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Research report

Modeling PTSD in the zebrafish: Are we there yet?

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HIGHLIGHTS

- PTSD is a rapidly growing anxiety disorder with deleterious symptomology.
- There is not an all encompassing, efficacious treatment for the disorder.
- We provide a brief overview of PTSD using human and rodent models.
- We summarize the available literature on stress using the zebrafish model.
- We present the benefits of creating a PTSD paradigm using zebrafish.

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ABSTRACT

Post-traumatic stress disorder is an anxiety disorder that can develop following one or more traumatic events that threaten one's safety or make the victim feel helpless. Currently there are an increasing number of cases in the population in part due to the number of soldiers returning from combat. The disorder is characterized by symptoms that include hypervigilance, sleep disturbances, social and cognitive degradation, and memory flashbacks. Most of the research has been centered on the human and rodent as subjects but recently another viable contender has emerged – the zebrafish (*Danio rerio*). The zebrafish is a strong comparative model with the ability to exhibit a wide variety of behaviors, complex learning, and neurobiological changes that can be extrapolated to the human condition. The zebrafish is an ideal organism to study pharmacological treatments as well as the neurological underpinnings of the disorder. Here we review a sampling of the human and rodent model literature on post-traumatic stress disorder focusing on symptomology, current treatments, and stress paradigms. We also make the argument for the inclusion of the zebrafish model in future studies investigating the causes, symptoms, and treatments of post-traumatic stress disorder.

1. Introduction

The discussion of post-traumatic stress disorder (PTSD), a debilitating anxiety disorder, is becoming more prevalent within the scientific literature [1,2]. With large numbers of soldiers returning from combat exhibiting symptoms of the disease, and a 6.8% lifetime prevalence among citizens from the United States, the need for research into not only the treatment of the disorder but also the etiology of PTSD has become paramount [3]. PTSD is an affliction that is characterized by the development of negative symptoms after experiencing or witnessing a traumatic event. Such symptoms include flashbacks, nightmares, emotional numbing, hypervigilance and arousal, and sleep disturbances (DSM-V; [4]). PTSD is often co-morbid with depression, substance abuse, altered sleep patterns, decreased cognitive abilities, and memory impairment [5].

While research into the topic is ever increasing, there remains the need for more exploration as a truly effective treatment, especially with regards to co-morbid disorders, remains elusive. Much of the research on the disorder is focused on the human condition: testing for biomarkers, moderators, co-morbid disorders, various symptomology, and potential treatments [6–8]. Additionally, researchers have also examined PTSD using several rodent models (Table 1). The use of rodents has allowed for a more extensive look into not only possible pharmaceutical treatments of the disorder but also the neurobiological underpinnings of the disease.

In recent years the zebrafish has gained favor as an additional model organism, in the study of both diseased and normal states. Zebrafish display robust behaviors, which have become increasingly simple to quantify due to advances in video-tracking tools.

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Table 1
Rodent stress tasks.

Task	Definition/procedure	Reference(s)
Attentional Set Shifting	A rat is placed into a start box before it is allowed to enter the testing environment. Using small terra-cotta pots that are filled with different material and scented with different fragrances, a rat must dig through the pots to get to a food reward, such as a cheerio. The object of the task is to learn which medium and which scents are connected to the reward. Once the rat learns these cues, the cues are changed and the rat must learn the new reward producing combination. The task can go through multiple rule changes, which forces the rat to change to a new cognitive set and forget the previous one. Such a cognitive change is rather demanding and relies on functioning frontal brain areas. Stress mediated performance deficits have been reported.	[66,71]
Elevated Plus Maze	A rat is placed into an elevated plus shaped maze with two open arms and two that are closed in by walls. The rat is then given time to explore the maze. Naturally, when given the choice to explore an open alley in a maze and a closed arm in the maze, the animal will spend significantly more time exploring the closed arm. The model focuses on the inner conflict in the rat to want to explore all areas and their innate fear of open spaces. Anxiolytic treatments tend to result in more open area exploration.	[64,65]
Fear Conditioning	A rodent is placed into a novel environment and is allowed to explore for at least 27 s. After this exploratory period has passed, a moderately painful foot shock is administered. The shock cannot be administered immediately upon entering the environment, as a sufficient amount of time must be given to allow for true conditioning to occur. In doing this, the fear of receiving the foot shock is associated with the new environment. By re-exposing the rat to the environment where the shock was administered, symptoms of PTSD are thought to be simulated.	[62]
Inescapable Tail Shock	The rat is restrained in a ventilated tube and receives 40 tail shocks at random intervals of 150–210 s over a 2-h period. These sessions happen once a day for 3 consecutive days.	[63]
Light/Dark Exploration Test	After a 1 h habituation to the testing room in their home cage, a rat is placed individually into the center of the white compartment facing the dark compartment of the L/D test apparatus, an open box with 1/3rd painted black (illuminated by dim red light) and 2/3rd painted white (illuminated by bright white light). These two areas were separated by a wooden partition. A 5 min testing session commenced where the rat was videotaped and the amount of time spent in each area was quantified. Increased stress would have the rat spending more time in the dark area of the box.	[70]
Novelty-Induced Hypophagia	After a training period of eight days where the rat is familiarized with a certain food such as graham cracker, the rodent is placed into a novel environment, one in which its never been before, for 15 min. The rat is then presented with the familiar graham crackers and allowed to eat. After the 15-min session, food intake is then measured by weighing the food bowl before and after testing. Hypophagia (reduction of feeding) is said to occur as an anxiety response to the novel environment. One of the benefits of this test is that substances such as antidepressants can be tested in relation to the anxiety response.	[67,68]
Social Interaction Test	Two rats of similar build, one experimental and one stimulus, are placed at opposite corners facing away from each other in a wooden box. For 5 min, the amount of time the experimental rat spends engaged in social behavior with the stimulus rat is collected. Anxiogenic treatments tend to decrease social interaction time.	[66]
Single Prolonged Stress	Rats are exposed to an extreme, singular stressful event. Such as being restrained for 2 h. After the restraint period, the rats, in groups of eight, are forced to swim for 20 min in 24 °C water. After forced swim, the rats are rendered unconscious via ethyl ether anhydrous. The rats regain consciousness in 5–15 min and then are returned to their housing unit. Their behaviors are quantified no less than seven days after the exposure. Typically, reactions are behavioral (flashbacks, hyperarousal, and avoidance of similar situations) and neurochemical (HPA axis).	[60,61]
Stress Re-Stress Model	Similar to the SPS task, the rats are restrained, forced to swim, and rendered unconscious via ether. Unlike the SPS task, they are then made to endure the same stressful event again seven days later.	[72]

They have a well-characterized, simple nervous system (compared to humans) and a fully characterized genome displaying a nucleotide sequence similar to most other vertebrates [9]. As such, zebrafish are a viable model for exploring the molecular and genetic mechanisms of behavior in addition to being a powerful comparative model to better understand PTSD [10–12]).

2. Human model

2.1. Symptomology

The manifestation of the PTSD is quite extensive and the diagnosis can include deleterious changes to memory and/or anxiogenesis. The symptoms most often associated with PTSD are flashbacks, nightmares, emotional numbing, increased startle response, and sleep disturbances (DSM-V; [4]). In addition, there is often an accompanying decline in working memory and, more specifically, emotional memory [8].

Cognitive deficits may be associated with not only neural networks that control emotional responses, but also the prefrontal cortex (PFC; [13]). Vasterling et al. [14] suggests that PTSD may have some connection to the frontal lobe, as those who suffer from PTSD perform in a fashion similar to frontal lobe injury patients on cognitive tasks. A decline in cognitive functioning may be related to stress induced malfunctioning of monoamine neurotransmission in the PFC [15]. In addition, Honzel et al. [8] report that individuals with PTSD perform similarly to control subjects on singular tasks but when they were asked to perform multiple tasks at one time, specifically those related to working memory, performance was significantly impaired. One explanation for this is that the emotional difficulties experienced by PTSD patients and their inability to regulate memories related to their trauma provided distraction or interference that is severe enough to cause declines in cognition [14].

Substance abuse is often comorbid with PTSD. Approximately 10–40% of substance abusers show signs of PTSD [16]. Individuals who suffer from both PTSD and alcohol dependence show more severe PTSD symptoms compared to those who do not have both disorders [17]. Fuehrlein et al. [7] reported that alcohol dependent individuals show more frequent drinking patterns than those with comorbid PTSD. However, those with comorbidity reported more alcohol-related symptoms on the Alcohol Dependency Scale (ADS; [18]) than the former. These symptoms include: staggering, frequency of hangovers, shaking, blackouts, etc. Patients suffering

from both alcohol dependence and combat related PTSD have higher drinking rates, and symptom severity was negatively correlated with alcohol dependence symptoms. Alcohol misuse in military populations has been reported to be between 22 and 40%, a relatively large percentage when compared to non-military population [19]. This may be because alcohol is used as a method of self-medication specifically to cope with stress and negative affect [20].

2.2. Pathophysiology of PTSD

Patients with PTSD exhibit reduced cortisol and increased catecholamine and corticotrophin-releasing factor (CRF) levels, compared to elevated levels of both cortisol and catecholamines in groups without the disorder [21]. These physiological irregularities consistently seen in PTSD patients implicate the hypothalamic-pituitary-adrenal (HPA) axis, which is essential in the regulation of hormones related to the stress response, such as cortisol [22]. Because cortisol is important in the return to homeostasis after experiencing a stressful event, a reduction of cortisol would make returning to homeostasis difficult, resulting in a longer trauma experience than those who experience elevated cortisol levels when presented with a stressor [23].

The three main human brain areas associated with PTSD are the amygdala, hippocampus, and PFC [24–26]. The amygdala is important in the encoding of emotional memories, and individuals with PTSD typically display activation on the left side of amygdala when recalling memories [27]. In rodents, when the amygdala is damaged, there is a lack of fear responding [28]. In adults with PTSD, there is often atrophy on the right side of the hippocampus, which is associated with deficits in the recall of memory [29]. Finally, damage to the PFC is likely related to the deficits in working memory seen in patients with PTSD. PFC deficits are linked to the development of flashbacks, exaggerated startle response, and other fear responses [30]. More specifically, individuals with PTSD display increased activity in the orbitofrontal, anterior temporal, and anterior cingulate cortices and decreased activity in the frontal left inferior cortex when asked to visualize traumatic memories [31].

2.3. Biological and individual differences

While external factors and experiences are traditionally at the root of PTSD individual differences provide for greater susceptibility for some populations. This is important as determining biological markers for PTSD vulnerability may help prevent the disorder from forming in already at risk populations, e.g. the military.

Clark et al. [32] found that soldiers with heterozygous Val/Met COMT are less likely to exhibit PTSD compared to soldiers with Met/Met or Val/Val COMT. Individual differences related to COMT expression and the likelihood of developing PTSD is linked to the function of COMT in the PFC and the subsequent connections throughout the limbic system and thus its relation to memory and stress responses [32,33].

Individual differences, also implicated in the susceptibility to PTSD, include the inability to regulate the fear response as well as an overactive fear response [34]. There are also individual differences in the sensitivity to anxiety and the processing of stressors. Anxiety sensitivity has been identified as an etiologic factor in the pathogenesis of anxiety disorders [35]. The internalization of stress increases the probability of developing disorders such as PTSD. Alternatively, individuals who externalize (commonly referred to as "blowing off steam") have an increased risk for negative social behaviors [36,37].

Similarly, in a study that examined the intrapersonal and interpersonal sources of resiliency (measured in both optimism and hope scales, self-esteem, and perceived social-support) in a group of civilians exposed to combat, civilians who displayed sources of resiliency presented with lower cases of PTSD [38]. Additionally, a ruminative response to stressful or negative events has been shown to make a person more sensitive to such events and the development of stress disorders, such as PTSD [39].

The glucocorticoid hormone, cortisol, plays a role in the natural stress response [22]. When an individual is exposed to a stressor, the HPA axis secretes cortisol, which allows the body to respond to the stressor and thus eventually allow itself to return to its baseline levels pre-stressor [40]. The baseline cortisol levels in patients with PTSD are lower vs. those without the disorder [41]. Furthermore, when individuals are tested for cortisol levels directly following a trauma, those who show a lower level of cortisol have a higher risk of developing PTSD [42]. However, there are also symptom-specific differences in cortisol levels between the sexes after experiencing a trauma [43]. The effects of low cortisol levels in triggering PTSD were shown in females (but not males) who experienced trauma. This could explain why women are more susceptible to developing PTSD and have a longer period of recovery [44].

Glucocorticoids, in addition to norepinephrine and endocannibinoids, have been suggested to play a role in the onset and potentiation of PTSD due to their role in consolidating and the retrieval of traumatic memories [45]. More specifically, glucocorticoids increase the consolidation of memories and disrupt memory retrieval [46]. Specific to epinephrine, Schelling et al. [47] found that patients who underwent cardiac surgery and received higher doses of the chemical reported more traumatic memories specific to their surgery compared to those who did not receive it or received lower doses. Conversely, patients who received an epinephrine antagonist exhibited significantly less traumatic memories related to their time in the hospital [48]. Norepinephrine mediates many behaviors, such as attention, arousal and stress response, as well as the encoding of emotional memories in the PFC and amygdala [27]. Notably, individuals who showed symptoms of PTSD due to cardiac surgery also had higher endocannibinoid levels in their plasma [49].

2.4. Current state of treatment

Traditional treatments include non-pharmaceutical techniques such as psychotherapy, cognitive behavioral therapy, and eye movement desensitization and reprocessing therapy [50]. There has also been the use of less traditional treatments, such as art therapy and video games [51]. The most common pharmacological intervention applies antidepressants (selective serotonin reuptake inhibitors – SSRIs, specifically), although with limited efficacy, as they typically only treat a subset of symptoms. Only about 20–30% of individuals with PTSD who are treated with SSRIs show full alleviation of symptoms [52].

Some exploratory studies have investigated the utility of both illicit and licit substances to treat PTSD. Consider, for example, the use of 3,4-methylenedioxy-N-methylamphetamine (MDMA) to relieve the symptoms of PTSD. Individuals exposed to MDMA-assisted psychotherapy typically report relief of PTSD [53,54]. While short-term professionally assisted treatment with MDMA is relatively safe, chronic use has detrimental, sometimes permanent, effects (including retro- and prospective memory deficits, higher order cognitive impairment, sleep disturbances, impairment to the immune system, cell death, and psychiatric disturbances) [55].

In addition, it has been shown that opiates and corticosteroids, morphine and prednisone respectively, can be used as a treatment and may also prevent the onset of PTSD [56]. Similar to opiates and corticosteroids, in a study that examined the effects of ketamine, an NMDA receptor antagonist anesthetic, on the development of PTSD, soldiers who were treated for combat-induced burns with ketamine were significantly less likely to develop PTSD than burned

soldiers who had not received ketamine during their treatment [57].

Benzodiazepines have also been used to treat the psychological distress associated with PTSD. While these drugs provide relief of PTSD symptoms, they are generally taken chronically, often along with opioids. Benzodiazepines also have notable risks, including increased risk of suicide, substance abuse/dependence, and/or poisoning when combined with other medications, and age-related risks [58].

Antipsychotics have been used to treat hyperarousal. Many of these treatments are used in conjunction with other medications. There is a limited amount of experimental data that support the efficacy in treating symptoms such as re-experiencing, irritability, hypervigilance, etc. [59]. There is a need for more research in this domain, to better understand the efficacy of treatments already available but also to aid in the development of new more effective medications with fewer side effects.

3. Rodent models

As was previously mentioned, the use of animal models in the study of PTSD is well documented, enabling a wider breadth of topics to be studied and more knowledge to be added to the available cache of information related to the neurobiology underlying PTSD. Several widely implemented paradigms (Table 1) include single prolonged stress exposure, fear conditioning, inescapable tail shock, elevated plus maze, social interaction test, novelty-induced hypophagia, and the stress re-stress model.

The single prolonged stress test mimics in rats the behavioral symptoms of hyperarousal, avoidance, and negative memories analogous to PTSD in humans [60]. The task exposes the rodent to a singular stressful event to later record the evoked behaviors. Related activation of the HPA axis can be observed as well as the decreases in the neurochemical activity in the mPFC, usually seen in human PTSD patients [61].

The fear-conditioning paradigm allows for an animal to explore a novel environment. At a given point a moderately painful foot shock is administered. Eventually, the fear of receiving the foot shock is associated with the new environment. By re-exposing the rat to the environment where the shock was administered, symptoms of PTSD can be observed, specifically those related to fear [62].

The inescapable tail-shock task requires that a rodent be restrained to receive repeated tail shocks usually over a multiple day period [63]. Another paradigm used in the literature is the elevated plus maze [64]. In control conditions, when rats are put into a maze and given a choice to explore either an open or a closed arm in the maze, they spend significantly more time exploring the closed arm [65]. The maze is elevated and plus-shaped, with two open arms and two closed in by walls. The task behaviorally assesses the inner conflict in the rat between wanting to explore all areas and their innate fear of open spaces. By manipulating experimental conditions (e.g. pre-session exposure to stressors or the application of anxiolytic/anxiogenic medications) one can see how anxiety mediates the rodent's choice to explore or not explore their environment.

It is well accepted that PTSD patients exhibit social withdrawal; the rodent analog of social withdrawal is the social interaction test. This task assesses the amount of time a rodent spends engaged in social behavior (sniffing other rats, grooming, following) with unfamiliar rats. Fear conditioned rats spend less time spent engaged in social interactions [66].

Hypophagia can be measured as a function of exposure to a novel environment, which is thought to be anxiety-provoking [67]. The efficacy of antidepressants on anxiety reduction can be tested as a function of food consumption [68]. SSRIs are often prescribed to treat PTSD, increases in serotonin are thought to underlie decreases in behavior that are often observed with anxiety [69]. Using the light/dark exploration test can also test the effectiveness of drugs. In this test, a rodent is placed individually into an open box with one section painted black (illuminated by dim red light) and the remaining section painted white (illuminated by bright white light). The amount of time spent in each area is quantified. Increased stress results in the rodent spending more time in the dark area of the box, whereas reduced stress (e.g. via exposure to an anxiolytic) increases time in the light side of the box [70].

As previously mentioned, one of the symptoms of PTSD is the cognitive deficits often associated with damage to the frontal areas of the brain. Performance on the attentional set-shifting test is thought to be mediated by the frontal area of the brain [71]. Using small pots that are filled with different material and scented with various fragrances, a rat must dig through the pots to get to a food reward. The object of the task is to learn which medium and which scents are connected to the reward. Once the rat learns these cues, the cues are changed and they must learn the new reward giving combination. The task can cycle through multiple rule changes which forces a change in mental/cognitive set. This is understood to be a rather difficult cognitive task and depends highly on well functioning frontal areas of the brain [71].

The stress-restress model elicits glucocorticoid responses in rodents that are comparable to those seen in humans with PTSD. This task increases sensitivity to negative feedback specific to the HPA axis. Such a response limits the effectiveness of allostatic control which makes it difficult for the organism to return to homeostasis after experiencing a stressful event. The inability to return to baseline following a traumatic event creates a longer experience with the stressor when compared to those who are able to produce a sufficient amount of cortisol in response to the stressor. Such a malfunction in the stress response is linked to the development of PTSD [23,72].

Cortisol is an essential component of the human stress response. Rats, however, utilize a different hormone, corticosterone, which serves in a similar capacity in that it allows for the energy reallocation, increased heart rate, breathing, and other components of the stress response [73]. In addition to this, rats have lower throughput, are more expensive to upkeep, have longer breeding and growth times, but are of a higher animal order when compared to alternative models such as the zebrafish.

4. Zebrafish model

There are many advantages to using the zebrafish model, which will be discussed in this section. Like humans, zebrafish release cortisol in response to stress. They are also more cost effective, have shorter breeding and growth times, and the model allows for significantly higher throughput. Moreover, there are observable behavioral phenotypes that can be compared to behaviors exhibited by humans. Zebrafish also exhibit individual differences (extroversion, biological make-up, sex differences, etc.), which would be ancillary to the research of PTSD prevention as it allows for the nuances of human existence to be accounted [74].

Recent studies report complex behavioral responses in zebrafish [75–79]. When exposed to stimuli that evoke fear or anxiety, zebrafish display a wide range of clear-cut quantifiable behaviors, including markedly reduced exploration, increased scototaxis (dark preference), geotaxis (diving/bottom dwelling), thigmotaxis (preference of peripheral areas), freezing (immobility) and erratic movements (sudden bouts of high-velocity darting with rapid successive turns) [80–82]. These behavioral phenotypes are strikingly analogous to those of both rodents and humans. Additionally,

Table 2
PTSD related genes in humans, rats, and zebrafish.

Gene (PTSD related)	Human	Rat	Zebrafish	Reference(s)
Catechol-*O*-methyltransferase (COMT)	Genetic predictor of vulnerability to PTSD. Soldiers with heterozygous Val/Met COMT were less likely to exhibit PTSD and its various symptoms compared to those soldiers with Met/Met or Val/Val COMT	Found in the ependymal cells of the cerebral ventricles choroid plexus and glial cells	Used in the inactivation of epinephrine, norepinephrine, and dopamine	[32]; [85]; [33]
Tryptophan hydroxylase (TPH) 1 and TPH2	Genetic predictor of vulnerability to PTSD. Control the production of serotonin, which is utilized in alertness, sleep, and mood	TPH1 expressed in pineal gland; TPH2 expressed in raphe nuclei. Indicated in major depressive disorders	Serotonin synthesizing enzymes found in the posterior recess of the caudal hypothalamus	[86,87]

several physiological biomarkers traditionally explored in stress research (e.g., *c-fos* expression and cortisol) are strongly correlated, functioning in similar or identical roles across these species [81,83]. There is also rather compelling evidence supporting change in brain monoamine levels mediated by social interactions [84].

While the genetics of PTSD is not fully understood, there are a few genes that do allow scientists to determine those who are at risk for the disorder. Moreover, there are similarities in gene expression and brain composition exhibited by both humans and zebrafish that are key to understanding the vulnerability, development, and progression of post-traumatic stress disorder (PTSD) (Table 2). In humans, the catecholamine-degrading enzyme catechol-O-methyltransferase (COMT) is a genetic predictor of vulnerability to the PTSD. Zebrafish also express this gene, and it has been successfully studied in the species [85].

COMT is not the only gene linked to risk factors associated with PTSD. Human tryptophan hydroxylase (TPH) 1 and TPH2 are both related to the risk of developing PTSD [86]. These genes control the production of serotonin, which is utilized in alertness, sleep, and mood. TPH1 and TPH2 are also exhibited by the zebrafish and can be examined using a reverse transcriptase-polymerase chain reaction (RT-PCR [87]).

The zebrafish's hypothalamic-pituitary-interrenal gland (HPI) axis is similar to the HPA axis in humans, both of which are central in stress responding [88]. When exposed to a stressor, both of these axes release cortisol, a glucocorticoid. As previously mentioned, glucocorticoids play a key role in the retrieval of traumatic memories in humans. Campolongo et al. [45] reported that an increase of glucocorticoids improve the consolidation of memories and disrupt memory retrieval. In zebrafish, glucocorticoids, such as cortisol, increase when the fish is exposed to a stressor, similar to the response exhibited by humans [89]. In addition to this, like in humans, increased glucocorticoids can negatively affect the consolidation and retrieval of memories in zebrafish [90,91]. Cortisol can be measured in zebrafish using cortisol ELISA kits, both commercial and homemade [92]. The similarities in the stress response between humans and zebrafish support the utility of the zebrafish as a model for stress and anxiety mediated behavior.

4.1. A summary of relevant zebrafish behavior tasks

Although there is currently no single established paradigm specifically for PTSD with zebrafish as the test subject, there have been many successful models that measure and elicit the stress response in the species (Table 3). These tasks use a wide variety of stimuli and techniques to not only initiate the response itself but also measure various behaviors that are indicative of stress responding in zebrafish. While these tasks might not be able to elicit PTSD in the zebrafish themselves, each of these tasks provides potential and salient logic to aid in the development of a task for PTSD using the zebrafish. Zebrafish exhibit many behaviors in the presence of a stressor, namely significantly less exploratory behavior, increased freezing behavior, increased erratic movements, increased shoaling behavior, and paling of the skin as well as physiological responses such as increased cortisol levels.

For instance, Barcellos et al. [89] measured the whole-body cortisol of zebrafish in response to two different types of predator exposure, direct and visual. Direct exposure being where the predator fish was kept in the same tank as the zebrafish and visual being where the zebrafish was kept in a separate tank but one with direct visual contact to the predator fish. The control group (when no predator was present either directly or visually) had significantly lower whole body cortisol compared to the conditions in which the predator was present. When the zebrafish had visual contact with the predator, this significantly lowered whole body cortisol, compared to the direct contact condition. Such increases in whole body cortisol may be attributed to the detection of the predator by visual, chemical, acoustic, and/or olfactory cues.

It should be noted, however, that solely seeing (either a computer animated visual or a live version) the predator, sans chemical stimulation, can also elevate cortisol in the zebrafish. Stress-mediated behaviors can be assessed by measuring the distance of the zebrafish from the presented image of a known predator, distance from the bottom of the tank, and the overall characteristics of swimming. The presentation of an image of a natural predator to the zebrafish results in less distance from the bottom, heightened freezing behavior, and increased erratic movement, all of which are indicators of stress [93].

As part of a heightened stress response, threatened zebrafish can release an alarm pheromone as a warning of impending danger to conspecifics. Speedie and Gerlai [94] report a dose-dependent stress response to the exposure of varying levels of alarm pheromone. This study measured not only erratic movement and freezing, but also tank dive and shoal cohesion, suggesting that there was an increase in erratic movement and shoal cohesion when presented with the alarm pheromone (indicating a stress response, but no effect on freezing behavior and tank dive for this particular chemical cue).

Bass and Gerlai [95] examined the role of predator avoidance in zebrafish when exposed to different types of stimulus fish including an allopatric predator, sympatric predator, allopatric harmless fish, and sympatric harmless fish. Predator avoidance is when an animal, in this case the zebrafish, actively avoids contact with a predator species. This task allows researchers to examine stress and fear responses, both of which are important in PTSD research. This study measured shoal cohesion, distance of the zebrafish from the stimulus fish, number of jumps, and thrashing behavior, all of which were seen to be indicative of predator avoidance. While the zebrafish did not show an increased amount of shoal cohesion (a decreased distance between individual zebrafish), there

Table 3
Zebrafish stress tasks.

Task	Definition/procedure	Reference(s)
Cue and Spatial Memory Tasks	Using a plus-maze, zebrafish are trained for 14 days to not only associate a food reward with a visual cue such as a red card placed in a certain arm of the maze but also to associate the location of a food reward with external spatial cues such as tables, shelves, and doors. After being exposed to an alarm pheromone or a predator, for 6 min, the zebrafish were put through the same memory tasks. Typically, this results in stress mediated deficits in task performance.	[90]
Habituation	Assessing spatial memory, a zebrafish is introduced to a novel environment, generally an unfamiliar tank. Measuring for erratic movement and tank dive during a defined period, zebrafish show increased exploratory behavior with repeated exposure to the novel tank. When zebrafish are treated with an anxiogenic substance during test trials, exploratory behavior is severely reduced. Stress impairs the animal's ability to habituate to a novel environment.	[82]
Light Dark Box	Generally an open, unrestricted tank is divided into two halves with one side being dark and one side light. The amount of time spent on either side as well as the transitions between one to the other are measured. Zebrafish prefer the dark side of the tank (scototaxis), a position of relative safety. This increases when there is a perceived threat. In normal control conditions there is some exploration into the light side, especially after a period of habitation.	[98]
Novel Object Approach	Centered on measuring the characteristic of boldness, multiple or a single zebrafish are placed into a tank that is cylindrical in shape. The fish is then presented with a novel object that it has never been introduced to before. The amount of time spent near the novel object as well as distance from the object is measured. The more time spent around the object as well as increased closeness to the object is seen as increased boldness which is indicative of a lessened stress response.	[99]
Novel Tank Test	Zebrafish are placed into a novel tank environment, one in which they've never been before, and the time spent at the bottom of the tank is measured (diving behavior; geotaxis). Spending an increased amount of time at the bottom of the tank, which is a position of relative safety, and not exploring toward the top of the tank is seen as a stress mediated response. Introducing the zebrafish to a novel environment itself causes anxiety in the species; however, as the animal habituates to the novel tank, behavior starts to change.	[97]
Open Field Test	Zebrafish are placed into a tank that has no barriers partitioning the inside, creating a completely open area. The time spent around the outside edges and in the corners of the tank is measured (thigmotaxis). As the zebrafish habituates to the tank, the more it will explore the center area, away from the outside of the tank. Increased time spent at the outside edges of the tank and not toward the center is seen as being indicative of a stress mediated response.	[98]
Predator Exposure	Multiple (numbers range from two at a time to 15) or a single zebrafish is exposed to a predator either directly (stocked in the same tank) or visually (with direct visual contact through a clear barrier or a computer animated image of the predator). Subsequent stress mediated behaviors are measured such as erratic swimming, freezing behavior, and distance from predator (predator avoidance). Physiological responses such as whole body cortisol may also be collected.	[89,95]; Blanchard et al. (2003); [93,94]
Shoaling Behavior	Zebrafish display a natural tendency to swim in groups or shoals. Multiple (generally seen at 5 or more) fish are kept together in a single tank and allowed to habituate for upwards of 20 min before being presented with a stress-inducing stimulus. The distance between individual zebrafish (shoal cohesion) is then subsequently measured and compared to a baseline.	[94,96]
Unpredictable Chronic Stress (UCS)	Using multiple types of stressing techniques over a short period of time, unpredictable chronic stress is achieved. The zebrafish may endure one of the following stress inducing techniques twice a day for a period of either seven or 14 days: restraint stress, extreme (both warm and cool) water temperatures, social isolation, crowding, predator exposure, low water levels, and multiple tank changes. Swimming patterns, shoaling, diving behavior, and epidermal color are measured.	[91]

was a difference in distance between the zebrafish and the stimulus fish, independent of the stimulus fish species. Also, there were significantly more jumps when the zebrafish was exposed to its natural predator (Indian leaf fish). This is understood to be a fear response.

A well-studied behavior in the zebrafish is its natural tendency to form shoals with conspecifics [96]. This group shows tighter cohesion, lessened distance between fish, when the fish are stressed or threatened. A loosened shoal with more distance between fish is indicative of a state with minimal perceived threat. Shoaling tasks can be performed by measuring the distance between fish when in the presence of a stressor, whether it be predator, chemical, or otherwise [93,94].

The measurement of stress-mediated behaviors in the zebrafish is not limited to the aforementioned predator related tasks. For example, Bencan et al. [97] performed an experiment in which a novel tank environment was utilized to exhibit diving behavior in zebrafish. When introduced to a novel environment, the zebrafish has a tendency to stay at the bottom of the tank, a position of relative safety, but as it becomes habituated to the new environment, it begins to explore the higher regions of the tank. Bencan et al. [97] found that zebrafish do indeed dive to the bottom of the tank when the tank is novel, an indicator that the fish is stressed and/or threatened, whereas when the tank is not novel, the tank dive response is significantly less (or nonexistent) than the former situation.

Somewhat similar to the novel tank test is that of the open field test. Instead of measuring how much time is spent at the bottom of the tank as an indicator of stress, the dependent measures are the time spent around the sides and corners of an open tank. More time spent at the sides of the tank is reflective of an increased stress response [98].

The novel object approach is another task in which the reaction to novelty can be measured. More specifically, the measurement is on a continuum often referred to as boldness. In the novel object approach task, the zebrafish is presented with a new, novel object and its time spent around the new object is used as the measure of boldness [99]. If a fish is exhibiting a stress response while presented with the novel object, it will spend less time around the object. As the zebrafish becomes less stressed it typically will spend more time in the proximity of the object.

The light dark box is another task in which the zebrafish stress response can be measured. This apparatus is divided into a light side and a dark side, and can assess the zebrafish's preference to spend its time in dark areas, especially during stressful events. Conversely, as the animal becomes less stressed, for example, through

the administration of an anxiolytic compound (or habituation), it will explore the light side of the tank, expressing a dampened stress response [98,100]

Gaikwad et al. [90] examined another way in which the behavioral effects of stress can be measured, using cue and spatial memory tasks. Using a plus-maze, zebrafish were trained to not only associate a food reward with a visual cue, a red card placed in a particular arm of the maze, but also associate the location of the food reward with cues such as doors, tables, etc. The zebrafish were also exposed to an alarm pheromone or their natural predator, the Indian leaf fish, evoking stress mediated responding. Such stressors significantly decreased both cue and spatial memory in the zebrafish, measured by the amount of time spent in the correct arm of the maze as well as the number of entries made therein.

In another set of experiments that measured spatial memory, Wong et al. [82] examined the habituation response to novelty. Impaired habituation is indicative of increased stress levels in the zebrafish. Zebrafish are able to habituate to conditioned place preference, light/dark boxes, and the startle reflex, all of which have uses in the study of stress responses. Wong et al. [82] found that through the measurement of tank dive and erratic movement in a novel tank test, control zebrafish showed increased exploratory behavior after repeated exposure to the tank. When zebrafish were presented with an anxiogenic substance (e.g. caffeine or pentylenetetrazol) the fish exhibited less exploration and transitions to the top of the tank, an indicator of increased anxiety. These memory tests are an important correlate to the memory dysfunction exhibited by human PTSD patients. Because zebrafish also display memory and learning deficits when they are exposed to a stressor, one can begin to study the underlying neural connections between memory, learning, and PTSD through similarly acting brain structures in zebrafish and humans.

Lastly, unpredictable chronic stress employs multiple types of stressing techniques many times over a relatively short period of time. As a result, zebrafish show increased time spent at the bottom of the tank, paled color, increased shoaling behavior, and decreased locomotion, all of which are indicators of stress [91]. These stressors include restraint stress, extreme (both warm and cool) water temperatures, social isolation, crowding, predator exposure, low water levels, and multiple tank changes.

While these tasks themselves may not induce PTSD, they are important to mention in order to understand the ability for fear and anxiety responses to be studied in PTSD. Using such tasks and modifying them in ways to allow for a chronic stress response can actualize a valid and reliable zebrafish PTSD model.

4.2. Limitations of the zebrafish models

While the zebrafish is capable of providing a strong comparative model to study PTSD, there are many possible limitations that should be addressed. First, there are genetic differences between the zebrafish and humans that could provide for difficulties in developing a PTSD paradigm in the former. For instance, although there is about a 95% homology between the genes shared between zebrafish and humans [101], there is the concern that zebrafish and humans have different genetic bases that control anxiety and fear responses.

There are CNS brain structures present in humans that are not as developed or present at all in zebrafish [101]. One such area is that of the PFC, which is instrumental in the development of PTSD in the human. Other areas include the amygdala and hippocampus, but these are arguably equivalent to the lateral and medial pallium, respectively [102,103]. Thus, while the exact human brain structures are not copied in the zebrafish, there are comparable structures that provide for similar biological and behavioral responses as would be produced by humans. Therefore, through examining the observable behaviors of the zebrafish and comparing them with previously identified fear and/or anxiety responses, we can infer whether or not a certain paradigm is successful in eliciting the desired response. Furthermore, we are able to collect stress hormones (such as cortisol) from the fish, to determine whether or not it responded sufficiently to the anxiety or fear-inducing paradigm to which it was exposed.

5. Conclusion

Here we demonstrate the utility and validity of the zebrafish model as it relates to the better understanding of stress mediated anxiety disorders. As previously stated, there is no single established paradigm specifically assessing PTSD with zebrafish as the test subject. However, when one considers the symptomology and neurobiology that underlies PTSD, researchers have successfully measured and analyzed many of the components (e.g. stress induced physiological and behavioral changes, negative alterations of cognition and increased arousal).

The field of zebrafish research has a firm foundation in the realm of genetics and developmental biology. The volume of research in this area, coupled with concerted efforts at replication and validation of the findings has effectively established the zebrafish model as an important one within these fields. A host of studies have expanded the utility of this model into the fields of neuroscience, cognition, and behavior. With the recent sequencing of the genome, along with the ease and availability of genetic mutant models, the opportunity exists to expand upon the knowledge of the genetic origins and the influencing factors of genes on neurobehavioral components.

Biochemical, histological, neurological, and anatomical data suggest that zebrafish are a viable model of human disease states and a solid candidate for the screening of pharmacotherapies [84,97,104]. In order to effectively assess the effects of experimentally administered drugs, it is imperative that we have a thorough baseline understanding of zebrafish behavior and performance [11,79,81,90]. Not only must these tests be developed, but they must also be empirically assessed and found to be both reliable and valid.

Like other commonly studied laboratory animals (e.g. rats, mice or primates), zebrafish were selected as a model because they possessed a set of characteristics with practical benefits that made this species amenable for use in research [105]. The fact that this species is a prolific breeder with embryos that are deposited externally and undergo development within a chorion with optical clarity, has contributed greatly to the value of the zebrafish in studies of embryology and development.

Zebrafish exhibit a relatively large degree of behavioral variety, are capable of fairly complex learning, and have a host of identifiable neurons, making them useful in behavioral, cognitive, and neurobiological studies, respectively. Their frequent and prolific breeding allows for easy maintenance of a stock population and a sufficient number of subjects to facilitate high-throughput chemical or genetic screens, making them useful to drug development, pharmacological testing, toxicology research, and a host of other biomedical areas.

Biomedical research will continue to rely on model organisms to help us better understand the human condition. We need only refer to the vast rodent literature to see the possibilities that may be in store for us as zebrafish researchers. If we move forward, the zebrafish will gain favor as a viable and useful model for testing the neurobiological underpinnings of anxiety disorders (broadly defined, not just limited to PTSD) and for predicting the efficacy of pharmacotherapies for psychological disorders.

5.1. PTSD in relation to multi-domain analyses

Brain disorders should be seen as complex, multi-dimensional diseases that have phenotypes that interact not only within the specified disorder but also across disorders [106]. These phenotypes can influence one another to create new phenotypes specific to a certain disorder. With PTSD, a disorder often comorbid with major depressive disorder, alcohol abuse/dependence, and cognitive deficits, such interplay affects the severity of the symptoms displayed. For example, those who suffer from both PTSD and alcohol dependence show more severe PTSD symptoms compared to those who do not have both disorders [5,17,107]. These symptoms come in the form of flashbacks, nightmares, emotional numbing, hypervigilance and arousal, and sleep disturbances (DSM-V; [4]).

It would thus be neglectful to rely on a single-domain style of research. With a disorder such as PTSD that has symptoms that cross multiple domains, only focusing on one, overarching domain would limit the ability of a model to be truly translational. For example, the stress-related symptoms of the disorder would fall under the affective domain, the cognitive and memory impairments under the cognitive domain, and the social impairments experienced by those afflicted with PTSD would fall under the social domain [108].

Through adapting to a multi-domain analysis in the formulation of a PTSD model using zebrafish, we can not only more fully understand the neural bases for the disorder but also study the symptoms so that we can better understand not only how they stand alone but also how they interact with one another and influence or are influenced by those disorders often seen occurring comorbidly with PTSD.

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