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Review Article

ZEBRAFISH AS AN EMERGING ALTERNATIVE TOOL FOR STUDYING ANXIETY DISORDERS

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ABSTRACT
Zebrafish are increasingly becoming popular as promising new model species for translational research in a variety of neurological fields. Because of their complex behaviors across all major neurobehavioral domains and strong genetic and physiological similarities to humans, zebrafish are well-suited to modelling many aspects of anxiety-related states. In this paper, we first summarized the behavioral models available in zebrafish, such as novel tank test, light/dark box test, open field test, and social preference test, and their efficacy in discovering anxiety-like indices in zebrafish, followed by highlighting the key neurotransmitter systems, such as glutamate and GABA. In addition, cortisol levels and gene expression were explored as well. Overall, this review discusses the benefits of using the zebrafish model for anxiety research and examines current research in the field.

Keywords: Neurological field, Translational research, GABA, Glutamate, Gene expression, Novel tank test.

1. INTRODUCTION
Anxiety disorders are widespread mental conditions including behavior, neural circuitry, physiology genetics and experience [1]. It is one of the most common mental disorders impacting 33.7% of people of all ages worldwide. However, good data on how this prevalence has evolved overtime is difficult to come [2]. The term anxiety comes from the Latin word 'anxietatis' which means 'demand, 'concern' [3]. It is characterized by feelings of distress, uneasiness, fear, panic attacks, difficulty in sleeping and concentration. While anxiety disorders are heritable and genetic factors play a role, environmental variables influence most of the risk of these illnesses [4]. An increase in anxiety disorders have been connected to stress, particularly at young age, substance addiction, circardian rhythm and microbiota [5-8]. Anxiety disorders attributed 6.1% of all suicides [9]. Higher activity in emotion-processing brain area in people with anxiety disorders may be caused by reduced transmission through gamma aminobutyric acid (GABA) or enhanced excitatory neurotransmission through glutamate [10]. To address these issues, we will discuss on how the zebrafish models can be used to mimic different features of anxiety disorders, as well as how they can help in the investigation of molecular and genetic mechanisms.

2. ZEBRAFISH GENERAL CHARACTERISTICS AND GENETICS
The zebrafish (*Danio rerio*) is an aquatic fish native to Southeast Asian countries, and it is one of the most frequent species of fish in tiny lakes and paddy fields [11]. Zebrafish are quickly gaining attraction as viable new stress model species, with complex behaviors across all major neurobehavioral domains and strong genetic and physiological similarities to human [12, 13]. Rodents have traditionally been used to discover new neuroactive medicines, particularly in the field of neurology [14]. These approaches have found to be beneficial, and they possess significant drawbacks (e.g., tedious, low-output, and costly) which severely reduces the opportunities for discovering new medications [15]. Zebrafish have significant benefits over rodent models for improved for drug discovery and screening in preclinical stage. These include: (i) Genetic tractability, (ii) The larval and adult stages are both tiny in size, (iii) Simple maintenance and housing, (iv) Fertilization and development occur relatively soon, (v) The embryos are translucent mark

zebrafish as a convenient model organism in biological research and (vi) Stable reproductive capacities in controlled laboratories. Adults, for instance, capable of reproducing after three months of age [16-18]. The embryonic transparency of zebrafish enables monitoring of diverse organ development and aids in tracing embryonic pattern of gene expression *in vivo* employing a variety of fluorescent dyes [19], having key properties for undertaking medium-to-high performance pharmacology and genomic testing [20].

Zebrafish hold great promise as an alternate model towards human diseases due to its very well-preserved genomes [21], more than 70 percent of zebrafish genes have a high degree of homology to its mammal' sequivalent [22]. Around 70 percent of zebrafish genes get a human equivalent, while 40 percent of human gene gets a direct link with a zebrafish analogue [21]. This clearly supports utilizing of zebrafish in the study of hereditary basis of human central nervous system diseases [23]. Despite the significant neurobiological variations among mammals and fish central nervous systems growing proof suggests that numerous major zebrafish brain areas have similar functions [24, 25]. In zebrafish the lateral pallium of the telencephalic region is involved in cognitive control, whereas the habenula is involved in anxiety [26, 27]. Furthermore, the zebrafish expresses all the neurotransmitter systems found in mammals, including dopaminergic, serotonergic, cholinergic, and non-adrenergic systems [28-30]. Zebrafish exhibit high cognitive capacities and extensive decision- making abilities, as well as high sensitivity to pharmacological intervention [31]. Zebrafish have phenotypes that are quite robust, making them suitable for researching neurobehavioral diseases, specifically anxiety-like and approach-avoidance behaviors [32]. As in humans, the zebrafish also respond to diverse stressors by increasing the release of cortisol (the major glucocorticoids) [33, 34]. These results support the use of zebrafish model in anxiety research.

3. ANXIETY DISORDER BEHAVIORAL MODELS

The following paradigms are used for assessing anxiety-like behavior in adult zebrafish to learn more about how zebrafish models may be used to identify neurobiological mechanisms.

3.1. Novel tank test

The value using zebrafish in modelling anxiety is determined by a variety of research protocol which study the aversion behaviors, locomotor activity and zone preference. These behaviors have traditionally been employed as proxy for anxiety-like behaviors in fish. Novel tank test was among the prominent zebrafish anxiety test [35-37]. This test exposes zebrafish into a new environment in order to elicit an anxiety reaction [38]. When a zebrafish is first introduced to a new environment, it dives to the bottom before progressively exploring the top [39]. Aside from geotaxis, anxiousness raises in the time to enter the tank's top sections as well as the number of irregular movements, an increase in frequency and duration of freezing, decrease in transitions, amount of time spent in the top container, swim speed, and exploration behavior [40-42]. Other research has found that bottom dwelling habit develops over time, when animals were tested in a tank with the same dimensions as their home tank, the effect was eliminated [43]. Nonnis S *et al.,* reported that using this paradigm, zebrafish exposed to acute ambient temperatures of 18°C, 26°C, and 34°C exhibit anxiety-like behaviors, with less exploration, time spent, distance travelled, and number of transitions in the top area at 18°C compared to 26°C and 34°C [44]. In addition, a study by de Abreu MS *et al.,* reported that in comparison to white, yellow, and red tanks, zebrafish at transparent tanks travelled less, made fewer top entrances, and spent less time in top according to additional research utilizing this behavioral experiment [45].

3.2. Light or dark box test

Light or dark box test is used to evaluate fish exploratory activity as well as anxiety-like behavior. Another novelty paradigm is the light or dark box, which is established on the adult zebrafish's actual choice for dark vs bright section (called scototaxis) [37, 40]. This method's behavioral parameters are comparable to that of the novel tank test (Table 1). In the light or dark open and open field tests, for example, light aversion behavior and aversion of the middle of an area were utilized as stress indicators in mice [46-48]. Increased activity in the light tank section implied anti-anxiety behavior, whereas activity in the dark tank section showed anxiety [49]. Rather than a preference for darkness, a preference for brightness was found [50]. Zebrafish showed an aversion to the dark section in the light/dark box test, spent much more time in the bright section. After restraint stress, the bright section was no longer preferred. Similarly, animals prefer the transparent wall of an open-tank than the opaque wall, and this preference decreased after restraint [51]. Additionally, highlighting the importance of color and illumination in light or dark box researchers found

that fish spend light levels, it prefers more time in the black section at lower illumination levels. At intermediate light levels, it is insensitive, but at higher light levels, it prefers the black section [52]. Prut L et al., reported that animals subjected to the white section for three consecutive 15 mins prior to testing displayed risen in swimming, thigmotaxis, and immobility in the white wall however no change in the locomotor activity[53]; aversion of the white section does not really seem to become used to the apparatus after numerous exposures [54]. Sireeni J*et al.,* reported that zebrafish behavior in this activity cannot be explain either by the avoidance of white section or approach to black section, confirming the hypothesis that scototaxis in zebrafish represents a confusion between method and aversion, as seen in rodent models namely the elevated plus maze and light or dark box test [55].

Table 1: Zebrafish behavioral models

Test	Test overview and parameters	Characteristics	Citations
Novel tank test	Zebrafish are singly kept in a small container full of water and divided into three equal zones designated with a border on sides after acclimatization. In addition to manual recording, the latency, time spent, frequency of entries in the top part of the container, number of freezes and time spent immobilized were video recorded for 6 min (s).	↑in latency to entran- ce, number of erratic movementsand dura-tion of freezing. ↓ in number of transi-tions,time spent exp-loratory behaviors. Change in swimming speed.	[41,42]
Light or dark box test	Zebrafish are placed individually in a two-equal sized compartmentsrectan-gular plastic tank filled with wateraf- ter acclimatization, in which one com-partment was lit by light source from fluorescent bulb above the container, while the another was darkened by an impenetrable top, impenetrable wall and impenetrable lower side. In addition to manual recording theamountof crossings among the compartment, amount of time spent (s) in each compartment and zone preference were video recorded for 5 mins.	↑ Scototaxis (preference for dark vs bright section)	[41-42,51]
Open field test	Zebrafish are individually placed in a novel arena filled with water to a depth of about 12 cm after acclimatization. By dividing the open field area into sub-compartments, the outer and inner zones were created. In addition to manual recording: time being spent on the apparatus edge or middle (s), total distance moved in each zone (m), velocity in each zone, amount of entries, number of freezes and the time spent in immobilized were video recorded for 5 mins [1,2].	↑ Thigmotaxis (Preference for the apparatus walls)	[41, 59-60]
Social preference test	Zebrafish are kept in groups in a container (e.g., 40L) maximally filled with water after acclimatization. This test has been used to see how zebrafish react to changes in coloring and patterning that are conspecific, heterospecific, or both. Transparent Plexiglas can be used to separate potential shoaling mates, allowing subjected fish to show variance in choice.	↑ time of shoal cohe- sion. ↓ latency to shoal co-hesion, social distan- ce, time away from shoal	[35,41,68-69]

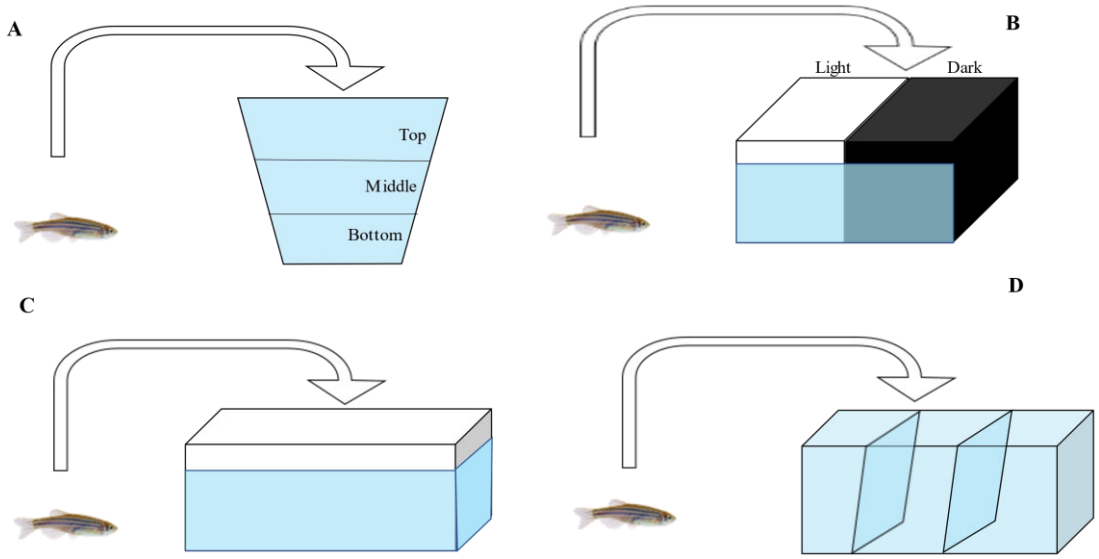
3.3. Open field test

Open field test measures basic locomotion and determine anxiety-like behavior. Although not without complications, the open field test is a typical test for measuring exploration drive and acclimatizes in rats [56]. Open field exploration has also been used to assess anxiety-like behavior in zebrafish [3]. Zebrafish exhibit non-associative learning, as well as fear-related

behaviors such as thigmotactic behavior [48, 57-58]. In the open field test, zebrafish showed considerable aversion to the middle area in the apparatus, preferring to spend their time in the outside area [47, 58-59]. Thigmotaxis is another term for this type of behavior. This type of action has also been observed in rats in the past, indicating an unfamiliar atmosphere can induce anxiety-like behavioral responses in zebrafish like thigmotaxis. By the end of the 5-min test session, the behavioral pattern of a great number of squares transverse, distance travelled, and high velocity had significantly decreased, indicating that zebrafish are capable of acclimatization, the type of non-associative learning, whereas time spent in the outer zone (transparent zone) had significantly increased overtime [51,60-61]. Wagle M *et al.,* reported that rodents spend more amount of time in a trials swimming close to the apparatus's borders (thigmotaxis zone) and spend less time at the center [62]. Engeszer *et al.,* interestingly reported fish with strong dark aversion (sda) traits had higher thigmotaxis (preference for the walls) than fish with variable dark aversion (vda) trait [63].

3.4. Social preference test

Social preference test assesses social behavior. Zebrafish are extremely sociable fishes that forage by shoaling, reproduce, and reduce predation risk. Even if zebrafish are raised in isolation, this tendency is intrinsic and begins early in development [11, 64] where, in rodent unpredictable social isolation is distressing [65]. In contrast to zebrafish tested in groups, when zebrafish exposed to novelty-based tests in isolated, they exhibit higher level of behavioral and cortisol stress indicators, as well as an additional in response to stress [66]. Chronic stressed zebrafish also have a longer period of shoal cohesiveness and a shorter latency to it [67]. Furthermore, after becoming habituated to novelty, the average zebrafish distance rises, with similar effects observed following ethanol treatment, indicating that lower social cohesion reflects anxiolytic responses [68]. Overall stress models rely on the environment since enriched surroundings relieve anxiety as well as improve zebrafish's well-being. Environment advancement lower the anxiety for both the isolated as well as the grouped fish, which is analogous to the effect seen in fish given with anti-anxiety medicines like diazepam, fluoxetine [35]. Hence, housing circumstances should be carefully evaluated in order to increase validity and data in translational models of anxiety, as they play a critical role in zebrafish behavioral and neuroendocrine responses.



(A) Novel tank test, (B)Light or dark box test, (C) Open field test, (D) Social preference test

Fig. 1: Paradigms used to assess anxiety-like behavior in adult zebrafish.

Zebrafish anxiety-like behaviors are highly sensitive, and profit from it being three-dimensional (3D) because of the added dimension. Consequently, whereas rat models are often explored in 2D dimensions, zebrafish paradigms provide greater dimensionality for assaying anxiety pattern [35, 69, 70]. Grossman *et al.,* found that

specific end point temporal representations can also be mapped for a more reasonable view of zebra fish behavior using 3D analysis. Cluster analysis may be used to find meaningful subgroup inside a large set of data, categorize scientific manipulation or behavioral outcomes due to the similarities of its changes, simplify behavioral data, and enhance the quality in addition to complementing 3D reconstruction [69].

4. NEUROTRANSMITTER SYSTEM IN ZEBRAFISH

Chemical messengers known as neurotransmitters start, enhance as well as control signal transmission among neurons and other cells in the body. The activity of neurons in the brain is based on the ratio of excitatory and inhibitory processes that affect somethingthat could happen separately or together. Furthermore, abnormal neurotransmitter production or function are linked to neurological and psychiatric illnesses, and experimental techniques utilizing transporters, receptors, and enzymes in these settings were described [70].

Since zebrafish and other vertebrates have similar neurotransmitter systems, they can be adopted for anxiety disorders method. Even though mammals and zebrafish have considerably greater resemblances in their neurotransmission, there are some notable variances, primarily in the amount as well as names of chromosomes producing proteins, due to the fish's homologous recombination event. The biosynthetic and metabolic processes in the brains of mammals and zebrafish are the same. A summary of various neurotransmission is provided below.

4.1. Glutamate

Glutamate is the primary excitatory neurotransmitter in the mammals and the teleost central nervous system (CNS) [71]. Neurodevelopment, learning, memory, basic thinking, and neurological illnesses and diseased states like seizures, dementia, cerebral neuropathies, motor nerve disorder, discomfort, and psychotic, and more recently, the stress response and anxiety disorders, are all affected by glutaminergic transmission control [72].

Elevated glutamate levels have been associated to anxiety disorders. Glutamate excitotoxicity at high concentrations have been linked to anxiety, which can lead to neuronal damage and/or death due to receptor overstimulation [73].

Zebrafish also have excitatory amino-acid transporters (EAATs) with high affinity that control glutamate levels as well as avoid excitotoxicity. According to the study by Agostini JF *et al.,* novel tank and light/dark test promotes anxiety-like behaviors in zebrafish through increasing glutamate levels, which leads to decreased glutamate uptake however, when treated, the zebrafish showed significant increase in glutamate uptake [74].

4.2. GABA

Gamma-amino butyric acid (GABA) is the most abundant inhibitory neurotransmitter in the central nervous system (CNS), with GABAergic neurons found all over the brain. GABA is an inhibitory neuro-transmitter that primarily regulates brain systems and post synaptic cell activity [75]. Anxiety disorders are linked with low GABA levels. GABA is abundantly synthesized inside the brain and spinal cord of zebrafish from interneurons [76]. A study by Assad N *et al.,* found that acute restraint stress causes anxiety-like behavior in zebrafish by lowering GABA release, which in turn results in low GABAA receptor activation. However, GABA therapy reduced the anxiety-like behavior [77].

5. ANXIETY MOLECULAR AND GENOMIC BIOMARKERS IN ZEBRAFISH

5.1. Cortisol analysis

Hypothalamic-pituitary-adrenal (HPA) dysregulation has already been linked with a variety of mental diseases, including anxiety, depression, and post-traumatic stress disorder [78, 79]. Clinical anxiety is characterized by an excessive worry and comprises numerous mental diseases on the 'anxiety spectrum' including acute mania, specific phobias, and social anxiety disorder [80, 81]. Cortisol, the major stress hormone in zebrafish, is set to release by interrenal cells following the activation of the hypothalamus-pituitary-interrenal (HPI) axis, as it is in humans [82, 83].

Psychosocial stressors have been used in several investigations to see if psychiatric problems are linked to changes in levels of cortisol and HPA response [79]. Anxiogenic stimuli, like net stressed, alarm pheromone, or caffeine, cause elevated levels of cortisol in zebrafish, which are associated with anxiety-related behaviors [84]. A study by Oliveira TA *et al.,* demonstrated that exposing live zebrafish to deceased zebrafish caused increases in whole-body cortisol levels and protective reflexes as part of an anticipated response to stress toward an ongoing threat [85]. In addition, Mezzomo NJ *et al.,* reported that zebrafish subjected to an acute net stressor exhibit an increase in whole-body cortisol levels when compared to control fish [86].

5.2. Gene expression

Cortisol and other stress hormones, as well as pro-inflammatory and anti-inflammatory cytokines can be used to measure HPA deficiencies and neuroinflammation (which has been linked to major stress-related disorder in humans) [32]. In response to repeated stress, zebrafish CRH and calcineurin mRNA expression was enhanced, and phospho-cAMP response element-binding protein was reduced, according to molecular analysis [66]. A study by Viscarra F *et al.,* reported there was no big difference in β-actin expression among the control and the stressed fish. Using standard RT-PCR, the level of mRNA expression of α4 nACh and α7 nACh receptor subunits in the brain of adult zebrafish exposed with net stressor was determined and found the level of expression of α4 and α7 receptor subunit of mRNA were significantly reduce [87].

Cytokines are important brain mediators in neuro-modulation, and they are especially useful for investigating modelling psychological effects [88, 89]. Kirsten K *et al.,* reported that the expression of pro-inflammatory cytokine genes: Interleukin-1beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) and anti-inflammatory cytokine gene IL-10 were not affected by acute stress [90]. In contrast, Song C *et al.,* the PUCS procedure in zebrafish induced the elicit anxiety-like behaviors. It also increased whole-body cortisol and pro-inflammatory cytokines and interleukinsIL-1β and IL-6, as well as the anti-inflammatory cytokine IL-10 [91]. These findings suggest that a single stressful-incident may not always be sufficient to trigger changes in pro-inflammatory cytokines, or that the post-stress assessment period (one hour) used to collect samples did not coincide to the change in cytokine expression.

6. CONCLUSION

Understanding and treating anxiety disorders requires studying the biochemical process to promote the forming of brain pathways as well as their action to control behavior. This review basically highlighted the significance of zebrafish over rodents. We mainly summarized the behavioral models available in zebrafish, such as novel tank test, light/dark test, open field test, and social preference test, and their efficacy in discovering anxiety-like indices in zebrafish followed by the key neurotransmitter systems, such as glutamate and GABA in this paper. In addition, cortisol levels and gene expression were discussed as well.

The following areas should be investigated further in order to make zebrafish models more useful for human anxiety investigations. The behavioral validation of anxiety models must be tougher and more comprehensive as the experiment was carried out using the approach outline above, which involved the addition of drugs to water. Because drugs can be rapidly absorbed through skin and gills, depending on an individual fish's surface area and gill activity, such trials cannot properly regulate the drug dose ingested. In the future, pharmacokinetic studies in zebrafish should be prioritized. It would be beneficial if the quantity of drug that reaches different targets was investigated further.Additionally, comparative studies of zebrafish and other vertebrates are important since locomotion, localization, and function of neurotransmitter signaling pathways can vary.Despite the above limitations, the results of investigation showed that zebrafish can be useful and applied in a variety of neurological fields.

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