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Perspectives on experimental models of serotonin syndrome in zebrafish

Adam Michael Stewart [a,b], Jonathan Cachat [a,c], Siddharth Gaikwad [a], Kyle S.L. Robinson [a,c], Michael Gebhardt [a,c], Allan V. Kalueff [a,c,*]

[a] Zebrafish Neuroscience Research Consortium (ZNRC) and ZENEREI Institute, 1441 Canal Street, Suite 311, New Orleans, LA 70112, USA
[b] Department of Neuroscience, University of Pittsburgh, A210 Langley Hall, Pittsburgh, PA 15260, USA
[c] Department of Pharmacology and Neuroscience Program, Tulane University Medical School, 1430 Tulane Avenue, New Orleans, LA 70112, USA

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ABSTRACT

Serotonin syndrome (SS) is a serious life-threatening disorder associated with elevated brain serotonergic function. With the growing use of serotonergic drugs, SS affects a large portion of general population, becoming a major biomedical concern. SS-like behaviors have also been reported in animals following administration of serotonergic drugs. Although clinical and rodent studies have provided significant insight into the etiology of SS, its exact mechanisms and risk factors remain poorly understood. The need to develop more efficient psychotropic drugs also requires extensive high-throughput screening of novel compounds using sensitive *in-vivo* tests. The use of zebrafish (*Danio rerio*) in neuroscience research is rapidly expanding due to their homology to humans, robust behavioral and physiological responses, genetic tractability, and low costs. Here we discuss the potential of zebrafish models to study SS-related phenotypes induced by selected serotonergic drugs. Overall, zebrafish exposed to serotonergic agents and their combinations exhibit a characteristic top dwelling (surfacing behavior) and hypolocomotion which may represent potential markers of SS-like states in zebrafish. This behavior in zebrafish models positively correlates with brain concentrations of serotonin, suggesting the developing utility of zebrafish (and other aquatic models) for studying SS. Future research is expected to foster high-throughput screening of drug interactions, and pharmacogenetics studies identifying zebrafish mutations implicated in pathological SS-like states.

1. Introduction

Serotonin (5-HT) syndrome (SS, serotonin toxicity or hyperserotonemia), is a serious pathological condition resulting from excess serotonergic activity in the brain (Bodner et al., 1995; Shioda et al., 2004; Gillman, 2006). SS is one of the most important neurotoxic syndromes, and commonly develops due to an overdose of serotonergic antidepressants or interactions between several serotonergic drugs (Boyer and Shannon, 2005; Boyer, 2012). Based on the degree of toxicity, SS manifests clinically as a characteristic triad of neuromuscular, autonomic and mental symptoms, which range from mild to life-threatening, and can develop rapidly, within hours after drug administration (Boyer, 2012). Early symptoms of SS typically include tachycardia, shivering, diaphoresis, mydriasis, tremor and hyperreflexia (Sternbach, 1991; Martin, 1996; Birmes et al., 2003). Progression from mild to moderate serotonin toxicity produces hypertension, fever, and mental status changes (such as confusion, hypomania, anxiety and agitation). Finally, severe SS cases involve muscular rigidity, hypertonicity, hyperthermia, seizures and coma (Sporer, 1995; Mills, 1997; Boyer, 2012).

The drugs evoking SS encompass a wide range of agents (see (Martin, 1996) for review), especially selective serotonin reuptake inhibitors (SSRIs) and monoamine oxidase (MAO) inhibitors (Martin, 1996; Birmes et al., 2003; Sun-Edelstein et al., 2008). SSRIs potently inhibit trans-membrane serotonin transporter (SERT), a critical regulator of brain serotonin levels (Hirano et al., 2005; Gutman and Owens, 2006). MAO is a key enzyme in serotonin metabolism, responsible for oxidative deamination of endogenous and exogenous amines, including serotonin (Gillman, 2006). Thus, inhibition of MAO and/or SERT markedly elevates synaptic concentrations of serotonin (Gillman, 2005, 2006). While SS may also occur following overdose of a single serotonergic agent, life-threatening SS is most often induced through the combination of several agents, especially MAO inhibitors, SSRIs and serotonin agonists or precursors (Sun-Edelstein et al., 2008).

Serotonergic drugs, especially SSRIs, are the most prescribed psychotropic medication (Krasowska et al., 2007; Mandrioli et al., 2012), which due to the lack of more specific therapy are currently used to treat a wide range of disorders from depression to repetitive behaviors (Zohar and Westenberg, 2000; Brambilla et al., 2005; Soorya et al., 2008; Homberg et al., 2010). With the growing use of serotonergic drugs (Spigset, 1999; Isbister et al., 2007), SS is affecting a large portion of general population, thereby becoming a

* Corresponding author. Address: ZENEREI Institute, 309 Palmer Court, Slidell, LA 70458, USA. Tel./fax: +1 240 328 2275.
E-mail address: avkalueff@gmail.com (A.V. Kalueff).

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major emerging biomedical concern (Sternbach, 1991; Martin, 1996; Sun-Edelstein et al., 2008) which requires further extensive clinical and pre-clinical translational research.

2. Current experimental models of SS

Although SS represents a common brain disorder, its pathogenesis remains poorly understood (Hodgman et al., 1997; Parrott, 2002; Shioda et al., 2004). Experimental models have been an indispensable tool in SS research, revealing striking similarities between animal (especially rodents) and clinical SS-like phenotypes (Fox et al., 2007a,b, 2009; Kalueff et al., 2007a,b, 2008, 2010). Typical motor SS-like phenotypes in rats and mice include hypertonicity or rigidity, tremor, forepaw treading, head weaving and twitches, tics/back muscle contraction, flat/low body posture, hind limb abduction and backward gaiting (Kalueff et al., 2007a,b, 2008). Similar to clinical SS symptoms, animal *autonomic* SS-like responses include temperature dysregulation (e.g., hyperthermia), Straub tail, piloerection, hypersalivation, hyperhidrosis and defecation (Kalueff et al., 2007a,b). In addition, paralleling human *mental* SS symptoms (agitation, confusion and hypomania), alterations in rodent motor activity have also been used as a marker of SS-like behavior (Isbister et al., 2007; Kalueff et al., 2007a,b, 2008).

Animal *in-vivo* screens are increasingly used to study SS and the effects of various psychotropic drugs and their interactions. For example, tranylcypromine (TCP; a potent MAO inhibitor) co-administered to mice with fluoxetine (a typical SSRI) elevates extracellular serotonin 40-fold and evokes robust SS-like phenotypes (Shioda et al., 2004). Similarly, administration of a serotonin precursor 5-hydroxy-L-tryptophan (5-HTP) and a MAO inhibitor clorgyline induce prominent SS-like behavior in rats (Nisijima et al., 2000, 2001). Recently, pharmacogenetic animal models of SS have been developed, including mice that lack the serotonin transporter (*SERT)* gene and display elevated extracellular serotonin and hypersensitivity to SS-evoking drugs (Kalueff et al., 2008; Fox et al., 2007b).

While clinical and rodent studies have provided significant insight into SS pathobiology and neurochemistry, there is an urgent need for novel efficient and sensitive experimental models of this disorder (Fox et al., 2007a,b, 2009; Kalueff et al., 2007a,b, 2008, 2010). Expanding the range of animal model species has been recently recognized as a crucial strategy of translational biopsychiatry research (Kalueff et al., 2007a,b), suggesting the potential value of using non-mammalian tests to complement the existing rodent models of SS.

3. Developing zebrafish models of neurotoxic syndromes

Zebrafish (*Danio rerio*) are rapidly gaining popularity in neuroscience research (Grunwald and Eisen, 2002; Bretaud et al., 2004; Guo, 2004; Gerlai et al., 2006; Lau et al., 2006; van der Ven et al., 2006; Yu et al., 2006; Alsop and Vijayan, 2008a,b; Zhdanova et al., 2008). They also have several characteristics that make them particularly suitable for modeling various brain disorders, including neurotoxic syndromes (Panula et al., 2006; Rubinstein, 2006; Barros et al., 2008; Best and Alderton, 2008; Flinn et al., 2008; Kokel and Peterson, 2008; McGrath and Li, 2008) and their pharmacogenetics (Zon and Peterson, 2005). For example, as a vertebrate species, zebrafish share substantial physiological homology with humans, possessing all major brain structures, key neurotransmitters, receptors and hormones (Panula et al., 2006; Alsop and Vijayan, 2008a,b). With the growing utility of zebrafish models (Egan et al., 2009; Maximino et al., 2013; Champagne et al., 2010), assays utilizing larval and adult fish become crucial for genetic research and drug discovery (Schneider, 1992; Yonan et al., 2003; van der Zwaag et al., 2009; Rihel et al., 2010). The widely recognized strength of *larval* models is in their high-throughput nature, ease of genetic manipulations, and well-defined neurological phenotypes (Lockwood et al., 2004; Rubinstein, 2006; Best and Alderton, 2008).

In addition to larval fish, a*dult z*ebrafish models are also becoming widely used to study brain functions and disorders (Egan et al., 2009; Champagne et al., 2010). These models possess high relevance of adult fish physiology to human brain disorders, well-developed physiological systems, sensitivity to environmental challenges, and a rich spectrum of quantifiable neurophenotypes (Nilsson and Fange, 1967; Hausler et al., 1992; Egan et al., 2009; Webb et al., 2009; Cachat et al., 2010; Grossman et al., 2010; Stewart et al., 2010a,b, 2011a,b,c). Mounting evidence indicates that both larval and adult zebrafish are highly sensitive to various serotonergic drugs (Gabriel et al., 2009; Sallinen et al., 2009; Maximino and Herculano, 2010; Stewart et al., 2011a,b,c; Maximino et al., 2013), as well as to pharmacological agents (such as ethanol and opioids (Gerlai et al., 2000; Bilotta et al., 2004; Wong et al., 2010)) that do not directly act on serotonin, but modulate its levels, and therefore can contribute to clinical and experimental SS-like states.

The need to develop more efficient psychotropic drugs also requires extensive high-throughput screening of novel compounds using sensitive *in-vivo* tests. The use of zebrafish in neuropharmacology research is rapidly expanding due to their robust behavioral and physiological responses, genetic tractability, and low costs. For example, based on our 2009–2012 estimates, an average experiment using adult zebrafish is 500–1000 times less expensive compared to mouse or rat study of similar design. Depending on specific laboratory conditions, housing and testing zebrafish for such studies requires ~10–20 times less space, compared to a rodent colony of the same size. Using larval zebrafish can lead to an additional ~10-fold reduction in research costs and space requirements, compared to adult models. Since high costs of preclinical testing are recognized as a major challenge for efficient drug discovery (Dickson and Gagnon, 2004; Rawlins, 2004), the latter practical considerations become an important factor in biomedical research.

Another reason to consider novel zebrafish-based models for SS (in line with famous views that "nothing in biology makes sense except in the light of evolution" (Dobzhansky, 1973)) is the value of targeting evolutionarily conserved 'core' mechanisms when modeling clinical brain disorders in species evolutionarily distant from humans. Based on the importance of evolutionarily conserved pathogenetic traits in translational neuroscience (Kas et al., 2007), we note that optimal animal models of brain disorders must be *evolutionarily relevant,* targeting shared behavioral and physiological phenotypes in a similar manner across multiple species. This approach enables a better focus on core, evolutionarily conserved (and therefore more fundamental and translationally relevant) aspects of brain pathology, including SS. Recognizing the potential of zebrafish models in preclinical and translational neuroscience research, here we discuss the developing utility of zebrafish models to study SS-related phenotypes.

4. Zebrafish serotonergic system

Serotonin is a key modulator of zebrafish normal and pathological brain mechanisms (Wang et al., 2006a,b; Maximino et al., 2011a, 2013, in press). Like other teleost fish, zebrafish possess a well-developed serotonergic system, functionally similar to mammals (Winberg et al., 1997; Arslan and Edmondson, 2010; Stewart et al., 2010a,b; Maximino et al., 2013, in press). The expression patterns, binding, and signaling properties of serotonin receptors and transporters also resemble those in mammals (Panula et al., 2010; Maximino et al., 2013).

A duplication event in the phylogenetic development of teleosts produced numerous genes that code for functional proteins, such as tryptophan hydroxylase (thp), the 5-HT1A receptor, and SERT (Bellipanni et al., 2002; Wang et al., 2006a,b; Norton et al., 2008; Lillesaar 2011; Maximino et al., 2013, in press), all relevant to central serotonergic processes. For example, zebrafish possess three copies of the tph gene (*tph1a*, *tph1b* and *tph2*) encoding thp, the rate-limiting enzyme in serotonin synthesis (Bellipanni et al., 2002). *Tph1a/b* genes are expressed in the pineal gland, retina, hypothalamus, and spinal cord (Bellipanni et al., 2002), while *tph2* is expressed in the raphe, reticular formation and pretectal area (Teraoka et al., 2004). Likewise, the duplicated *SERT* gene is expressed in a complementary fashion with both its isoforms (*SERTa* and *SERTb*) (Wang et al., 2006a,b; Norton et al., 2008) displaying similar pharmacological profile. While *SERTa* is more homologous to mammalian *SERTa* (widely distributed throughout the brain, and binding with high affinity to serotonin reuptake inhibitors), *SERTb* seems to be limited to the medulla and retina, and therefore is less likely to be implicated in the serotonergic drug response (Wang et al., 2006a,b; Norton et al., 2008). Moreover, while there are 14 receptors of serotonin in humans, the two of them (5-HT1a and 5-HT2) most strongly implicated in SS (Sternbach, 1991; Nisijima et al., 2001) are both possessed by zebrafish (Airhart et al., 2007; Norton et al., 2008; Schneider et al., 2012).

Zebrafish also have functional MAO, exhibiting a strong affinity profile for serotonin (Maximino et al., 2013). While two distinct isoforms of MAO (A and B) are present in humans, zebrafish have only one isoform (zMAO) (Anichtchik et al., 2006; Aldeco et al., 2011) which is sensitive to both MAO-A and B inhibitors (Aldeco et al., 2011) and shares high sequential similarity to human MAO-A and MAO-B (Arslan and Edmondson, 2010; Aldeco et al., 2011).

In addition to central serotonergic regulation, the sympathetic system contributes to many of the symptoms associated with SS (Lane and Baldwin, 1997; Mills et al., 2004; Boyer and Shannon, 2005). Noradrenergic pathways are highly conserved in zebrafish, with noradrenaline primarily governing the sympathetic branch of the autonomic nervous system (ANS) in all vertebrates (Vincent et al., 1998; Maximino and Herculano, 2010). Importantly, vertebrate sympathetic noradrenergic transmission is modulated by serotonin, and has a demonstrated sensitivity to various serotonergic drugs (Meehan et al., 1986; Szabo et al., 1999; Szabo and Blier, 2001, 2002). Zebrafish also demonstrate β-adrenergic control of their heart rate, and actively regulate cardiac output to changes in their environment using both the parasympathetic and sympathetic branches of the ANS (Mann et al., 2010). Given the importance of cardiac responses in clinical SS (Lane and Baldwin, 1997; Mills et al., 2004; Boyer and Shannon, 2005), and the unique ability of zebrafish models to allow non-invasive video-recording of their cardiac activity in free-swimming larval and adult fish (Milan et al., 2006; Chan et al., 2009; Mann et al., 2010), the possibility of using neuropharmacological *in-vivo* zebrafish screens combined with assessing relevant physiological SS-like responses, becomes particularly promising.

5. Potential SS-like phenotypes in zebrafish

Despite the growing use of zebrafish in biomedical research, establishing aquatic/zebrafish models of SS is a challenging task. Conceptually, the validity of such models would derive from the characterization of SS-like states and phenotypes, dissecting them from other phenotypes which may overlap in behavioral profile (e.g., anxiety or sedation), finding similarity between zebrafish and rodent/clinical phenotypes, and showing the serotonergic nature of identified responses.

Considering the history of SS research may be an interesting starting point for this discussion. While rodent serotonin-related behavior has long been recognized in the literature in mid-20th century (Woolley et al., 1957; Bogdanski et al., 1958; Brodie et al., 1960), the first clinical symptoms of excess serotonin were only reported in 1960 (Oates and Sjoerdsma, 1960) in patients receiving a MAO inhibitor and serotonin precursor tryptophan. In 1982, T. Insel's group first used the term "SS" to formally describe clinical effects of serotonin excess and introduce SS as a specific brain disorder (Insel et al., 1982). After these two clinical works, "connecting the dots" between animal and human phenotypes became a logical next step, enabling numerous subsequent studies to recognize SS as a common pathology, and establish translationally relevant parallels between clinical SS and animal SS-like behaviors (Spanos and Yamamoto, 1989; Mills, 1997; Kalueff et al., 2007a,b; Sun-Edelstein et al., 2008; Shioda et al., 2010; Fox et al., 2011).

In animals, the exact SS-like behaviors and their quantification has been a subject of long-term debate, with rodent and primate models generally satisfactory mimicking motor and autonomic human SS responses (see (Kalueff et al., 2007a,b) for detailed review). Thus, what can be SS-related responses in zebrafish? To address this question, we have performed a thorough unbiased analysis of data on various phenotypes evoked in zebrafish by serotonergic agonists and drugs known to potentiate human and animal serotonergic system (Table 1). These analyses produced an extensive repertoire of experimental data, consistently demonstrating marked surfacing behavior, hypolocomotion and elevated serotonin levels in adult zebrafish following treatment with different serotonergic drugs (Table 1). Based on this evidence, we suggest that such responses may represent SS-like phenotypes in adult zebrafish models. Moreover, we have previously discussed the unique advantage of zebrafish models based on the availability of both larval and adult zebrafish models. Are potential SS-like responses similar between adult and larval zebrafish? Mounting evidence again suggests that this may be the case, since, for example, a similar surfacing and hyperserotonergic phenotype as well as developmental neurotoxicity has been observed in larvae following MAO inhibition (Jie et al., 2009; Sallinen et al., 2009) (Table 1).

In addition to SS-like effects of high doses of serotonergic drugs (known to evoke SS in humans), zebrafish evidence further supports the promise of these models to study potential drug interactions known to contribute to clinical SS. For example, serotonergic drugs that do not evoke surfacing behavior by themselves (e.g, low doses of tranylcypromine (TCP) (Stewart et al., 2012), mescaline (Collins, 2012) or 3,4-methylenedioxy-*N*-methylamphetamine (MDMA) (Stewart et al., 2011a,b,c)), may do so in combination with each other – exactly as in clinical instances of SS. For example, many hallucinogens increase serotonin activity and may interact with other serotonergic agents, such as SSRIs or MAO inhibitors, to evoke clinical SS (Parrott, 2002; Vuori et al., 2003) or animal SS-like states (Silbergeld and Hruska, 1979; Bankson and Cunningham, 2001). As shown in Figs. 1 and 2, a lower dose of fluoxetine (an SSRI) coupled with TCP (blocking the degradation of serotonin (Jie et al., 2009)) or low dose LSD (drug acting as an agonist on several serotonin receptors (Wing et al., 1990; Backstrom et al., 1999)), can produce the SS-like top dwelling, otherwise not present in zebrafish if either drug acted alone.

As already mentioned, similar to mammals, zebrafish possess a β-adrenergic control of heart rate and a robust sympathetic response (Vincent et al., 1998; Maximino and Herculano, 2010). For example, the tachycardia (that accompanies clinical SS) has been shown to be specifically mediated by the sympathetic response in zebrafish (Mann et al., 2010). Paralleling sensitivity of mammalian noradrenergic transmission to serotonergic drugs (Meehan et al., 1986; Szabo et al., 1999; Szabo and Blier, 2001, 2002), zebrafish show a similar response. Notably, the increased heart rate in larvae

Table 1
Summary of serotonergic phenotypes observed in larval (L) and adult (A) zebrafish, including marked surfacing behavior, hyperlocomotion and elevated serotonin (see similar profiles in other aquatic species in Table 2). SERT – serotonin transporter, SSRI – selective serotonin reuptake inhibitor, SRI – serotonin reuptake inhibitor, MAO – monoamine oxidase, ↑ – increased, 0 – no effects, ↓ – decreased.

Agent	Pharmacological profile	Exerted effects			Models, additional phenotypes and references
		Top dwelling	Hyper-loco-motion	Brain serotonin levels	
Buspirone	5-HT_{1A} agonist	↑A			Novel tank (Bencan et al., 2009, Maximino et al., in press) and group behavior task (Gebauer et al., 2011)
Lysergic acid diethylamide (LSD)	5-HT agonist	↑A*			*Novel tank: shorter latency to enter the top, more top transitions, top duration, and reduced freezing duration following acute exposure (Grossman et al., 2010; Stewart et al., 2011a,b,c); increased top duration following repeated exposure (Stewart et al., 2012)
Mescaline	$5HT_{1A/2A/B/C}$ agonist	↑A	↓ A		Novel tank: increased top transitions, also reducing immobility and disrupting the patterning of swimming in a dose-dependent manner (Kyzar et al., 2012)
Quipazine	Non-selective 5-HT agonist		↓L*		*Increased the number of swimming episodes with normal duration and tail-beat frequency in larvae (Brustein et al., 2003)
SB 224289	5-HT_{1B} agonist	↑A			Novel tank: increased top entries and time in top (Maximino et al., in press)
3,4-methylene-dioxy-N-methyl-amphetamine (MDMA)	5-$HT_{1A/2A}$ agonist; SERT blocker	↑A*			*Novel tank (at higher doses 40-120 mg/L); also increased brain *c-fos* expression (Stewart et al., 2011a,b,c)
Fluoxetine	SSRI	↑A* (acute) 0 or ↑A*** (chronic)	↑L** (acute) ↓L**** (chronic)		*More top entries and less freezing (Egan et al., 2009; Wong et al., 2010). **Down-regulated SERTa/b and 5-HT_{1A} receptors in the spinal cord, but not in the brain (Airhart et al., 2007); ***Inactive after immersion (Stewart et al., 2011a,b,c), but not after intraperitoneal injection (Maximino et al., in press); ****following high dose (10 mg/kg) (Maximino et al., 2011b, Maximino et al., in press)
Fluoxetine + LSD	SSRI + 5-HT agonist	↑A			Fig. 2
Citalopram	SSRI	↑A			Novel tank (without anxiolytic behaviors in plus maze) (Sackerman et al., 2010)
Desipramine	Non-selective SRI	↑A			Novel tank (without anxiolytic behaviors in plus maze) (Sackerman et al., 2010)
Deprenyl	MAO inhibitor	↑L*	↑L	↑L	*Increased vertical (surface) place preference in cylindrical chamber; also increased heart rate (Sallinen et al., 2009)
Selegiline	MAO-B inhibitor		↑L*	↑L	*Spontaneous locomotion, postural impairment, and increases heart rate in larvae (Sallinen et al., 2009)
Tranylcypromine (TCP)	MAO inhibitor	↑A*	↑L**		*Novel tank: shorter latency to top, increased top transitions, and reduced freezing duration following acute (Stewart et al., 2011a,b,c) and chronic (Stewart et al., 2012) exposure; **developmental neurotoxicity with "sluggish" locomotion (i.e., slow swim and slow escape action) (Jie et al., 2009)
TCP + fluoxetine	MAO inhibitor + SSRI	↑A			Fig. 1
Ethanol	Positive modulator of serotonergic activity	↑A	↑A	↑A*	Novel tank (Egan et al., 2009; Stewart et al., 2010a,b; Cachat et al., 2011; Mathur and Guo, 2011), dose-dependent hypolocomotion (Gerlai et al., 2000; Gerlai et al., 2006; Mathur and Guo, 2011); *following high dose (1.00%) of ethanol (Chatterjee and Gerlai, 2009)

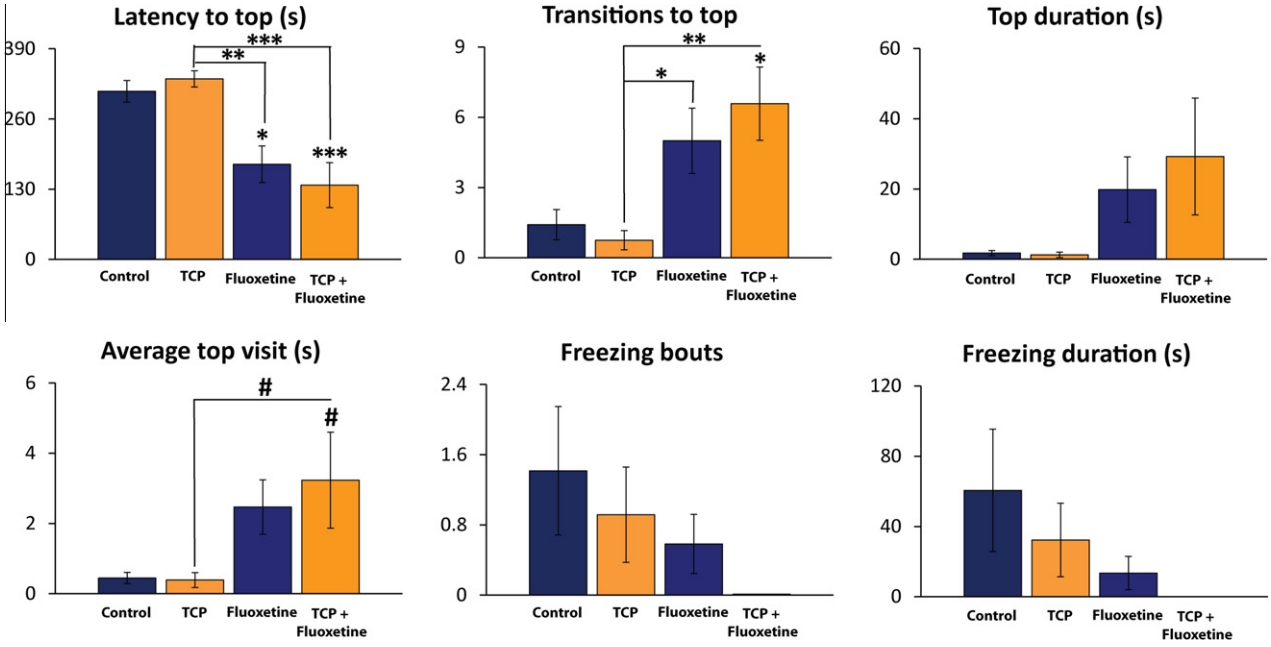

Fig. 1. Effects of acute (60 min) fluoxetine (1.2 mg/L) and tranylcypromine (TCP, 1 μg/L) exposure on zebrafish behavior in the novel tank test. Data are presented as mean + SEM ($n = 12$ per group), $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$, vs. control; post-hoc Tukey test for significant ANOVA data. Adult (5–7 months) male and female wild type short-fin zebrafish were housed in groups of 20–30 fish per 40-L tank, with tanks filled with filtered facility water maintained at 25–27 °C. Behavioral testing was performed between 11.00 and 15.00 h using tanks with water adjusted to the holding room temperature. Following pre-treatment with the experimental drug, zebrafish were subjected to the 6-min novel tank test, a 1.5-L trapezoidal tank (15 height × 28 top × 23 bottom × 7 cm width), resting on a level stable surface, and divided into two equal virtual horizontal portions (top and bottom) by a line marking the outside walls (Egan et al., 2009). Zebrafish behavior was recorded by trained observers (inter-rater reliability >0.85), who were blinded to the treatments and manually scored different behavioral endpoints, including the latency to the upper half (s), transitions to, and time spent (s) in the top, and the frequency and duration (s) of freezing bouts, as described previously (Egan et al., 2009; Grossman et al., 2010). Average top visit (s) was also assessed, calculated as the top duration divided by the number of top entries. Drug exposure was performed by immersing individual zebrafish in a 1-L plastic beaker prior to the testing in the novel tank. Drug doses and treatment times were chosen based on previous studies conducted by our group demonstrating the behavioral effects of these compounds. Control fish were exposed to drug-free vehicle for the same treatment time. Exposure to 1.2 mg/L fluoxetine and 1 μg/L TCP evoked a robust surfacing behavior following acute administration, with shorter latency to enter the top ($F_{(3,\ 44)} = 10.917$, $P < 0.005$), increased number of top entries ($F_{(3,\ 44)} = 6.3433$, $P < 0.001$), and longer average top visit duration ($F_{(3,\ 44)} = 3.256$, $P < 0.05$). Post-hoc analysis revealed that the number of top entries and average top visit were only significantly different vs. controls in fish administered both fluoxetine and TCP in combination, while fish exposed to only fluoxetine or only TCP showed markedly less surfacing behavior.

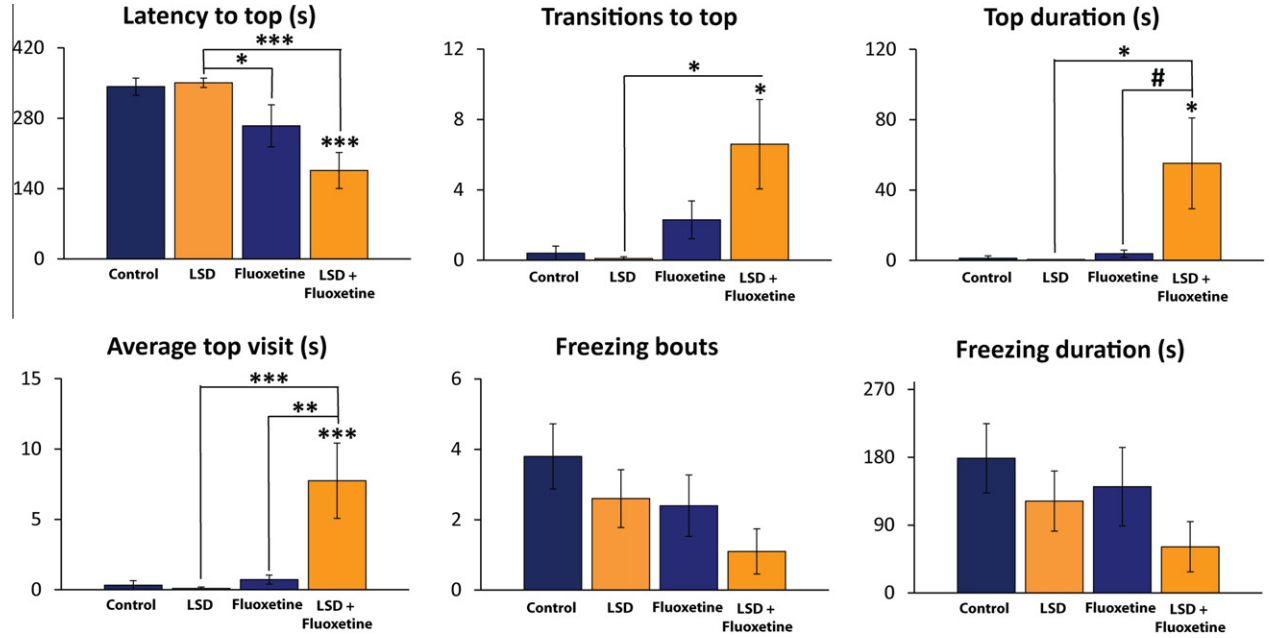

Fig. 2. Effects of acute (60 min) fluoxetine (1 mg/L) and lysergic acid diethylamide (LSD, 15 μg/L) exposure on zebrafish behavior in the novel tank test (experimental details as described in Fig. 1). Data are presented as mean + SEM ($n = 9$-10 per group), $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$ vs. control; post-hoc Tukey test for significant ANOVA data. Exposure to 1 mg/L fluoxetine and 15 μg/L LSD evoked a robust surfacing behavior following acute administration, with shorter latency to enter the top ($F_{(3,\ 35)} = 9.95$, $P < 0.005$), increased number of top entries ($F_{(3,\ 35)} = 4.53$, $P < 0.01$), greater time spent in the top ($F_{(3,\ 35)} = 4.12$, $P < 0.05$), and longer average visit duration ($F_{(3,\ 35)} = 7.167$, $P < 0.001$). Post-hoc analysis revealed that these endpoints were only significantly different vs. controls in fish administered both fluoxetine and LSD in combination, while fish exposed to only fluoxetine or only LSD showed markedly less surfacing behavior.

Table 2
Summary of serotonergic phenotypes in various aquatic species zebrafish, including surfacing behavior and hypoactivity (see Table 1 for similar profiles in zebrafish). Ac - acute treatment, Ch – chronic treatment, ↑ – increased, ↓ – decreased.

Agent	Species	Exerted effects		Models, additional phenotypes and references
		Top dwelling	Hypo-locomotion	
Serotonin	Betta splendens		*Ac	*Reduced aggression (Clotfelter et al., 2007)
8-OH-DPAT (5-HT1A agonist)	Betta splendens		*Ac	*Reduced aggression (Clotfelter et al., 2007)
LSD (5-HT agonist)	Betta splendens	↑Ac, Ch		(Abramson and Evans 1954; Abramson et al. 1963)
	Betta splendens	↑Ac	↑Ac	(Arbit 1957)
	Betta splendens		↑Ac	(Chessick et al. 1964)
	Guppy, white cloud		↑Ac	(Chessick et al. 1964)
	Large carp	↑Ac		(Abramson et al. 1962)
	Goldfish	↑Ac		(Gettner et al. 1964; Gettner et al. 1973)
Methysergide (partial 5-HT agonist, antagonist at some 5-HT receptors)	Goldfish, betta splendens	↑Ac		(Gettner et al. 1964; Gettner et al. 1973)
MDMA (5-$HT_{1A/2A}$ agonist; serotonin transporter blocker)	Weakly electric fish		↑Ac	(Capurro et al. 1997)
Fluoxetine (SSRI)	Striped bass	↑Ch		(Gaworecki and Klaine 2008)
	Mosquitofish	↑Ch	↑Ch	(Henry and Black 2004; Henry and Black 2008)
	Betta splendens		*Ac, Ch	*Reduced aggression (Lynn et al., 2007)
	Chinook salmon		↑Ch	(Clements and Schreck 2007)
	Coral reef fish		*Ac, Ch	*Reduced aggression (Perreault et al. 2003)
	Goldfish		↑Ch	(Beulig and Fowler 2008)

following treatment with MAO inhibitors such as deprenyl and selegiline, coupled with a hypersertonergic phenotype (Sallinen et al., 2009), suggest a response profile consistent with clinical (Lane and Baldwin, 1997; Mills et al., 2004; Boyer and Shannon, 2005) and rodent (Nisijima et al., 2000, 2001; Shioda et al., 2010) reports on SS.

In addition to behavioral and autonomic responses potentially relevant to SS in zebrafish models, it is critical to parallel the observed phenotypes with well-validated physiological biomarkers of SS, such as elevated brain serotonin. From this point of view, demonstration of elevated levels of extracellular serotonin, such as through high precision liquid chromatography (HPLC), becomes critical for validating an SS-like phenotype. In line with this notion, HPLC findings in mouse models of SS consistently show elevated in brain extracellular serotonin in response to treatment with serotonin-enhancing drugs (Fox et al., 2008, 2011). HPLC neurotransmitter analyses in zebrafish have traditionally been limited due to their small brain size (rendering the amount of neurotransmitter one needs to detect as quite low). However, recent studies have demonstrated the applicability of HPLC assays to zebrafish (Lopez Patino et al., 2008; Chatterjee and Gerlai, 2009; Sallinen et al., 2009), and were recently extended to their responses to serotonergic modulation, revealing a marked increase or decrease in extracellular 5-HT content depending on the drug and behavioral challenge used (Maximino et al., in press). From this perspective, positive correlation between zebrafish surfacing and brain concentrations of serotonin is consistent with the noted utility of zebrafish for modeling SS.

6. SS-like states in other aquatic species

As already mentioned, the brain serotonergic systems are highly conserved among vertebrates (Winberg et al., 1997; Panula et al., 2006), which becomes particularly relevant to dissecting shared behavioral and physiological serotonin-related responses in fishes. Are there parallels between behavioral responses to serotonergic drugs in zebrafish and other fish species? Mounting evidence (summarized in Table 2) strongly supports their high sensitivity to serotonergic drugs, and the relevance of suggested fish phenotypes (increased surfacing with hypoactivity; Table 1) to SS-like states, revealing a striking similarity across fish species. Together with evidence of toxic effects of serotonergic drugs in various aquatic organisms (Brooks et al., 2003; Perreault et al., 2003; Henry and Black, 2008; Morando et al., 2009; Winder et al., 2009), this further supports a rapidly growing potential of zebrafish and other aquatic models in SS-related research.

7. General discussion and future directions of research

As a potentially life-threatening condition, combined with the increasing use of serotonergic drugs, SS warrants increased attention. With a myriad of pharmacologic agents that can potentially cause SS, constructing useful animal models is a key step in understanding the activities of various implicated drugs. Zebrafish can greatly enhance this effort due to their as role as practical and sensitive models. Our analyses suggest that the surfacing behavior and hypolocomotion, commonly observed in fish following serotonergic drug exposure (Tables 1 and 2), may represent a characteristic behavioral phenotype associated with elevated serotonin levels – the hallmark of SS-like states in humans and rodents. While increased top dwelling in the novel tank may also indicate anxiolytic profile and increased exploration, the surfacing behavior exhibited here represents an SS-like state in the context of a more complex syndrome. For example, while several serotonergic drugs (e.g., fluoxetine, SB 224289) evoke marked surfacing behavior in the novel tank, they also reveal an anxiogenic response at the same dose in the light/dark box (Maximino et al., in press), which may be in line with agitation, confusion and anxiety frequently observed (as part of 'mental' symptoms) in clinical SS (Karki and Masood, 2003; Boyer, 2012). Likewise, in several larval assays, the reported increased levels of extracellular serotonin were also accompanied by a sympathetic response (i.e., increased heart rate), suggesting an autonomic mobilization incompatible with reduced anxiety (Sallinen et al., 2009), but strikingly resembling tachycardia observed in clinical SS (Sternbach, 1991; Isbister et al., 2007; Boyer, 2012).

Dissection of surfacing behavior often reveals a phenotype characterized by a marked top dwelling duration exhibited apart from several other typically covarying novel tank measures. For example, reduced bottom swimming and immobility leading to a distinct surfacing behavior, is coupled with a reduction in the number of top entries, such as in combined fluoxetine and TCP exposure (Fig. 1) as well as high dose MDMA treatment (Stewart et al., 2011a,b,c). Collectively, this raises the possibility of using

the 'average duration of top entry', assessed as the total top duration divided by the number of top entries, as an additional behavioral parameter relevant to SS (see details in Figs. 1 and 2).

Of considerable importance is the choice of drugs that can reveal the zebrafish potential to model SS. For example, the dose for LSD (15 μg/L, Fig. 2) was markedly below the typical dose necessary to evoke a hallucinogenic effect (e.g., 150–250 μg/L), thus the stereotypic "trance-like" state was not produced here. Instead, the robust surfacing behavior evoked appears to be due to the innate neurotoxic effects of the serotonergic drug overdose, as opposed to a disorientation or hallucinogenic-like state. While not addressed here, the potential role of non-serotonergic drugs in modeling SS-like states should also be considered. For example, methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a neurotoxin which depletes striatal dopamine levels, and has been shown to increase levels of serotonin as well as evoke SS-like motor responses in monkeys (Boulet et al., 2008), also producing a marked surfacing behavior in zebrafish (Bretaud et al., 2004).

In addition to the above-mentioned SS-like behaviors, incorporating additional potential biomarkers may be necessary. For example, skin coloration responses (mediated in various species by melanophore aggregation/dispersion) emerge as promising tools in drug discovery and high-throughput screening (Dooley et al., 1994; Jayawickreme et al., 1998; Carrithers et al., 1999; Virador et al., 1999). Since serotonin is associated with the dispersion of melanophores in various vertebrate species (e.g., amphibians) (Miller, 1989; Potenza and Lerner, 1994; Teh and Sugden, 2001), and given pigment changes in fish exposed to drugs acting on the serotonergic system (Abramson and Evans, 1954; Arbit, 1957; Cachat et al., 2012), the development of pigment-sensitive zebrafish *in-vivo* screens may reflect serotonergic toxicity and SS-like states. Furthermore, genetic animal models may prove particularly useful for SS-related neurophenotyping. For example, transgenic zebrafish expressing highly visible melanophores, have been recently established as a potential model of Alzheimer's disease (Newman et al., 2010). Thus, the development of a pigment-sensitive transgene line of zebrafish may further enhance zebrafish behavioral models of SS. Moreover, given the ease of genetic manipulations in this species, zebrafish-based models may also be valuable for investigations into the genetic mechanisms and receptor mediation underlying the behavioral sensitivity to serotonin-enhancing drugs, similar to rodent SS models (Fox et al., 2007a,b, 2010; Kalueff et al., 2007a,b).

Future zebrafish SS models may also help high-throughput screening of novel serotonergic compounds and identifying their potential molecular targets. For example, recent clinical investigations have focused on establishing multi-affinity profiles for a wide array of serotonergic drugs relative to various receptors, transporters, and ion channels (Jensen and Roth, 2008; Ray, 2010). Application of *receptorome* and other 'omics'-based approaches to zebrafish research, to characterize the diverse patterns of drug interactions with different classes of receptors, will aid the identification of zebrafish mutations implicated in pathological SS-like states, as well as help establish their sensitivity profiles to drugs involved in evoking such states (Jensen and Roth, 2008; Setola, 2009; Ray, 2010; Stewart et al., 2011a,b,c).

Finally, SS-based zebrafish models may also be used to solve other biomedical tasks. For example, the lack of novel, more efficient and selective psychotropic drugs is currently recognized as one of the main challenges in contemporary biomedicine (Spedding, 2006; Agid et al., 2007). Thus, while potent serotonergic compounds are more likely to produce adverse toxic effects (such as SS), zebrafish SS-related screens may be used to solve an opposite problem – enable a more targeted serotonergic drug discovery based on identifying agents most potently evoking SS-like fish phenotypes. Likewise, zebrafish SS models may help uncover novel therapeutics for treatment of serotonin toxicity. For example, in severe cases of SS requiring rapid intervention, chlorpromazine is the only 5-HT2A antagonist available for i.v. use in humans. However, while its administration can aggravate the cardiotoxic or epileptogenic properties of other drugs (e.g., venlafaxine or tricyclic antidepressants) (Gillman, 2005), safer treatment alternatives are needed, and their search may be fostered by active use of sensitive preclinical aquatic models such as zebrafish. In line with this, SS-based zebrafish screens may also reveal potentially valuable adjunctive treatments for use with standard 5-HT2A antagonists, perhaps complementing or replacing current adjunctive agents, such as benzodiazepines and beta blockers (Koniari et al., 2008).

In summary, this report outlines rationale and conceptual framework for developing experimental models of SS in zebrafish and other aquatic species. The utility of this model and its behavioral biomarkers is in their potential application as a sensitive and high-throughput tool for screening novel serotonergic drugs and/or zebrafish mutants potentially relevant to the development of serotonin toxicity.

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