDT, and increased in the midbrain after exposure to the NTT (Maximino et al., 2013b). There is a tight, positive correlation between serotonin turnover and scototaxis/dark preference (ZBC 1.127) in the LDT, and extracellular serotonin levels are directly correlated with scototaxis/dark preference, thigmotaxis (ZBC 1.173) and risk assessment in the LDT, while being inversely correlated with bottom-dwelling in the NTT (Maximino et al., 2013b). Nonetheless, these results are suggestive of a "dual role" of serotonin in the control of zebrafish defensive behavior in the LDT and NTT, increasing it in the first and decreasing it in the latter. This is reminiscent to the "dual role" of serotonin proposed in rodents, with the neurotransmitter increasing anxiety-like behavior and decreasing panic-like behavior (Graeff et al., 1997). In support of this hypothesis, strain differences in neurochemistry and behavior have been observed: in relation to blue shortfin (BSF), skin mutants from the *leopard* strain show decreased whole-brain tissue 5-HT and increased anxiety in both the LDT and NTT — effects which are rescued by treatment with fluoxetine (5 mg/kg), suggesting a misregulation of serotonin uptake (Maximino

et al., 2013c). It has also been observed that BSF animals show decreased expression of *serta* and decreased whole-brain serotonin in relation to zebrafish from the AB strain (Pan et al., 2012). AB fish also show less geotaxis and are less active in a group preference task, but do not present differences in predator avoidance behavior in relation to BSF fish (Gerlai et al., 2009). In wild-caught zebrafish, females have lower forebrain serotonin turnover than males (Dahlbom et al., 2012) and display less boldness (ZBC 1.18) than males (Winberg et al., 2011). Whether these differences are correlative or causative remains to be tested.

Acute exposure to a conspecific alarm substance (CAS) produces a fear-like alarm reaction in zebrafish (ZBC 1.5), characterized by increased geotaxis, erratic swimming and freezing (Egan et al., 2009; Mathuru et al., 2012; Speedie and Gerlai, 2008); CAS also increases scototaxis and inhibits nocifensive behavior (ZBC 1.104) in BSF and LFS animals (Maximino, 2011; Maximino et al., submitted for publication), effects which are accompanied by an ~80% increase in extracellular serotonin level and are blocked by acute fluoxetine treatment (Maximino et al., submitted for publication). Repeated CAS exposure decreases mRNA for *pet1* and *serta* (but not *tph2*) in the brains of

Table 1
Effects of drugs which increase extracellular 5-HT levels on larval and adult zebrafish behavior in models of fear/anxiety, as well as on physiological variables.

Assay	Drug	Treatment	Effect	Reference
Five-fish bouncing ball assay (larvae)	Fluoxetine	Acute, 6.5 μM	-Impairment of escape -No effect on thigmotaxis	Maximino et al. (2013b)
Locomotion (larvae)	Deprenyl	Developmental (0–7 dpf), 1–100 μM	-Hypolocomotion -Top-dwelling -Decreased thigmotaxis -Increased heart rate -Increased extracellular 5-HT	Sallinen et al. (2009)
376 nM, and	Tranylcypromine	Developmental (0–72 hpf), 25–200 μM	-Hypolocomotion -Apoptosis of neurons	Jie et al. (2009)
Novel tank test	Tranylcypromine	Acute, 3.76 μM	-Decreased geotaxis	Stewart et al. (2011c)
		Acute, 376 nM	-Decreased freezing	Stewart et al. (2011c)
	Desipramine	Acute, 82.8 μM	-Decreased geotaxis	Sackerman et al. (2010)
	Citalopram	Acute, 247 μM	-Decreased geotaxis	Sackerman et al. (2010)
	Fluoxetine	Acute, 323.3–3233 nM	-No effect	Stewart et al. (2011c, 2013)
		Acute, 161.60–323.3 nM	-Decreased geotaxis	Iturriaga-Vásquez et al. (2012)
		Acute, 3.88 μM	-Decreased geotaxis	Stewart et al. (2013)
		Acute, 2.5–5 mg/kg	-Decreased geotaxis -Decreased freezing -Increased extracellular 5-HT	Kizil and Brand (2011)
		Acute, 10 mg/kg	-Increased locomotion	Maximino et al. (2011b, 2013b)
		Chronic, 323.3 nM	-Decreased geotaxis -Decreased erratic movements -Decreased whole-body cortisol	Egan et al. (2009)
		Chronic, 323.3 nM	-Decreased geotaxis in HSB -Increased *oxtl*, *npy* and *isg15* mRNA -Decreased *ucn3l*, *prl2*, GAT, and *nrbf2* mRNA -Downregulation of genes involved in lipid and steroid metabolism -Upregulation of genes involved in amino acid and organonitrogen metabolism	Wong et al. (2013)
	R-fluoxetine	Chronic, 95.4 nM	-Decreased geotaxis in HSB	Wong et al. (2013)
	S-fluoxetine	Chronic, 95.4 nM	-Decreased geotaxis in HSB	Wong et al. (2013)
	Sertraline	Chronic, 1 μg/day, p.o.	-No effect on geotaxis	Gould (2011) and Gould et al. (2007)
	5-HTP	Acute, 300 mg/kg	-Decreased geotaxis -Decreased freezing -Increased 5-HT turnover	This article, Fig. 3
Aquatic plus-maze	Desipramine	Acute, 82.8 μM	-No effect	Sackerman et al. (2010)
	Citalopram	Acute, 247 μM	-No effect	Sackerman et al. (2010)
	Sertraline	Chronic, 1 μg/day, p.o.	-Decreased scototaxis -Decreased SERT binding sites in the OT and periventricular hypothalamus -Decreased AChE activity	Gould (2011) and Gould et al. (2007)
Light/dark test	Moclobemide	Acute, 5–10 mg/kg	-No effect	Maximino et al. (2011b) and Araújo et al. (2012)
	Fluoxetine	Acute, 2.5 mg/kg	-Increased scototaxis -Increased latency to white -Increased thigmotaxis -Increased risk assessment -Increased extracellular 5-HT	Maximino et al. (2013b)
	Fluoxetine	Acute, 5 mg/kg	-No effect in LSF -Decreased scototaxis in *leo* -Decreased thigmotaxis in *leo* -Decreased risk assessment in *leo*	Maximino et al. (2011b, 2013b,c)
	Fluoxetine	Acute, 10 mg/kg	-Increased locomotion	Maximino et al. (2011a,b, 2013b)
	Fluoxetine	Chronic, 10 mg/kg	-Decreased scototaxis	Maximino et al. (2011b)
	5-HTP	Acute, 300 mg/kg	-Increased scototaxis -Increased thigmotaxis -Increased risk assessment -Increased 5-HT turnover	This article, Fig. 3

RIKEN Wako strain zebrafish, but does not decrease the alarm reaction (Ogawa et al., 2014).

Further support to the "dual role" hypothesis is lent by pharmacological manipulations. Intraperitoneal injection of the TPH inhibitor *para*-chlorophenylalanine (pCPA; two doses of 300 mg/kg, separated by 24 h, with the last dose injected 24 h before behavioral testing) increases geotaxis, inhibits habituation in the first minutes, and increases homebase behavior (ZBC 1.76) in the NTT; the same treatment, however, decreases scototaxis, thigmotaxis and risk assessment in the LDT (Maximino et al., 2013b). Treatment with reserpine (32.86 and 65.72 μM), a VMAT inhibitor which depletes dopamine and noradrenaline as well as serotonin, induces a delayed phenotype of skin darkening (Nguyen et al., 2013), increased latency to top and freezing, and "droopy tail" (ZBC 1.49) 7 days after a single treatment (Kyzar et al., 2013). Larvae treated with the TPH inhibitor *para*-chlorophenylalanine (pCPA, 25 μM) for 24 h between 1 and 2 dpf do not show touch responses (ZBC 1.177), and, while spontaneous swimming appears at 5 dpf as in non-treated larvae, they present hypolocomotion (Airhart et al., 2007). At the end of the treatment, the mRNA levels for the 5-HT_{1AA} receptor are diminished in the brain and spinal cord, while SERTA is diminished in the spinal cord; interestingly, at 7 dpf (five days after treatment offset), 5-HT_{1AA}R levels in the spinal cord, but not in the brain, are restored, while SERTA levels in both the brain and the spinal cord are increased in relation to control animals. Since *tph2* in the superior raphe has been implicated in arousal (Yokogawa et al., 2012), both hypolocomotor (in larvae) and anxiolytic-like effects (in adults) could be explained by decreased generalized arousal, but the increase in geotaxis and inhibition of habituation argue against this explanation.

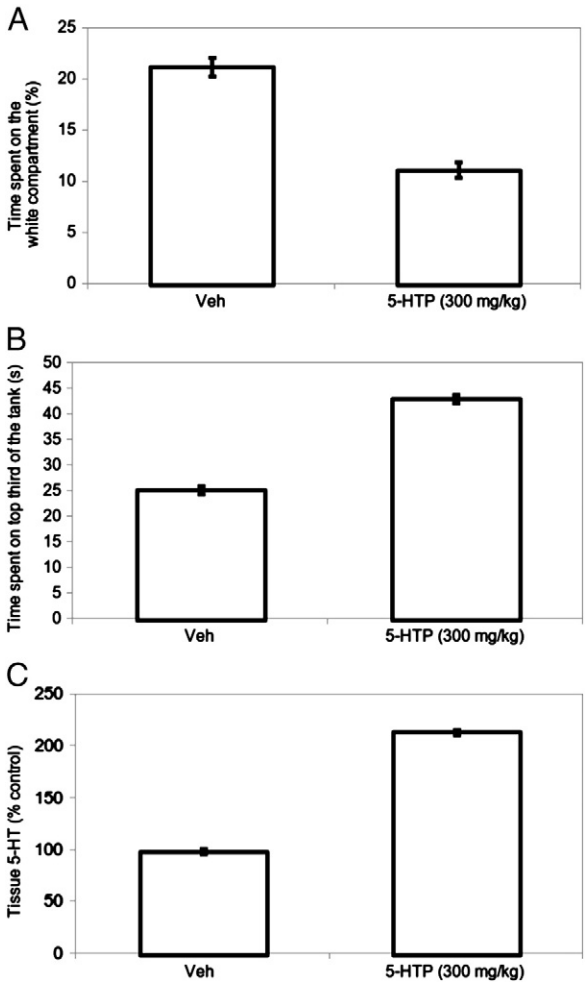

Fig. 3. Effects of 5-HTP (300 mg/kg, i.p.) on (A) scototaxis ($t_{[df = 22]} = 8.8$, $p < 0.0001$, Maximum Predictive Value = 2.64), (B) geotaxis ($t_{[df = 22]} = -15.5125$, $p < 0.0001$, Maximum Predictive Value = 4.66), and (C) brain 5-HT concentrations ($t_{[df = 8]} = -22.50$, $p < 0.0001$, Maximum Predictive Value = 11.02) in adult longfin stripped (LFS) zebrafish.

If the pharmacological depletion of serotonin increases anxiety-like behavior in the NTT and decreases it in the LDT, treatments which increase serotonin do not produce such straightforward results (Table 1). Treatment with the 5-HT precursor 5-hydroxytryptophan (5-HTP, 20 mg/kg, 60 min before test) increases scototaxis and decreases geotaxis, while at the same time increasing serotonin turnover ~2.3 fold (Fig. 3). Monoamine oxidase inhibitors are clinically effective in the treatment of depression and panic disorder, but not generalized anxiety disorder (Baldwin et al., 2011). In larval zebrafish of the Turku strain treated with the preferential MAO-B inhibitor deprenyl (1–100 μM) from 0 dpf to 7 dpf a hypolocomotor effect is shown, accompanied by surfacing behavior (i.e., top-dwelling) (ZBC 1.164) and decreased thigmotaxis (Sallinen et al., 2009). At 10 and 100 μM, heart rate was increased. Treatment at 7 dpf for 2 h with 100 μM, a concentration which decreases MAO activity at 7 dpf to about a third of control values, is sufficient to produce hypolocomotion. This MAO inhibitory activity is accompanied by highly increased serotonin levels (up to 169% of control values after 0–5 dpf treatment with 100 μM, and up to 977% of control values after 0–7 dpf treatment with 100 μM); treatment with pCPA (1500 μM, from 1 to 5 dpf) restores the elevated 5-HT levels as well as the hypolocomotor effects of deprenyl, but had no effect by itself. Using immunohistochemistry for 5-HT, the authors were able to demonstrate that 5-HT-like immunoreactivity was highly elevated in areas innervated by serotonergic neurons; this effect was prevented by treatment with fluvoxamine (100 μM) 2 h before euthanasia, suggesting a SERT-dependent process (Sallinen et al., 2009). The dramatic elevation of serotonin levels, as well as the autonomic and hypolocomotor effects, suggest that, at these concentrations and treatment schedules, deprenyl induces serotonin toxicity (see Section 3.3 for further discussions on the topic).

While developmental deprenyl treatment produces hypolocomotion, surfacing and decreased thigmotaxis, acute treatment with the MAO-B inhibitor tranylcypromine decreases latency to top and increases top transitions at 376 nM, and reduces freezing duration at 3.76 μM in adult BSF zebrafish (Stewart et al., 2011a,c). A much lower concentration (7.5 nM) does not produce effects in the NTT, but, in combination with fluoxetine (3.88 μM), tranylcypromine increases top transitions and average top visit duration (Stewart et al., 2013). In contrast, treatment of BSF zebrafish with the MAO-A inhibitor moclobemide (5 or 10 mg/kg) does not alter behavior in the LDT (Araújo et al., 2012; Maximino et al., 2011b). These results suggest an anxiolytic-like effect of MAO inhibitors in the NTT, without effects on the LDT.

Waterborne treatment of PETCO zebrafish with desipramine (82.8 μM), which show ~15 times more affinity for zebrafish SERTA than human SERT (Severinsen et al., 2008), decreases the time spent in the half third of the tank in the NTT, but does not alter scototaxis in the aquatic plus-maze (Sackerman et al., 2010); citalopram (247 μM), which shows a similar affinity for zebrafish SERTA and human SERT (Severinsen et al., 2008), produces an even more dramatic effect in this model, and is also without effect in the aquatic plus-maze (Sackerman et al., 2010). The prototypic SERT inhibitor, fluoxetine, produces very mixed results, presenting a hormetic dose–response profile. While the affinity of this ligand for any of the zebrafish SERT isoforms, either in vivo or in vitro, has not been determined, in wild-type larvae (7 dpf) escape responses in the five-fish bouncing ball assay are impaired by acute fluoxetine (6.5 μM), without effects on thigmotaxis either with or without stimulation (Richendrfer et al., 2012); interestingly, an opposite effect was observed with diazepam (17.6 μM) treatment. In adults, acute treatment with 323.3–3233 nM racemic fluoxetine in BSF zebrafish failed to produce any effects on the NTT (Stewart et al., 2011c);

combined treatment of the highest concentration with a non-effective concentration of LSD (46.4 nM) decreased geotaxis (Stewart et al., 2013). At both lower (161.6 and 323.3 nM) and higher concentrations (3.88 μM), however, geotaxis is decreased (Iturriaga-Vásquez et al., 2012; Stewart et al., 2013). Intraperitoneal injection produces a similar profile: low doses (2.5 and 5 mg/kg) decrease geotaxis and freezing and promote habituation in the last 3 min in LSF zebrafish tested in the NTT, with a higher dose (10 mg/kg) producing hyperlocomotor effects (Maximino et al., 2013b). In the LDT, 2.5 mg/kg increase scototaxis, the latency to enter the white compartment, thigmotaxis and risk assessment in LSF zebrafish (Maximino et al., 2013b), with 5 and 10 mg/kg not altering scototaxis, and 10 mg/kg also producing hyperlocomotor effects (Araújo et al., 2012; Maximino et al., 2011b, 2013b). Interestingly, in the *leopard* strain, which shows increased geotaxis (Egan et al., 2009), fluoxetine (5 mg/kg) rescues the increased scototaxis and risk assessment observed in the LDT in relation to LSF animals (Maximino et al., 2013c); the *leopard* strain also shows decreased whole-brain tissue 5-HT levels, increased serotonin turnover, and increased MAO activity in relation to LSF (Maximino et al., 2013c).

While acute treatment with SSRIs produce mixed effects on different tests, chronic treatment is usually less variable. Treatment with sertraline (1 μg/day, p.o.) for 21 days decreases SERT binding sites in the optic tectum and periventricular hypothalamus, decreases acetylcholinesterase activity, and decreases scototaxis in the aquatic plus-maze, without effects on geotaxis in the NTT (Gould, 2011; Gould et al., 2007). Treatment with racemic fluoxetine for 2 weeks produces marked effects on both the LDT (5 mg/kg; Maximino et al., 2011b) and in the NTT (323.3 nM; Egan et al., 2009). In both cases, these effects were observed in BSF zebrafish; in male zebrafish from a selectively bred High Stationary Behavior (HSB) line – which shows increased freezing and decreased thigmotaxis in the open tank, increased geotaxis and freezing in the NTT, increased sensitivity to CAS, increased scototaxis in the LDT, and increased latency to feed in undisturbed and disturbed conditions (Wong et al., 2012) – chronic racemic fluoxetine (323.3 nM) dramatically decreases bottom-dwelling in the NTT, without altering freezing (Wong et al., 2013); moreover, treatment with either R-fluoxetine or S-fluoxetine (95.4 nM) produced a similar effect, but drug effects were not different between each other, suggesting that the behavioral effect of chronic fluoxetine is not stereospecific (Wong et al., 2013). Moreover, fluoxetine increases the expression of genes which dampen the stress response (e.g., *oxtl*, *npy*) and decreases the expression of genes which induce cortisol responses (e.g., *ucn3l* and *prl2*), and a microarray analysis revealed that racemic fluoxetine altered the expression of genes associated with steroidogenesis (Wong et al., 2013). Consistent with this observation, BSF zebrafish treated with racemic fluoxetine show decreased whole-body cortisol levels (Egan et al., 2009). Nonetheless, as we will see below, the relationship between serotonin and the HPI axis is more complicated than that.

3.2.2. 5-HT_{1A} and 5-HT_{1B} receptors in zebrafish defensive behavior

While the 5-HT_{1A} receptor duplication event was retained in zebrafish, drug effects are remarkably conserved (Table 2). At 6 dpf, scotophobia is markedly reduced by treatment with the 5-HT_{1A} partial agonist buspirone at a concentration of 59.24 μM (Steenbergen et al., 2011). In adult whole-brain homogenates, buspirone displaces [^{3}H]8-OH-DPAT with an inhibition constant K_i of 1.8 nM (Barba-Escobedo and Gould, 2012); specific binding for [^{3}H]8-OH-DPAT was defined as ~175 fmol/mg protein in the hypothalamus, ~275 fmol/mg protein in the optic tectum, and ~230 fmol/mg protein in the telencephalon (Connors et al., in press). In adult zebrafish (AB strain), waterborne buspirone (24 and 36 μM) decreased scototaxis in the LDT (Lau et al., 2011), while a higher concentration (237 μM) decreased scototaxis in the aquatic plus-maze (Connors et al., in press). In BSF zebrafish, buspirone (7.11 μM) also decreased scototaxis, without altering locomotion or the latency to enter the dark compartment (Gebauer et al., 2011). A similar effect is observed after intraperitoneal injection in BSF

Table 2
Behavioral and physiological effects of 5-HT1A- and 5-HT1B-acting drugs on zebrafish behavior in models of fear/anxiety.

	Drug	Effect	Comments	References
Group behavior task	Buspirone (7.11 and 11.85 μM)	-Decreased bottom-dwelling -No effect on shoaling -No effect on thigmotaxis		Gebauer et al. (2011) and Maaswinkel et al. (2013)
Social preference	Buspirone (23.7 μM)	-Promotes social interaction preference	Animals originally did not prefer the conspecific chamber	Barba-Escobedo and Gould (2012)
Novel tank test	Buspirone (11.85–118.49 μM)	-Decreased bottom-dwelling (immediately after exposure) -Increased bottom-dwelling (3½ h after exposure) -Hypolocomotion (3½ h after exposure) -Increased freezing (3½ h after exposure)	Delayed effect suggests either rebound anxiety or 'dizzyness'	Bencan et al. (2009) and Maaswinkel et al. (2012)
	Buspirone (25 and 50 mg/kg)	-Decreased geotaxis -Increased habituation		Maximino et al. (2013b)
	WAY 100,635 (0.003 and 0.03 mg/kg)	-Decreased geotaxis -Increased habituation -Decreased erratic swimming -Increased homebase (smaller dose)	Hormetic dose–response	Maximino et al. (2013b)
	SB 224,289 (2.5 and 5 mg/kg)	-Decreased geotaxis -Increased habituation -Increased homebase (smaller dose)	Hormetic dose–response	Maximino et al. (2013b)
Aquatic plus-maze	Buspirone (237 μM)	-Decreased scototaxis		Connors et al. (in press)
Light/dark test	Buspirone (7.11–36 μM)	-Decreased scototaxis	BSF and AB zebrafish	Gebauer et al. (2011) and Lau et al. (2011)
	Buspirone (25 and 50 mg/kg)	-Decreased scototaxis -Decreased thigmotaxis -Decreased risk assessment	BSF and LFS zebrafish	Araújo et al. (2012) and Maximino et al. (2011b, 2013b)
	WAY 100,635 (0.003 and 0.03 mg/kg)	-Decreased scototaxis -Decreased thigmotaxis -Decreased risk assessment		Maximino et al. (2013b)
	SB 224,289 (2.5 and 5 mg/kg)	-No effect on scototaxis -Increased risk assessment (lower dose)		Maximino et al. (2013b)

and LFS wild-type zebrafish at doses of 25 and 50 m/kg (Araújo et al., 2012; Maximino et al., 2011b, 2013b); moreover, buspirone also decreases thigmotaxis (ZBC 1.173), freezing (ZBC 1.68) and risk assessment in the LDT (Maximino et al., 2013b). In wild-type (non-described phenotype) adult zebrafish, buspirone (14.81–118.49 μM) decreases bottom-dwelling in the NTT (Bencan et al., 2009). Intraperitoneal injection in LFS zebrafish at doses of 25 and 50 mg/kg decreases bottom-dwelling, while 50 mg/kg also decreases freezing and promoted habituation (ZBC 1.72) in the last 3 min in the NTT (Maximino et al., 2013b). In the group behavior task (GBT), buspirone (7.11 and 11.85 μM) decreased bottom-dwelling without altering shoal cohesion (ZBC 1.141), color response (ZBC 1.19) or thigmotaxis (Gebauer et al., 2011; Maaswinkel et al., 2012, 2013). While shoal cohesion was not altered by this concentration in the GBT, buspirone (23.7 μM) promotes social interaction preference (ZBC 1.152) in PETCO animals, which normally show no preference towards a stranger conspecific in relation to an empty blue box (Barba-Escobedo and Gould, 2012).

Given the high density of 5-HT_{1A}-like receptors at presynaptic sites in mammals, it is generally believed that decreases in 5-HT release following the activation of autoreceptors are responsible for the anxiolytic-like effects of buspirone (DeVry, 1995). There are many evidences that are at variance with this hypothesis. First, transgenic mice expressing the 5-HT_{1A} receptor under the control of a *tph2* promoter show restored negative feedback and hypothermia mediated by 5-HT_{1A} receptors, but no differences in anxiety-like behavior in relation to 5-HT_{1A}R knockout mice (Piszczek et al., 2013), and animals with transgenic decrease or increase in the expression of 5-HT_{1A}Rs in the DRN show differences in stress reactivity, but not anxiety-like behavior (Richardson-Jones et al., 2010). Second, 5-HT_{1A} antagonists, which usually do not alter 5-HT release in vivo, decrease anxiety- and depression-like behavior in rodents (Cao and Rodgers, 1997a,b, 1998; Griebel et al., 1999; Rex et al., 2008; Rodgers and Cao, 1997). Third, systemic injection of drugs which are preferential agonists at postsynaptic sites (Assié et al., 2010) and microinjection of 5-HT_{1A}R agonists and antagonists at postsynaptic sites (Broiz et al., 2008; File and Gonzalez, 1996; File et al., 1996; Roncon et al., 2012; Soares and Zangrossi, 2009; Viana et al., 2008; Zangrossi et al., 1999) alter anxiety-like behavior in rodents. Since, in zebrafish, both 5-HT_{1A} receptor isoforms are present in serotonergic nuclei, probably as autoreceptors (Norton et al., 2008), it is difficult to test whether the reported effects of buspirone are mainly pre- or post-synaptic.

To complicate matters even further, buspirone shows a complex pharmacological profile. As a partial agonist in a system which shows receptor reserve, buspirone acts as a full agonist at autoreceptors and as a full antagonist at heteroreceptors (Meller et al., 1990). Moreover, systemic administration of buspirone (3 mg/kg) in rats decreases nitric oxide synthase 1 (NOS-1) immunoreactivity in the DRN without altering 5-HT or tyrosine hydroxylase immunoreactivity (Jahanshahi et al., 2010). Finally, treatment of rat brain sections with 10 μM 8-OH-DPAT recruits $G\alpha_{i3}$ but does not alter forskolin-stimulated cAMP accumulation, while treatment with the same concentration of buspirone recruits $G\alpha_{o}$, $G\alpha_{i2}$ and $G\alpha_{i3}$, as well as inhibits forskolin-stimulated cAMP accumulation (Valdizán et al., 2010).

All these data suggest a potential pharmacological effect of 5-HT_{1A} antagonists. In zebrafish whole-brain homogenates, WAY 100,635 displaces [^{3}H]8-OH-DPAT with a K_i of 1034 nM, three orders of magnitude higher than that in mice (Barba-Escobedo and Gould, 2012). In spite of this lower affinity, WAY 100,635 exerts behavioral effects in zebrafish at doses which are lower than or inside the range of doses that affect rodent behavior (Maximino et al., 2013b). In the NTT, a low dose (0.003 mg/kg) not only decreased bottom-dwelling, promoted habituation and decreased erratic swimming (ZBC 1.51), but also increased homebase behavior, while a higher dose (0.03 mg/kg) decreases bottom-dwelling and erratic swimming (Maximino et al., 2013b). In the LDT, both doses decrease scototaxis, while the higher dose decreases thigmotaxis and risk assessment (Maximino et al., 2013b). Interestingly, in goldfish (*Carassius auratus*), WAY 100,635 (29.74 nM) improves active avoidance acquisition (Beulig and Fowler, 2008).

Table 3
Effects of treatment with the 5-HT_{1A} antagonist WAY 100,635 on extracellular serotonin levels after exposure to the LDT or NTT. Values represent mean ± S.E.M., and are relative to control values.

Behavioral test \WAY 100,635 dose	0 mg/kg	0.003 mg/kg	0.03 mg/kg
LDT	182.3 ± 12.1%	179.4 ± 16.1%	191.2 ± 11.8%
NTT	113.4 ± 8.7%	121.1 ± 9.8%	109.2 ± 12.1%

As referred above, acute buspirone decreases NOS-1 immunoreactivity in the DRN of rats (Jahanshahi et al., 2010), a species in which some NOS-1-containing cells do not produce 5-HT and some do produce (Lu et al., 2010; Okere and Waterhouse, 2006; Wang and Nakai, 1995; Xu and Hökfelt, 1997); in zebrafish, co-localization of nitric oxide and 5-HT has been demonstrated in the posterior tuberculum and caudal hypothalamus (Holmqvist et al., 2007). Nitric oxide appears to play a tonic regulatory role on serotonergic neurons to stimulate basal and NMDA-induced 5-HT release (Smith and Whitton, 2000). Pre-treatment with 8-OH-DPAT blocks the hyperlocomotor effect of the peroxynitrite donor SIN-1 microinjected in the DRN of rats (Gualda et al., 2011). At the postsynaptic side, Zhang et al. (2010) have shown that NOS-1 is required for both the anxiolytic-like effects of the 8-OH-DPAT and the anxiogenic-like effects of NAN-190 at the hippocampus. Interestingly, we have observed (Maximino et al., unpublished data) that pre-treatment with the NOS inhibitor L-NAME blocks the anxiolytic-like effect of WAY 100,635 in the LDT.

In guinea pigs, WAY 100,635 (1.0 and 3.0 mg/kg) decreases depression-like behavior in the forced swim test, but these doses do not alter 5-HT release in the medial prefrontal cortex; it does, however, increase serotonin turnover in the cortex, ventral hippocampus and raphe (Rex et al., 2008). It has been proposed that WAY 100,635 increases serotonergic function only when activity in the raphe is high, but not when it is low (Rex et al., 2008). Therefore, the effects of WAY 100,635 geotaxis and scototaxis on these tasks might be due to blockade of 5-HT_{1A} autoreceptors, disinhibiting serotonin release. Nonetheless, treatment with both doses of WAY 100,635 did not modify the effects of exposure to the LDT or the NTT on the extracellular 5-HT levels (Table 3).

In the mammalian dorsal raphe, 5-HT_{1A}Rs are located in the cell bodies of serotonergic neurons, while 5-HT_{1B} receptors are located mainly in axon terminals located at other regions as well as in collaterals sent to raphe neurons (Guimarães et al., 2008, 2010). These receptors have been shown to regulate the activity of SERT (Riad et al., 2000) in the raphe, microinjection of 5-HT_{1B}R agonists decrease extracellular 5-HT levels, but 5-HT_{1B}R antagonists have no effect by themselves (Hagan et al., 2012). Thus, as is the case with 5-HT_{1A} autoreceptors, 5-HT does not seem to produce a tonic inhibition over 5-HT_{1B} receptors (Adell et al., 2002). In LFS zebrafish, treatment with the 5-HT_{1B}R antagonist SB 224,289 produces a hormetic dose–response profile, with smaller doses (2.5 mg/kg) producing a higher reduction of bottom-dwelling in the NTT than the higher dose (5 mg/kg), promoting habituation in the first half of the trial, and increasing homebase time (Maximino et al., 2013b); both doses also decrease erratic swimming. Interestingly, injection of 2.5 mg/kg, but not 5 mg/kg, increased risk assessment in the LDT, without altering other measures; the higher dose did not produce any behavioral effect in this test (Maximino et al., 2013b).

3.2.3. Serotonergic regulation of neuroendocrine stress responses

An interaction between the serotonergic system and the hypothalamic stress axis has been proposed as the basis for stress integration in different species (Lanfumey et al., 2008). While chronic fluoxetine

treatment reduces whole-body cortisol levels in zebrafish (Egan et al., 2009), in chinook salmon *Oncorhynchus tshawytscha* intracerebroventricular injection of corticotropin-releasing factor induces hyperactivity that is potentiated by co-administration of fluoxetine, and the 5-HT_{1A}R antagonist NAN-190 attenuates it (Clements et al., 2003) — suggesting a role for 5-HT_{1A} receptors in that response. In zebrafish and goldfish, 5-HT_{1A} receptors are expressed at all levels of the HPI axis, but the mRNA levels in the preoptic region and the head kidney are 12- to 16-fold higher than in the pituitary (Lim et al., 2013; Norton et al., 2008). In goldfish, intraperitoneal injection of the 5-$HT_{1A/7}$ agonist 8-OH-DPAT (100 and 400 μg/kg) *increases* cortisol levels (Lim et al., 2013), while in Arctic charr 8-OH-DPAT dampens the increase in plasma cortisol caused by handling and injection (Höglund et al., 2002). In both cases, plasma ACTH levels were not altered, and in goldfish 8-OH-DPAT treatment does not alter mRNA levels of *crf* in the preoptic region or *pomc* in the pituitary (Höglund et al., 2002; Lim et al., 2013). In vitro, cortisol release from the goldfish head kidney is increased by 8-OH-DPAT, an effect which is blocked by WAY 100,635 and merely delayed by the 5-HT_7 receptor antagonist SB 258,719; moreover, superfusion with the 5-HT_4R agonist cisapride also increases cortisol release in vitro, an effect which is blocked by the 5-HT_4R antagonist GR 125,487 (Lim et al., 2013). These results suggest that 5-HT_{1A}R- and 5-HT_4R-mediated cortisol responses are due to the activation of these receptors directly in steroidogenic cells in the interrenals, and suggest that the behavioral effects of drugs acting at the 5-HT_{1A} receptor are independent of their neuroendocrine effects.

While 5-HT can mediate cortisol release, glucocorticoids can also modulate the serotonergic system by blocking the uptake$_2$ system (Hill et al., 2011), or by altering the expression of serotonergic proteins such as the 5-HT_{1A} receptor (Ou et al., 2001). Interestingly, the zebrafish mutant *gr*^{s357}, which was identified on the basis of lack of visual background adaptation response (ZBC 1.26) (Muto et al., 2005), emerged as an important model of glucocorticoid–serotonin interactions. In these mutants, a single base-pair change completely disrupts the transcriptional capacity of the glucocorticoid receptor (GR), and therefore the only effects which cortisol can exert are nongenomic (Ziv et al., 2013). Whole-body cortisol levels are elevated in these mutants in a gene dose-dependent way (Griffiths et al., 2012; Ziv et al., 2013); however, these levels are not altered in adult homozygous animals by confinement stress – which elevates plasma cortisol in heterozygous and non-carrier animals – or dexamethasone (25 μM) – which reduces plasma cortisol in heterozygous and non-carrier animals (Ziv et al., 2013). *pomca* transcript levels are also increased in the pituitary of larvae and in the lateral tuberal nucleus of adult *gr*^{s357} animals, and this overexpression is not reduced by treatment with betamethasone-17-valerate (25–30 μM) in larvae and confinement stress in adults (Griffiths et al., 2012; Ziv et al., 2013). *crh* levels are increased in the preoptic region and lateral tuberal nucleus of the homozygous animals in relation to heterozygous, but confinement stress increased *crh* expression in the latter but not in the first (Ziv et al., 2013). These results are highly suggestive of a lack of negative feedback in the HPA axis in *gr*^{s357} mutants, and led the authors to propose these mutants as model organisms to study depression and other affective disorders.

The drawback with that proposal is the behavioral alterations observed in these animals, which resemble anxiety-like states, but have little face validity as assays for depression. *gr*^{s357} larvae show decreased spontaneous activity, without altered circadian activity rhythm, and increased acoustic startle magnitude in relation to wildtype animals (Griffiths et al., 2012). Adult *gr*^{s357} animals show decreased *serta* expression in the superior raphe (Ziv et al., 2013), and treatment of mutant, but not wildtype, larvae with fluoxetine (4.6 μM between 5 dpf and 6 dpf) rescued activity and startle response levels, but did not decrease whole-body cortisol levels (Griffiths et al., 2012). Adult mutants exposed to an open-field test (OFT) show aberrant habituation of freezing responses, which are not different from heterozygotes or wild-type animals at the first day of testing, but do not decrease – and actually *increase* – freezing after three tests separated by 4–7 days (Ziv et al., 2013). Moreover, at the third test, mutants show decreased thigmotaxis in relation to heterozygotes and wild-type animals. Treatment with diazepam (5 μM for 30 min) rescued the freezing response without altering the expression of *gr*, *mr* or *serta* mRNA (Ziv et al., 2013). More interestingly, sub-chronic treatment with fluoxetine (0.8 μM), but not with the catecholamine reuptake inhibitor bupropion (3 μM), also rescued the freezing and wall-avoidance responses, again without normalizing plasma cortisol levels — further reinforcing the hypothesis of independence between behavioral and neuroendocrine effects of these drugs. When animals are exposed to chronic social isolation for 2 weeks, whole-brain *serta* and *mr* mRNA levels were increased in both mutants and control animals, and administration of fluoxetine (0.8 μM) during this protocol blunts these increases and reduces plasma cortisol levels (Ziv et al., 2013). However, as pointed above, *serta* levels are diminished in the superior raphe of *gr*^{s357} animals, while pretectal expression is unaltered, and therefore, it is likely that this longer fluoxetine treatment produces its therapeutic effects by normalizing *serta* in these areas, as well as by attenuating the mineralocorticoid receptor-mediated stress responses.

3.3. Serotonin toxicity in zebrafish?

Excessive serotonergic activity in the central nervous system and at peripheral serotonin receptors – due to therapeutic drug use, drug interactions, overdose, or recreational use of drugs which act mainly at the serotonin transporter – can lead to serotonin toxicity (also called serotonin syndrome) (Boyer and Shannon, 2005). In humans, serotonin toxicity is marked by autonomic (hyperthermia, hypertension, tachycardia, nausea, diarrhea), somatic (overrresponsive reflexes, myoclonus, and tremor) and cognitive symptoms (hypomania, hypervigilance and agitation) (Boyer and Shannon, 2005; Dunkley et al., 2003). SERT knockout mice present the somatic and autonomic alterations, as well as hypolocomotion and increased anxiety-like behavior (Kalueff et al., 2007); moreover, co-treatment of rodents with tranylcypromine and fluoxetine elevates extracellular serotonin levels ~40-fold and evokes serotonin toxicity (Shioda et al., 2004). In zebrafish larvae, treatment with deprenyl from 0 to 5 dpf elevates serotonin levels, at 7 dpf, to about 1000% of control levels, associated with hypolocomotion, tachycardia, and surfacing behavior (Sallinen et al., 2009). This led Stewart et al. (2013) to propose that the effects of SSRIs and MAO inhibitors on the NTT (i.e., decreased latency to top, more top entries, decreased freezing) actually represent the elicitation of surfacing behavior, since no effect was observed, in a wide array of concentrations of racemic fluoxetine in BSF zebrafish, on bottom-dwelling (Stewart et al., 2011c). Moreover, the combination of high concentrations of racemic fluoxetine with an inactive concentration of tranylcypromine or LSD potentiates the behavioral effects of fluoxetine, without altering bottom-dwelling in the case of tranylcypromine (Stewart et al., 2013). Thus, in zebrafish, serotonin toxicity would be characterized by surfacing behavior and decreased freezing, a behavioral profile that was also observed by intraperitoneal injection (Maximino et al., 2013b). In this case, SSRIs and MAOis would not produce an 'anxiolytic' effect in the NTT (Maximino et al., 2012) but rather these alterations would be symptoms of serotonin toxicity. Some complications arise from this thesis, as in at least two cases low, but not higher doses/concentrations, of fluoxetine decrease bottom-dwelling and freezing (Iturriaga-Vásquez et al., 2012; Maximino et al., 2013b). Moreover, at 2.5 mg/kg (the dose which also increases scototaxis; Maximino et al., 2013b), fluoxetine increases extracellular serotonin levels by only ~50% (Maximino, 2014), a value much smaller than that observed after developmental deprenyl exposure (Sallinen et al., 2009) and after co-treatment of rodents with fluoxetine and tranylcypromine (Shioda et al., 2004). These results argue against the hypothesis of serotonin toxicity at low fluoxetine doses, but do not necessarily discard it at *higher* fluoxetine doses, or by the combination of fluoxetine with other serotonergic drugs (Stewart

et al., 2013). At the present moment, both hypotheses – that acute fluoxetine is 'anxiolytic' in the novel tank test, and that this effect actually reflects serotonin toxicity – are weakly supported and lack face and construct validity. Both hypotheses must be tested by analyzing brain serotonin content after these treatments, and would be greatly strengthened by a more thorough behavioral analysis – including using other tests which are thought to model anxiety-like behavior – and especially by the analysis of autonomic arousal, including, e.g., alterations in swimbladder activity, heart rate, circulating catecholamines, or behavioral reactivity (see Section 3.1).

3.4. Serotonin and aggression

In addition to its role in mediating arousal, fear and anxiety, and stress, serotonin has also been implicated in the modulation of zebrafish aggressive behavior. The temporal sequence of agonistic behavior during dyadic fighting is highly structured and somewhat stereotypical (Oliveira et al., 2011). The first phase consists of an appetitive element, with both animals in the dyad exhibiting display (ZBC 1.45), circle (ZBC 1.32), and biting (ZBC 1.17) behaviors; this appetitive phase is usually followed by a resolution phase, in which chases (ZBC 1.28)/flights (ZBC 1.61) ensue.

Treatment with serotonergic drugs has been shown to alter the appetitive phase of aggression in other teleost species. In the Siamese fighting fish *Betta splendens*, chronic (14 or 28 days) treatment with fluoxetine (130 nM, but not smaller or higher concentrations) increased the latency to initiate an aggressive display and decreased the frequency of aggressive behavior in the mirror test (Kania et al., 2012). Acute treatment with fluoxetine (8.7 μM) also inhibits aggressive behavior in male bettas (Lynn et al., 2009). Interestingly, in WIK zebrafish, acute fluoxetine (8.7 or 13 μM) does not alter aggressive behavior, while treatment with WAY 100,635 (37.9 nM) greatly increases it (Filby et al., 2010). In the solitary, territorial weakly electric fish *Gymnotus omarum*, 8-OH-DPAT decreased attack rate, but not display latency; moreover, after administration of this drug the winner could no longer be predicted by size asymmetry. In a resident–intruder paradigm, injection of 8-OH-DPAT did not alter contest outcome, attack rate, or latency of resident *Brachyhypopomus gauderio*, which only shows agonistic displays during the reproductive season (Zubizarreta et al., 2012). While zebrafish are gregarious — and therefore their social ecology resembles more that of *B. gauderio* than *G. omarum* or *B. splendens*, the WIK strain does not show a marked social preference (Barba-Escobedo and Gould, 2012), which could explain the pharmacological similarity with *G. omarum*.

After resolution, all agonistic behaviors are initiated by the winner, whereas the loser displays flight and submissive behavior (ZBC 1.162), including staying near the bottom or top of the tank (Oliveira et al., 2011). Winners show increased serotonin and dopamine turnover in the prosencephalon, while losers show increased serotonin and dopamine turnover in the optic tectum (Teles et al., 2013). Moreover, a negative correlation between 5-HT turnover in the diencephalon and submissive behavior was found (Teles et al., 2013). The outcome of this dyadic fight leads to the formation of dominant–subordinate relationships which are very stable, and have been observed in zebrafish for at least 5 days (Oliveira et al., 2011; Pavlidis et al., 2011). The activity of the serotonergic system is altered by these interactions; dominant animals of the Türku strain show an upregulation of whole-brain mRNA levels for TPH3 (Pavlidis et al., 2011), and dominant WIK male zebrafish, *tph1b* and *htr1aa* are overexpressed in the hypothalamus, while in females *htr1a*, *tph2* (instead of *tph1b*), *mao* and *serta* are overexpressed in this region (Filby et al., 2010). Moreover, in the telencephalon of dominant females both *tph1b* and *tph2* are overexpressed (Filby et al., 2010). On day 5, *tph2*, *serta* and *mao* are overexpressed in the hypothalamus of dominant males in addition to those which were already represented in the first day, and *tph1b* is overexpressed in the telencephalon (Filby et al., 2010). Therefore, at the fifth day synthesis, uptake and metabolism of serotonin should be higher in the hypothalamus, while synthesis should be higher in the telencephalon of dominant animals. In the solitary *Gymnotus omaromum*, however, both dominant and subordinate animals show lower telencephalic 5-HT levels than controls, while in the gregarious *B. gauderio* subordinate males show increased 5-HT levels in the telencephalon in relation to controls (Zubizarreta et al., 2012). Moreover, in subordinate wild-type zebrafish, hindbrain 5-HT turnover is higher than in dominant animals, while no differences are found between dominant and subordinate animals in the forebrain (Dahlbom et al., 2012); in this experiment, however, the forebrain included telencephalon, optic tectum and hypothalamus, and therefore alterations in serotonin activity in the latter area (as predicted by the gene expression experiments of Filby et al., 2010) cannot be assessed.

3.5. Zebrafish models of depression?

Alterations in the brain of subordinate fish are reminiscent of what is observed in rodents in social defeat models (Berton et al., 1998; Huhman, 2006), suggesting that the analysis of behavioral, neural and genomic responses of subordinates can be used to model depression-

Table 4
Behavioral and physiological effects of manipulations which might produce depression-like behavior in zebrafish.

Manipulation	Behavioral effects	Neurochemistry/endocrinology	Reference
CUS	GBT: -Increased geotaxis -Increased coloration -Increased *or* decreased shoaling LDT: -Increased scototaxis	-Decreased brain *gr* -Increased brain *crf* -Increased brain *bdnf* -Upregulation of brain mitochondrial proteins -Increased whole-body cortisol	Piato et al. (2011) and Chakravarty et al. (2013)
Social defeat	N/T	-No effect on brain *gr* or *crf* -Increased whole-body cortisol[a] -Increased hindbrain 5-HT turnover[b] -Decreased brain *tph3* -Decreased hypothalamic *tph1b*, *tph2*, *htr1aa*, *serta and mao* -Decreased telencephalic *tph1b*	Dahlbom et al. (2012), Pavlidis et al. (2011), and Teles et al. (2013)
Immune stimulus	NTT: -Hypolocomotion -Increased immobility -Increased geotaxis Shoaling: -No effect	-No effect on brain purine metabolism -No effect on whole-body cortisol	Kist et al. (2011, 2012)

[a] Difference in relation to controls, but not dominant animals.
[b] Difference in relation to dominant animals.

like states in fish. Some steps have been made towards eliciting depression in zebrafish, including these social defeat models (Dahlbom, 2013; Dahlbom et al., 2012; Teles et al., 2013), HPA axis mutants (Griffiths et al., 2012; Ziv et al., 2013), animals exposed to chronic unpredictable stress (Kist et al., 2011), models of sickness behavior, and animals exposed to learned helplessness paradigms (Lee et al., 2010) (See Table 4). CUS, for example, increases the expression of *crf* and decreases *gr* in the brain of zebrafish, while increasing whole-body cortisol levels, after 7 or 14 days of exposure (Piato et al., 2011). After 7 days of CUS, animals also show increased geotaxis, shoaling and coloration response in the GBT, as well as learning impairments in the inhibitory avoidance task (Piato et al., 2011). Interestingly, though, after 14 days of CUS animals show increased geotaxis and coloration, but either decreased or increased shoaling and hypolocomotion in the GBT (Chakravarty et al., 2013; Piato et al., 2011), increased scototaxis in the LDT (Chakravarty et al., 2013) and no learning impairments (Piato et al., 2011). Besides increased *crf* levels in the brain after 14 days of CUS, *bdnf* mRNA is also increased, as well as mitochondrial proteins (Chakravarty et al., 2013).

Sickness behavior induced by an immune stimulus has been proposed as a model of depression (Dantzer, 2009), and the interfascicular part of the rodent DR has been identified that is highly responsive to peripheral immune system activation (Lowry et al., 2007). Moreover, a peripheral immune stimulus can also increase extracellular 5-HT in the brain (Baganz and Blakely, 2013). In zebrafish, waterborne exposure to MC-LR derived from *Microcystis aeruginosa* increases bottom-dwelling (50 and 100 nM) and immobility time and decreases total locomotion (100 nM), without altering social interaction or whole-body cortisol levels (Kist et al., 2011). In sticklebacks infested with *Schistocephalus solidus*, serotonin turnover is increased in the hypothalamus and brainstem, while 5-HT and NE levels are reduced in the telencephalon (Øverli et al., 2001). So far, the evidence is still weak to judge whether an immune stimulus can emulate depression-like behavior in fish species.

The main difficulty in assessing whether these manipulations can induce depression-like behavior in the zebrafish is methodological, and rests on the lack of behavioral studies with enough construct validity. While it has been argued that chronic elevations in circulating cortisol, associated with hypolocomotion, represent endophenotypes of mood alterations in zebrafish (Kalueff et al., 2014; Nguyen et al., This issue), other dimensions of mood disorders have not yet been assessed. Among these dimensions, it has been argued that the central feature of depression is anhedonia (Anisman and Matheson, 2005), a notably difficult construct to model. In zebrafish, social preference has an hedonic component (Al-Imari and Gerlai, 2008; Barba-Escobedo and Gould, 2012), which could produce complementary evidence for a depression-like state in zebrafish. Moreover, pharmacological isomorphism has not been assessed in these models — for example, in none of these models were SSRIs tested.

3.6. Serotonin and psychedelics

Psychedelic drugs are a subclass of hallucinogens which act primarily by altering serotonergic neurotransmission (Hanks and González-Maeso, 2013); among the effects of such drugs, activation of the 5-HT_{2A} receptor is a common target of lysergic acid diethylamide (LSD), mescaline, and psilocybin. In contrast, dissociatives act as noncompetitive NMDA receptor antagonists, delirants act as competitive muscarinic receptor antagonists, and some stimulants act as SERT inhibitors/transport reversers (Abraham et al., 2002; Hanks and González-Maeso, 2013). In zebrafish, these categories can be discriminated by their behavioral and, to some extent, endocrinological profile (Neelkantan et al., 2013). In the NTT, LSD (which binds to 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}, 5-HT_{5A} and 5-HT_6 receptors, as well as to D_2 dopamine receptors (Abraham et al., 2002; Appel et al., 2004; Hanks and González-Maeso, 2013; Ray, 2010; Roth and Driscoll, 2014)) produces top-dwelling at concentrations above 155 μM and decreases freezing at concentrations above 77.5 μM; in addition to those variables, LSD (775 μM) also increases vertical drift (ZBC 1.184) and freezing in the surface in the observation cylinder and T-maze, and disorganizes behavioral sequences in the observation cylinder (Grossman et al., 2010; Stewart et al., 2011c). An intermediate concentration (310 μM) decreases inter-fish distance in a shoaling test (Grossman et al., 2010), while the highest concentration tested (775 μM) decreases inter-fish distance in this test but does not alter conspecific preference in a social preference test (Green et al., 2012). In the OFT, the same concentration decreases the number of entries in the center without altering time on center or total locomotion (Grossman et al., 2010). Moreover, the same concentration also increases the average duration of entries in the white compartment in the LDT, albeit without altering the time spent in this portion of the tank (Grossman et al., 2010). Finally, LSD increases whole-body cortisol after open-field exposure, but not after exposure to the NTT or the LDT (Grossman et al., 2010; Stewart et al., 2011b). Similarly, mescaline (79 μM), which acts at 5-HT_{1A} and 5-HT_2 receptors, as well as showing dopaminergic activity (Abraham et al., 2002; Appel et al., 2004; Hanks and González-Maeso, 2013; Ray, 2010; Roth and Driscoll, 2014), decreases bottom-dwelling in the NTT and increases the frequency of top swimming episodes, as well as the probability of transition between bottom and top swimming, and decreases inter-fish distance in the shoaling test; moreover, mescaline did not reduce whole-body cortisol levels (Kyzar et al., 2012). Ibogaine, a psychedelic hallucinogen which stabilizes SERT in an inward-facing conformation that is associated with the production of transporter-mediated currents, activates 5-HT_{2A}, 5-HT_{2C} receptors, μ-, κ- and σ-opioid receptors (Abraham et al., 2002; Appel et al., 2004; Hanks and González-Maeso, 2013; Ray, 2010; Roth and Driscoll, 2014) produces a more complex phenotype, decreasing latency to top in the NTT at 32.21 and 64.43 μM, but increasing freezing, locomotion and erratic movements at 32.21 μM. In the NTT, ibogaine also increased the transition probability from top to bottom swimming, from erratic movement to bottom swimming, and from erratic movement to top swimming. In the LDT, ibogaine (64.43 μM) decreases scototaxis; in the OFT, ibogaine (32.21 and 64.43 μM) increases meandering (ZBC 1.97) and increases homebase behavior. This drug also does not alter conspecific preference, but increases inter-fish distance at both concentrations. Moreover, ibogaine increases body coloration at both concentrations, and increases approaches and contacts in the mirror test; in this test, ibogaine also disorganizes behavioral sequences. Finally, despite the marked behavioral alterations, ibogaine does not increase whole-brain *cfos* expression or whole-body cortisol levels (Cachat et al., 2013).

Interestingly, dissociative drugs produce a behavioral phenotype in zebrafish that is qualitatively different from that produced by psychedelics. The most prominent phenotype that is observed is the appearance of "circling" behavior (ZBC 1.32), which is observed after administration of PCP (Kyzar et al., 2012), ketamine (Riehl et al., 2011; Zakhary et al., 2011) or MK-801 (Echevarria et al., 2008; Seibt et al., 2010; Sison and Gerlai, 2011). Notably, serotonergic psychedelics such as mescaline do not seem to produce this effect (Grossman et al., 2010; Kyzar et al., 2012).

Finally, methylene-dioxy-methamphetamine (MDMA, "Ecstasy"), a substituted amphetamine, has been tested in zebrafish (Green et al., 2012; Stewart et al., 2012). Substituted amphetamines induce an inwardly-directed sodium current in the SERT that couples to 5-HT efflux (Hilber et al., 2005). In adult BSF zebrafish, MDMA reduces bottom-dwelling and freezing (207–620.95 μM) and impairs intra-session habituation (51.75–620.95 μM), as well as upregulating *cfos* expression in the brain (Stewart et al., 2012) and increasing inter-fish distance in a shoaling assay (Green et al., 2012). In the weakly electric fish *G. omarum*, MDMA (5 mg/kg) decreases aggressive behavior and spontaneous swimming activity and increases the amplitude of the novelty response (Capurro et al., 1997). D-Amphetamine induces

dopamine, but not serotonin, release via a transporter-mediated mechanism (Leviel, 2001); when administered to BSF zebrafish D-amphetamine (36.98 and 73.96 μM) greatly increases bottom-dwelling, without affecting erratic swimming or freezing (Kyzar et al., 2013); the same is observed with the dopamine transporter blocker methylphenidate (214.3 and 428.62 μM) (Iturriaga-Vásquez et al., 2012), suggesting that the behavioral effects of MDMA are serotonin-specific. Similarly, MDMA treatment (413.97 μM) does not alter skin coloration, while amphetamine treatment (73.96 μM) produces skin darkening (Nguyen et al., 2013).

4. Future directions

The present review attempted to raise some important questions regarding the role of the serotonergic system in behavioral arousal, defensive behavior, aggression and hallucination-like states in zebrafish. In the course of our review, a picture in which a somewhat different neuroanatomical organization of this system is associated with conserved function has emerged. This paradox is not easily solved, and it is probable that this task will necessitate further developments in at least two fields.

The first field is that of model validation. Most of the work reviewed here is theory-agnostic, in the sense that a few selected endpoints are analyzed in terms of drug effect, but very little is known about the measures themselves. For example, homebase behavior is an exploratory strategy that has been observed in rodents (Eilam and Golani, 1989) and zebrafish (Stewart et al., 2010) alike, but whose function is poorly characterized in both species. Since, from a face validity point of view, it resembles perseverative behavior that is observed in obsessive-compulsive disorder, it has been proposed as a model for this disorder (Albelda and Joel, 2012); nonetheless, drugs with an anxiolytic-like profile, as well as hallucinogenic drugs, can increase homebase behavior. In another example, decreased geotaxis can be interpreted either as a reduction in anxiety-like behavior and a serotonin syndrome-like signal (Sections 3.2 and 3.3).

Clearly, while it has advanced greatly since Gerlai's (2003) call for more attention to zebrafish behavior, our understanding of what these behavioral models actually model is very incipient. An important clue to this conundrum might lie in a return to the evolutionary and ecological approach championed by the Blanchards (Blanchard and Blanchard, 1988). While the non-theoretical "data mining" approach to behavioral phenotyping has produced important results (Cachat et al., 2011; Rihel and Schier, 2011; Rihel et al., 2010) and has a great potential for increasing throughput and decreasing anthropomorphism in the selection of behavioral endpoints (Crabbe and Morris, 2004), perhaps now the time is ripe for more attention to the evolutionary, comparative and ecological contexts in which behavior emerges in experimental situations — that is, to embody construct validity within an evolutionary framework (Alleva et al., 1995; Kalueff and Stewart, in press; Kalueff et al., 2014; Maximino et al., 2010b; McNaughton and Zangrossi, 2008; Stewart and Kalueff, in press). The first steps were already made in the direction of understanding stimulus control in commonly used tasks such as the NTT (Ahmed et al., 2012; Blaser and Goldsteinholm, 2012; Blaser and Rosemberg, 2012; Luca and Gerlai, 2012) and the LDT (Blaser and Peñalosa, 2011; Blaser et al., 2010; Maximino et al., 2010a; Stewart et al., 2011b), leading to the conclusion that the LDT best represents an approach–avoidance conflict, while the NTT represents escape from the surface (Maximino et al., 2012). As far as those experiments got, however, we still do not know whether anxiety and fear are different states in zebrafish (Braithwaite et al., 2011; Jesuthasan, 2011; Kalueff et al., 2012).

From the point of view of the pharmaceutical industry, a focus on construct validity might seem costly (van der Staay, 2006; van der Staay et al., 2009; Willner, 1991). History, however, demonstrates that it has paid off in the long term, at least in terms of discoveries relating to the serotonergic system (McNaughton and Zangrossi, 2008; Rodgers et al., 1997). Buspirone and other 5-HT_{1A} agonists did not produce any effect on "classical" spatiotemporal measures of anxiety-like behavior in the plus-maze (i.e., time spent in the open arms) in spite of their clinical efficacy; only when "ethological" measures (e.g., head-dipping, stretched-attend postures) were introduced did the effects appear (Guimarães et al., 2010; Rodgers et al., 1997). It might be the case that the same happens with zebrafish models.

Moreover, the scope of zebrafish models is still very small (Kalueff and Stewart, in press; Kalueff et al., 2014; Norton and Bally-Cuif, 2010). While the tasks described in the present article already provided a plethora of data on the role of serotonin in anxiety/fear and aggression, little is known about other behavioral functions modulated by this monoamine, including functions associated with depression and impulse control. Tasks to assess impulse control (Parker et al., 2013) are already available, but tasks to assess anhedonia or cognitive bias have not yet been explored.

A second field of future development is that of fine-grained functional neuroanatomy. Systemic drug administration does not allow for the differentiation between, for example, pre- and post-synaptic effects of a given drug, which is important given the existence of autoreceptors. In rodents, for example, microinjection experiments determined that 5-HT_{1A} activation is "anxiolytic" in some regions, while being "anxiogenic" in others (Guimarães et al., 2010). Moreover, 5-HT innervation has been observed in fish swimbladder and gills (Jonz and Nurse, 2005; Lundin and Holmgren, 1989; Sundin et al., 1995), which could produce false positives. Techniques for intracerebroventricular administration of drugs (Barbosa et al., 2012; Kizil and Brand, 2011) are available. Moreover, pharmacogenetic ablation of specific structures and projections has been applied to study the role of, e.g., habenulointerpeduncular projections (Agetsuma et al., 2010) and DRN neurons (Yokogawa et al., 2012). Similar constructs could be made by crossing, e.g., *Tg(−3.2pet1:eGFP)*^{ne0214} or *Tg(emrpg:gb:eGFP)* zebrafish with lineages expressing, e.g., tetanus toxin light chain, creating animals with specific lesions in serotonergic nuclei of either the raphe or the hypothalamus; constructs using transgenic expression of nitroreductase could make the lesion time-specific, without leading to developmental compensations. Finally – especially in larvae – light-controlled 5-HT receptors could be engineered, leading to the time- and region-specific activation of such receptors.

Interestingly enough, while zebrafish presents two copies of different genes in the serotonergic system, this duality has yet to be explored by using, e.g., knockdown strategies to understand the role of each phenocopy ('ohnologues') in the control of behavior. mRNA morphants are widely present in zebrafish, and, although their applicability seems to extend more to larval zebrafish, novel alternatives for adult animals can be delivered towards the brain without much difficulty (Kizil and Brand, 2011).

When these hurdles to development are solved, zebrafish promises to be an important model organism in the study of the behavioral and physiological roles of serotonin. Until then, much work still has to be done.

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