

ARTICLE

ZEBRAFISH: A VERSATILE BEHAVIORAL STUDY MODEL

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ABSTRACT

The popularity of zebrafish as a model organism can be accounted for by a number of attributes, including the relative ease of rearing and breeding in captivity, rapid development, short generation time, and availability of genomic resources, including the complete zebrafish genome sequence. Zebrafish is described as a versatile behavioral model since it is known to adapt to a wide range of changes in its surroundings without much difficulty and provides us with a reliable means of reproducing different aspects of human behavior and studying them. Though it is not only used for neurobehavioral studies, but also for several other purposes in an equally efficient manner. In this paper, we focused on recent developments made in behavioral studies using zebra fish. We tried to bring home the idea that zebra fish can be subjected to behavioral studies that may allow us to peek into the basic mechanism of some of the commonly seen behaviors of vertebrates like anxiety, social behavior, learning etc. along with the current research trends in this aspect.

INTRODUCTION

KEY WORDS
Zebrafish, behavioral study, neurotransmitter, age.

An animal model with biological and/or clinical relevance in the behavioral neurosciences is a living organism used to study brain-behavior relations under controlled conditions, with the final goal to gain insight into, and to enable predictions about these relations in humans and/or a species other than the one studied, or in the same species under different conditions from those under which the study was performed [1]. Of the many species used in biomedical research, specific animals are preferred in certain areas. Non-human primates are used in research on vaccines, infectious, cardiovascular, and neurological diseases, aging, reproductive biology, gene therapy, drug addiction, xenotransplantation (cross-species transplants), and vaccine and toxicity testing [2]. Zebrafish are vertebrates. Like humans, they have a backbone. This means that they are more closely related to humans than commonly used invertebrate models such as insects and worms (*Drosophila* - fruit flies and *Caenorhabditis elegans* - nematodes) which do not have backbones. Because zebrafish are more closely related to humans, they are more likely to be similar to them in many biological traits than a more distantly related organism. These biological traits would include genes, developmental processes, anatomy, physiology, and behaviors. This is an advantage that invertebrate lab animals do not share with humans. The invertebrates are more appropriately used in comparisons at the cellular or biochemical level of organization where they share many features with humans. The embryos develop quickly. They go from a single cell to something that is recognizable as a tiny fish within 24 hours which mice take 21 days. Although zebrafish and humans are obviously very different, their embryonic development is remarkably similar. Furthermore, it is becoming clearer that all vertebrates follow an evolutionarily-conserved developmental program. This conservation extends even to the molecular level where similar genes perform similar functions in many different species [3]. Zebrafish is a tropical fresh water fish native to the Himalayan region. Its lifespan in captivity is around two to three years, although in ideal conditions, this may be extended to five years. As a model biological system, the zebrafish possesses numerous advantages for scientists. Its embryonic development is very rapid, and its embryos are relatively large, robust, and transparent, and able to develop outside their mother. Furthermore, well-characterized mutant strains are readily available. Zebrafish is a small (3-4 cm long) freshwater teleost species that can be easily kept and bred in the laboratory [4]. A female can produce 200 eggs per spawning and the fry grow quickly and reach sexual maturity within 2-3months. Zebrafish has been successfully used in developmental genetics and recently neurobiologists have also started to study this species [5]. Zebrafish are highly prolific, resilient, and one of the lower order vertebrate species in which Complex brain function and behavior may be studied in the laboratory [6]. About, 70% of protein-coding human genes are related to zebrafish genes, and 84% of the genes known to be associated with human disease have a counterpart in the zebrafish genome. The behavioral repertoire of zebra fish is complex and should allow the development of a range of behavioral paradigms. Thus, an effort has been made in this article to highlight some of the key futuristic studies involving zebrafish to enunciate its potential as a remarkable study model for complex human behavior.

ZEBRAFISH: A PROFICIENT STUDY MODEL

The Zebrafish and behavioral studies

In this paper, we discuss some of the attempts made by several scientists all over the world to understand the neurological conditions of zebra fish by relating them to several behavioral parameters measured using a wide variety of methodologies; different stimuli bringing out different responses like anxiety, stress etc., also the effect of neurochemicals, age-related studies and their behavior in a social environment.

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Anxiety

The ultimate goal of most anxiety-related studies has been to develop zebra fish models of pathological processes and to investigate the mechanisms of fear and to eventually translate the findings to the human clinic. Different approaches have been made to study fear, stress and anxiety-related responses in zebra fish. Here, we will be seeing the different methodologies and parameters used to measure the levels of anxiety and other interesting outcomes of the experiments done till date in this aspect [7].

Three dimensional robotic models have been used, for example, to measure the anxiety-fear responses in zebra fish, live predatorial stimulus was created by designing a robot mimicking the morphology and locomotion pattern of a natural predator of zebra fish-Indian leaf fish *Nandus nandus*.

Another stimulation was in the form of an attack by a heron (a bird silhouette), impacting the water surface. In the presence of predatorial stimuli, the fish give the avoidance response. Also, the zebra fish responded to the predator fish irrespective of the color and pattern of the predator, but more to its tail frequency. Anxiety-related studies were also conducted using alarm substances, which is known to induce fear responses in a range of fish species. Alarm substance is formed in specialized epidermal club cells and released when there is a skin injury. This is detected by the neighboring fish using their chemoreceptors (olfaction), followed by decrease in the distance between fish in the shoal (a group of fish) and then moving away from the predator. Two motor patterns are known to change in response to alarm substance or other fear-inducing stimuli, erratic movement and freezing. Freezing was defined as complete immobility whereby only the gills and occasionally the eyes move. In nature, when erratic movement is performed at the bottom of small creeks or lakes, it stirs up the debris and the cloud formed covers the fish. The zebra fish show an increase in erratic movement. It is likely that in nature zebrafish exhibit the strongest alarm reaction when an injured fish is nearby and thus the concentration of alarm substance in the water detected by the neighboring fish is high. If the injury occurred farther away, the predator is unlikely to be nearby, and the smaller concentration of the alarm substance elicits a diminished alarm reaction. The presence or absence of the predator did not have any effect on the action of the alarm substance. Increased shoal cohesion is also thought to be an anti-predatory response, since fish in a shoal represent a more difficult target because the predator's attention is divided [8]. In another study [9] acute alarm pheromone exposure resulted in a significantly longer latency to explore the upper portion of the tank, frequency of freezing and erratic movements also increased. Around 7 ml of alarm pheromone solution extracted from euthanized zebrafish was added acutely to a novel test tank, 22 fish were subjected to this acute treatment. For prolonged exposure the fish were exposed to alarm pheromone in the pre-treatment beaker for 30min after which they were transferred to novel test tank, 20 fish were used for prolonged exposure. Prolonged alarm pheromone exposure did not result in any significant behavioral differences between the control and experimental groups. The results confirm that alarm pheromone each evoke relatively simple, yet robust anxiety-like behavioral responses in zebrafish. Interestingly, prolonged alarm pheromone-exposed zebrafish showed no significant behavioral differences compared to control fish. Thus, alarm pheromone appears to only be effective acutely, reflecting its natural use as a danger signal to nearby shoals. Chlorpyrifos is a common organophosphate pesticide(OP) used in the U.S. and some OPs have been found in detectable levels in air, dust and food samples and even in children's urine samples. To study the neurobehavioral disorders caused due to this exposure, zebrafish larvae are used to examine the effects of sub-chronic levels of chlorpyrifos on anxiety-related behavior during their development. Zebrafish larvae develop rapidly, hatching from their chorions by 2–3 days post fertilization (dpf), and fully develop all adult organs within their first week. By 4–5 dpf, larvae inflate their swim bladder and become free-swimming, exhibiting a number of behaviors such as avoidance, darting, scoots, and startle response. Thigmotaxis, a preference for the edge, is an anxiety-related behavior in zebrafish larvae. The highest dose of chlorpyrifos administered, 1 μ M, had a highly toxic effect on movement, body morphology, and body size. At this dose, the larvae exhibited twitching behavior but could not swim or move normally; therefore, they were not used for behavioral analysis. At this concentration, zebrafish larvae exhibited tails that curled upward and shorter than normal body lengths [10].

In one of the experiments, images were used as stimuli since olfactory cues (like alarm substance) are difficult to turn on and off as and when required and using a live predator may create an error variation (like if it actually attacks the prey fish). The images included the original sympatric predator, the Indian leaf fish, another sympatric predator, the needle fish, a bird silhouette moved on the side or above the tank, an expanding dot mimicking rapid approach of an object shown on the side and from above the tank, as well as non-fear inducing images including a single and a group of zebrafish. Another type of behavior observed in response to fear-inducing stimuli was jumping, a single fast jump with the use of the caudal fin. Similarly, 'leaping' also occurred (the attempt to perform a forceful and fast swim-like jumping) against the glass. Freezing is usually different from floating, which can occur anywhere in the tank but freezing occurs only at the bottom of the tank or when the fish is in contact with an object. Another form of active swimming is thrashing. It is a forceful swim that appears as if the subject was trying to swim through the glass of the tank and manifests as circular motion directed towards the glass. The fear response to needle fish was not that effective except for a few jumps and leaps. Even the image of Indian leaf fish or the dot on the side were not as effective as the dot approaching from above in eliciting fear responses. Fish respond to approaching predators with fleeing, fin erection display, or can even ignore the predator depending on the distance between them and the predator, or on the level of satiation of the predator and many other factors including access to escape routes. The reduction in mobility could also be due to non-

threatening stimuli and time spent by the fish at the bottom of the tank could depend upon external factors [11]. When given free choice between a black and a white chamber, zebrafish reliably demonstrate a preference for the black chamber, and in analogy to rodent models, it has been suggested that the degree of preference may be useful as a measure of anxiety. According to a hypothesis zebrafish may have its light/dark preference varied with the circadian clock [12]. The hypothesis was tested by recording the light/dark preference in a tank was divided into two compartments, one was illuminated by LED light and the other was covered with matte black paper on all sides and the top. The LED light was at the top of the experiment tank. A video camera was positioned in front of the tank. The behavior of zebrafish was recorded from 8:00am on the first day to 20:00pm on the third day. Between 9:00 and 20:00 during the third daytime melatonin was added so that the final concentration of melatonin in the tank was 0.1 mM. On day 1, the fish displayed a preference for the light area of the aquarium between 8:00am and 18:00pm. This preference was reversed to dark tendency when the time was 20:00pm. The preference for dark region continued till 2.00 am next day and thereafter started to decline reaching the minimum value at 8.00 am. The same preference continued for the next 24hr. 0.1mM melatonin significantly increased the mean proportion of time in the dark to over 70% at 10:00am. These results indicate a clear circadian-like tendency of staying in the dark area also melatonin treatment indicate that Melatonin could mediate the circadian clock of zebrafish. Behavior of zebrafish influenced by melatonin is dose dependent. Zebrafish adults displayed higher activities during the daytime, as they need to detect predators, to get food or to find conspecifics. In the nighttime, fish displayed brief periods of inactivity, often associated with a drooping caudal fin, suggesting a sleep-like state. During the night time, they prefer dark backgrounds to minimize their visibility and their locomotor activities. This study can aid in providing new insights in understanding the metabolism mechanism underlying the neurobehavior, and facilitate studies related to the neurobiology of normal and pathological behavior. There is still doubt regarding whether the zebrafish find the white compartment aversive or it is that they like dark compartment more. To check this a study [13] was conducted wherein avoidance of the white compartment was studied by analysis of the time spent in the white compartment of the apparatus and shuttle frequency between compartments and thigmotaxis, freezing and burst swimming in the white compartment. This study consisted of 3 experiments, in the first experiment a preference tank was divided into 2 compartments black and white. Fish were first kept in a central compartment for 5min habituation period then were allowed to make an initial choice between the two compartments. The behavior was video recorded for 15min. 3 such trials were conducted. During the inter-trial time of 15min the fish were kept in the acclimation tank from where they were taken. Experiment 2 was same as experiment 1 only difference was that the inter-trial time was 24hr. 5 trials were done. In experiment 3- 10 fish were individually transferred to the white compartment and were not allowed to leave the compartment for 15 min. After this treatment they were again subjected to experiment 1. The results indicate that forced exposures (post-confinement trials) did not alter the time spent in the white compartment or general locomotor activity (shuttle frequency), but it decreased the proportion of time that animals spent burst swimming, sticking to the walls, or freezing while in the white compartment. The results could indicate that exploration of the white compartment is driven primarily by fear/anxiety than by novelty.

Preference for dark place may be a measure of anxiety but which measures can be considered as a measure of anxiety is not clearly understood, in a study [14] Subjects were observed individually in a single session lasting approximately 2 h. The session included a preliminary black/white preference test, forced exposure to black and to white, and a final black/white preference test. Each one of the test lasted for 30 minutes. Subjects spent on average 34% of the pre-test in white and on average 36% of the post-test in white compartment. 21 of the 62 subjects spent 50% or more of the preference tests in the white compartment, while 41 spent less than 50%. Most of the fish preferred the black chamber to the white chamber as predicted which replicates the results of previous experiments. Freezing is the primary response of zebrafish to stimuli that are avoided this is evident from high levels of freezing observed in fish which avoided white chamber in pre-test and were subjected to it in the second test. So freezing can be proposed to be the most reliable behavioral measure of anxiety.

Zebrafish is a shoaling fish and a study [15] was conducted to check how shoaling is related to anxiety and stress The study focused on the effects of a variable number of *Danio rerio* fish subjects, ranging from 1 to 8, in the light/dark box preference test. There were four groups in this experiment and a different number of subjects was used in each group: the control group had only one subject inside the apparatus during the test, whereas the experimental groups had 2, 4 and 8 subjects. The subject's behavior is recorded with a video camera during 15 min after a 5min habituation period. There was reduced white avoidance behavior in the group of eight subjects reflects the role of shoaling which is a defense mechanism in reducing anxiety and stress. However the test results show that four subjects inside the apparatus is not enough to cause the anxiolytic effects of shoaling in the light/dark test. The absence of significant difference between the control group and the test groups with two and four subjects suggest feasibility to run the light/dark test with up to four subjects.

Anxiolytic effects

In novel environments, zebrafish swim to tank bottoms and dark backgrounds, which is attributed to anxiety associated with the threat of predation. AB, WIK, PETCO and Globish strains of zebrafish were treated with different drugs like nicotine, desipramine, chlordiazepoxide, yohimbine, citalopram, DMSO and ethanol and subjected to two novel environments: dive tank top and light-dark plus maze. The dive tank anxiety list is based on the tendency of zebrafish to remain in side and bottom contact with solid

boundaries in a novel tank and the aquatic light/dark plus maze is based on the tendency of zebrafish to seek dark backgrounds (or avoid light backgrounds) in unfamiliar environments. In the novel dive tank test, WIK line spent significantly more time in the top 2/3 of the dive tank than the others. This might be due to absence of predation pressure in their natural Indian Wild population environment. A moderate dose of nicotine increased the amount of time the fish spent at the top of the tank while higher amounts decreased mobility, indicating the possibility of sedation at higher doses. Chlordiazepoxide failed to increase zebrafish dwelling at the top but increased the amount of time on the light side and the serotonin reuptake inhibitor citalopram increased the amount of time spent by the fish in the upper sections of the tank. Exposure to ethanol or anxiolytic drugs reduces the light avoidance behavior. Zebrafish can be used for drugs or toxin screening. Update of water-soluble drugs into zebrafish brain through bath exposure produces brain drug concentrations that are roughly 1/1000 of bath concentrations [16]. To understand the behavioral and physiological stress in zebra fish, tank dive test and treatment with pharmacological substances were performed. Alarm pheromones and caffeine used were anxiogenic (causing anxiety) and fluoxetine and ethanol were anxiolytic (reducing anxiety). The extent to which acute and chronic exposure to caffeine and fluoxetine caused behavioral modulations was also observed. Alarm pheromone increased the frequency of erratic movements and freezing behavior. Caffeine increased the latency (hesitation) to enter the upper half, also increased erratic movements. Chronic exposure to fluoxetine caused lower latency to enter the upper half and treatment with ethanol increased the amount of time spent in the top. Also, zebrafish physiological stress responses were measured using a cortisol assay. Chronic exposure to fluoxetine showed reduced levels of cortisol, which indicates that it reduces anxiety [17].

Alcohol studies

Zebrafish is a potential model for analysis of drug addiction, e.g. alcoholism. Ethanol can easily cross biological membranes and affects virtually all body organs. Behaviorally, acute administration of alcohol in humans leads to disinhibition and euphoria. The use of animal models with similar or related behaviors may provide insights into molecules involved in mediating the biological effects of ethanol. Zebrafish are more structurally homologous to humans, and their genes are about 70–80% identical to human counterparts thus making it convenient to identify human orthologues of zebrafish genes. Although zebrafish have been widely used for studies of development, their use in the study of the effects of ethanol has been limited.

Study has been reported on basic behavior tests that account for characteristic behavioral patterns of zebra fish upon alcohol treatment where fish were tested in 6 behavioral paradigms-locomotor activity, group preference, aggression, anti-predatory model, light/dark preference, pigment response [4]. About 4 concentrations of ethanol were employed - 0.00%, 0.25%, 0.50%, and 1.00%. For locomotor activity fish treated with 0.25% and 0.50% showed significant increase in activity as compared to fish treated with 1.00% or control fish also fish treated with 0.50% alcohol spent more time in the upper layer of water than other fish. Fish treated with 0.00% or 1.00% alcohol spent significant amount of time in lower layer of the tank. Zebra fish generally swims near the surface of water. It only goes to the bottom when there is a threat from predators like birds. Control fish swam near the bottom indicating anti-predatory behavior but fish administered to 0.50% were generally in the upper layers initially this indicates that alcohol has anxiolytic i.e. anxiety reducing effect. For group preference alcohol significantly reduced the preference for conspecific in a dose dependent manner, for anti-predatory model 0.25% showed the strongest jump response. For light/dark preference test during the 1st minute of observation all fish avoided the dark component. But after 10 minutes of habituation 0.00% and 0.25% fish didn't show any preference or avoidance for any compartment but 0.50% and 1.00% showed avoidance for dark compartment [4]. This suggests that alcohol likely to affect central neural mechanisms rather than perception. For pigment response Alcohol enhanced the color of zebra fish, there was a linear relationship between alcohol concentration and skin color. It was speculated that alcohol may directly act on chromophore cells and change color or indirectly act by affecting the central neural mechanisms [4]. So it can be concluded that lower doses of alcohol had facilitatory role and higher doses had inhibitory dose and Alcohol likely to affect central neural mechanisms rather than perception.

Another study has been reported [5] where the aim was develop a method for inducing voluntary ethanol intake in individual zebra fishes which can be used as a model in future studies, and to characterize the effects of ethanol intake on different behaviors and the expression of hypothalamic orexigenic peptides, galanin (GAL) and orexin (OX) [5]. Gelatin-ethanol meals were prepared which consists of melted gelatin mixed with 2 day old brine shrimp plus ethanol (in three different concentrations) which resulted in three different types of meals with ethanol concentration being 0%, 10%, 20%. Gelatin-ethanol meals were prepared fresh daily. The increased intake of ethanol-gelatin may not due to an ethanol-induced change in appetite. Instead, zebrafish may have a preference for the pharmacological effects or the taste of ethanol-gelatin, at least at the 10% concentration, which in humans and rodents is perceived as containing both sweet and bitter taste components. The ethanol-gelatin caused a significant increase in locomotion and a decrease in anxiety, as indicated by increased exploration in zebrafish that ingested the 20% ethanol-gelatin. Voluntary intake of ethanol-gelatin stimulates the expression of the orexigenic peptides, GAL and OX, in the hypothalamus of zebrafish. This effect was found to be anatomically specific, occurring for GAL in the ventral and caudal zones of the periventricular hypothalamus, with a significantly greater effect in the caudal compared to ventral zone, and for OX (+60%) in the anterior periventricular hypothalamus.

Another study focused on acute effects of alcohol on larval zebrafish: a genetic system for large-scale screening [18]. The effects of acute treatment of alcohol on locomotion, thigmotaxis (wall seeking tendency in an open field) and melanocyte morphology in larval zebrafish were studied. Two strains of zebrafish were used AB (originated from Oregon, USA,) and WIK (originated from Germany). Larval zebrafish were obtained from these strains through natural mating. 7-day old fry (larval zebrafish) were used in this study. Larval zebrafish exhibits acute sensitivity to ethanol in a dose- and time-dependent manner. They initially become hyperactive, and as ethanol accumulates, they become hypoactive and sedated. This is similar to what has been observed in humans and other animal models. Also since the activating time of ethanol took several minutes the effect of ethanol on behavior is likely to be mediated by CNS than by chemosensory pathway because chemosensory pathway takes very less time. Future forward genetic analysis to identify mutations that show altered sensitivity to ethanol shall provide important insights into the genes involved in regulating the behavior. Another experiment with larval zebrafish included studying the swimming pattern of 5- dpf (days post fertilization) zebrafish larvae which were exposed to light in a particular sequence which was bright dim-bright. This experiment was to check a hypothesis that untreated fish will show more activity at the bright-dim transition as compared to dim-bright transition whereas ethanol treated fish will show more activity on both light transitions. 27 AB strain (9-10dpf) larvae were taken of which 15 were control i.e. they were not subjected to ethanol treatment rest 12 were exposed to 12% ethanol for 30 min prior to experimentation. Fish were first acclimated to bright light for 5 minutes and then abruptly switched to dim light for 15 minutes, followed by a transition to bright light for 5 minutes. Fish were placed in a standard 24-well plate (one fish per well). For bright-dim transition 9 out of 15 control fish responded within 5000 milliseconds of the change and 11 out of 12 ethanol treated larvae responded within the same time frame. For Dim-bright transition - Only six out of 15 responded however all but one ethanol treated fish responded with latency less than 5 seconds. The results confirm the hypothesis, also the ethanol treated fish were quicker to respond to light transition as compared to control fish. The larval zebrafish is an excellent model for investigating locomotory kinetics and drugs with anxiolytic properties and change in melanocyte morphology.

Learning

There is lack of research on alcohol dose-effect and how it acts on the brain. The drug seeking behavior caused by different alcohol doses in short and long term uses and the effects of different alcohol doses on a learning task [Fig. 4] with a cognitive element was tested in an study were lower doses (0.10%) resulted in learning behavior and no seeking behavior was generated [19]. Higher doses (0.25% and 1.00%) impaired the associative performance and induced search for the drug. low alcohol doses (0.10%) were able to learn to associate stimuli at least 2 days in advance of the control group. On the other hand, 0.25% and 1.00% chronic alcohol treatment inhibited learning behavior. Only 0.25% and 1.00%, both in acute or chronic use, generate alcohol seeking behavior. The 0.25% group showed worse learning performance than control and 0.10% groups. This study confirms the importance of zebrafish as a model for drug throughput screening. Zebrafish can be used for designing models to reverse drug seeking behavior such as punishment or reinforcement associated to withdrawal in order to weaken the brain reward systems. Also studies which show changes in brain because of low and high alcohol doses must be done.

In another study associative learning in zebrafish in the plus maze was studied [20]. Two classical learning tasks in a plus maze were employed. In the first task zebrafish were required to associate a visible cue with food reward irrespective of the location of this pairing. The visual cue was a red plastic cue card as it is expected to be clearly distinguishable for the tetra chromatic zebrafish. In the second task, zebrafish were required to find the fixed location of the food reward. The location of this reward was not marked by an intra-maze visible cue but instead it was supposed to be identified based upon external visual cues that surrounded the maze. Both groups showed some preference toward the target arm. This may be because the target arm was the only location where food was accessible and thus zebrafish may have spent slightly more time there actually eating the food. The zebrafish that received the visual cue-food reward pairing responded with strong preference towards the visual cue alone during the probe trial, and the fish in the unpaired group did not. Zebrafish appeared active, explored the plus maze, and exhibited no signs of fear. Zebrafish is capable of attaining good performance in these associative learning tasks. The significant increase of time in the target arm demonstrated that zebrafish of the paired groups have learned and remembered the association between the single visual cue and the food reward (simple associative learning), and in the following task, the location of the food reward (spatial learning).

Neurotransmitter studies

Dizocilpine also known as MK-801, is an uncompetitive antagonist of the N-Methyl-D-aspartate (NMDA) receptor, a glutamate receptor discovered by a team at Merck in 1982. Glutamate is the brain's primary excitatory neurotransmitter, it has been utilized in the analysis of mammalian learning and memory. The zebrafish is novel vertebrate study species that has been proposed for the analysis of the mechanisms of learning and memory.

In a study, the suitable dose of MK-801 that does not elicit performance impairing effects, a concentration that may be appropriate for the analysis of learning and memory in zebrafish [21]. It was investigated whether MK-801 can disrupt motor function (important for navigating through the maze), visual perception (whether the drug can disrupt the ability to see the target stimulus), and motivation (whether the drug can

reduce/alter shoaling tendencies) in zebrafish. Four concentrations of MK-801 hydrogen maleate was dissolved in system water: 0 μ M, 2 μ M, 20 μ M and 100 μ M. The drug was administered at three different time points: (a) fish received the drug during the 30 min long behavioral session, (b) 30 min period immediately before the behavioral session, and (c) 30 min 24 h before the behavioral session. 3 behavioral tests were conducted: open tank, light-dark preference, and group preference. All tests were conducted between 10:00am and 4:00pm. The behavior of the fish were video recorded. The recording session was for 30min. MK-801 in the currently employed dose range (0–100 μ M) appeared fairly safe, led to no increased mortality or morbidity, and rarely resulted in significant behavioral changes. Most of the motor and posture patterns of zebrafish remained unaltered by MK-801 in the open tank. Results suggest that even in the larger rectangular tank, circling may be induced by MK-801. The behavioral and neurobiological mechanisms underlying this drug response are unknown at this point. Lower doses of MK-801 are unlikely to alter vision and the motivation to swim close to conspecifics. Results suggest that the highest safe dose that is unlikely to affect motor function, visual perception, and/or motivation to respond to conspecifics of zebrafish is 20 μ M, and MK-801 at this dose will not affect these crucial performance factors irrespective of whether the drug is present during or if it has been administered prior to the behavioral test. Therefore it is suggested that 20 μ M may be an appropriate concentration for the analysis of the potential learning and memory impairing properties of MK-801 in zebrafish. In another study [22] effects of acute exposure of dizocilpine [(+) MK-801] on behavior was studied. 3 experiments were conducted. Experiment 1- In this experiment- 3 round chambers were taken of which one contained deionized (control) water, other two containers had 2 μ M and 20 μ M (+) MK-801 hydrogen maleate respectively. In each tank 10 fish were kept. After 5min habituation period observations were made. Experiment 2- Before experiment male and female fish were subjected to 0, 2, 20, 200 μ M of (+) MK-801 for 1hr. Subjects were then placed in observation chambers and activity was measured. Swimming activity was monitored by counting the number of line crossings in a 30-s observation period every 6 min. Experiment 3- In this experiment fish was placed at the start point of the T-maze and allowed to freely explore the maze for 5 min. The time of entry into the EC (Enriched chamber) was noted only after a fish spent 20 consecutive seconds in the chamber. Additionally, total time spent in the EC was recorded to determine chamber preference. Readings were taken at 27 (Trial 2) and 48 (Trial 3) h post dosing. Measurements of circling, swimming activity, and preference indicate (+) MK-801 exposure associated modification of behavior in zebrafish. Treated fish circled almost continuously, while control fish were active but rarely completed a 360° rotation of the test chamber. There was a trend for control fish to reduce the time that it took for them to reach the EC during the second and third trials. In contrast (+) MK-801-treated fish did not express a preference for the EC, nor were there significant differences in time to reach the chamber across trials. A preference for the chamber may provide the necessary motivation for route acquisition. The results of these experiments suggest the utility of further investigation of zebrafish as a potential model organism for assessing both normal and dysregulated glutamate systems.

Another category of behavioral studies includes analyzing the effect of neurochemicals on several characteristics of zebra fish. Since all of the embryonic development occurs outside the mother, zebrafish are being widely used to study neurodevelopmental defects associated with toxicant exposure and neurological diseases [23].

In one of such experiments, the effects of neurotransmitters like DOPAC and 5-HIAA on the serotonergic and dopaminergic systems of zebra fish and how it reflects on the maturation of shoaling in zebra fish. This development is compared among two different strains of zebra fish. Shoaling behavior is defined by the amount of Inter-Individual Distance (distance between each focal fish), which is expressed relative to body length. HPLC was performed to find out the amount of dopamine and serotonin and their respective metabolites DOPAC and 5-HIAA since previous studies indicated that dopaminergic system is involved in shoaling in zebrafish and serotonergic system is involved in fear- which induces shoaling. The differences in shoaling behavior of the strains AB and TU were studied on different days post-fertilization. These differences might be due to differential development or growth rates. But when the body length was measured, identical growth was recorded in both strains. From 7 dpf to 87 dpf, zebrafish reduced their inter-individual distance within the studied ten member shoals from about 14 body lengths to about 6-7 body lengths. The rapid increase of shoaling in TU coincided with the step-wise increase of dopamine and DOPAC levels seen after 40 dpf. The steady increase of shoaling seen in AB coincides well with the linear age-dependent increase of dopamine and DOPAC obtained from this strain [24]. In another study [25] animated images of conspecific are shown to fish conspecific means member of the same species, presentation of conspecific stimulus fish has been shown to be rewarding in zebrafish. Dopamine plays important roles in motor function and reward. Zebrafish have dopamine receptors homologous to mammalian counterparts, and dopamine receptor antagonists. Animated images act as a visual stimulus and the effects of it are monitored and the amount of dopamine, DOPAC, serotonin and 5HIAA extracted from the subject's brain immediately after the stimulus presentation using HPLC with electrochemical detection are quantified. Instead of animated images live conspecifics can also be used but animated images have an advantage that they are consistent and experimentally well controlled. Experimental subjects presented with zebrafish images all significantly decreased their distance to the computer screen as compared to the no stimulus group. Fish presented with zebrafish images for 10 or 15 min had significantly higher dopamine levels as compared to the other three groups. Fish that received the scrambled image had significantly lower levels of 5HIAA than all other fish, while other groups did not differ from each other. DOPAC levels significantly increase in response to the presentation of zebrafish images but not in response to the scrambled images. Serotonin levels significantly decrease in response to the presentation of the scrambled without the zebrafish images fish had no preference towards any side of the

tank. However upon presentation of conspecific images the distance from the screen decreased. Elevated dopamine and DOPAC levels induced by the sight of conspecific images are the result of increased dopaminergic function, which is due both to elevated dopamine release (synaptic transmission) and to increased dopamine synthesis. It is the appearance and not the mere presence of the social stimulus that engages the dopaminergic system. Future study in this field should focus on questions like How other neurotransmitter systems may be involved in shoaling in zebrafish and also importantly what neural circuits, and in general which brain areas, may be involved? In another experiment, different types of neurochemicals were tested on zebra fish. Chlorpyrifos exposure [23] has been shown to impact dopaminergic, serotonergic and noradrenergic systems. Early developmental exposure to a specific dose of CPF has been shown to cause a learning impairment. Nicotine and pilocarpine exposures were used to determine the contribution of the receptor systems to the CPF-induced effects. Startle response and habituation over a period of 10 minute trials were used as a measure of the effects of these chemicals. The startle response was induced by a tap. Developmental chlorpyrifos exposure caused an overall increase in startle response in adult zebrafish but only different higher doses of nicotine and pilocarpine increased startle response, though the response had more significance than the DMSO controls, showing that CPF exposure causes the fish to not get habituated to the tap-stimulus.

Social behavior related studies

Social behavioral study using zebrafish can be done to gain more insight regarding autism and schizophrenia where social behavior impairments are prominent [26].

Zebrafish (*Danio rerio*) are visually drawn to conspecifics (related to the same species) [26] and adults instinctively aggregate into shoals. They are highly social animals that live in groups with structured social relationships including shoaling, dominance hierarchies and territoriality [27]. Shoaling is one of the most important social behavior exhibited by zebra fish. It is thought to provide the individual fish with multiple benefits including access to mates, efficient foraging and defense against predators [28]. Shoaling is usually measured in terms of body lengths. The performance among zebrafish strains: inbred (AB) or wild-crossbred (WIK) from Zebrafish International Resource Center, to golden and short-fin from Petco stores, was compared to study the social interaction and novelty preferences among zebrafish [26]. AB is the oldest inbred strain, WIK is recently obtained from wild-caught zebrafish, short-fin zebrafish are outbred and golden form occurs due to a pigment mutations in short-fin.

Social interaction test: In this study, colorless water-filled acrylic box with stranger fish and blue water-filled acrylic box without a fish were placed at opposite ends. AB fish spent more time with the stranger fish and Petco short-fin zebrafish spent more time in the empty blue box than the other strains. This behavior of Petco short-fin zebra fish could be attributed to their environment during juvenile imprinting [26].

Social novelty preference test: Instead of blue box, a colorless box containing a 'new' stranger zebrafish was used while the old stranger was placed at the opposite end. AB and golden mutant strain preferred the new stranger while Petco short-fin fish spent an equivalent amount of time with both the old and new stranger zebrafish [26].

Social preferences: Shoaling preferences emerge during juvenile phase and are visually mediated, so that when given a choice between shoal mates with different coloration patterns, individuals prefer to shoal with those sharing the same coloration pattern as the fish with whom they were raised. Once established shoaling preference remains stable and it is not reversed by changing their social environment.

Social recognition: Zebrafish use both visual and olfactory cues in social recognition. Studies showed that visually mediated recognition is based on a mechanism of phenotype matching against a learned template in early life. Olfaction also plays a role in species recognition as well as kin recognition in zebrafish, through a process of phenotype matching.

Social learning: Groups of zebrafish learn an avoidance response to an electric shock faster than single individuals. It has also been shown that zebrafish can learn escape routes from trained demonstrators, and that the presence of demonstrators in groups of naive individuals increased the escape response [27].

There also have been other experiments which demonstrate social learning among zebra fish. One of them was to find out whether zebrafish at the center of their social networks have a greater impact on group behavior than less socially connected individuals. 'KEY' individuals are the most central to the groups because they interact readily with most of the other individuals, whereas 'NON-KEY' fish either stay at least two body lengths away or remain in the proximity of one other fish. Both the key and non-key fish were recognized and taken into another tank with an opaque screen leaving a gap enough for them to pass through to the other side. When the key and non-key zebrafish were placed in different groups in similar tanks, a wooden stick was introduced into the tank to induce avoidance behavior. This was done in three trials. The group with the key fish was faster to cross to the other side than the other groups with the Non-key fish in all the trials. This supports the idea that group motion is, in part, an indirect consequence of social relationships [29].

Age related behavioral studies

These studies consist of observing different behavioral parameters that change with age. For example, locomotor behaviors of zebra fish larvae have been observed. These studies focused on activity and space use in zebrafish larvae from 4 dpf to 7 dpf. Two experiments were conducted. In Experiment 1, the same larvae were observed from post-fertilization day 4. The main aim of Experiment 1 was to document any developmental trend in general activity levels in 4 to 7 dpf zebrafish larvae and to determine any age-dependent preferences for spatial location and orientation during this period. In Experiment 2, different groups of larvae from the same egg collection were observed at 4, 5, 6 and 7 dpf. The larvae were placed in 96 micro-well plate. Both experiments found significant behavioral differences in resting and a lesser extent of activity between 4 dpf and older larvae. There was a biased preference for the edge region and an outward facing orientation across all four ages. But there is a possibility that activity in 4 dpf larvae may be more variable than at any other age [30].

In another type of such studies, shoaling behavior has been observed. Here, age-dependent changes of shoaling behavior in freely moving groups of zebrafish have been analyzed. Shoaling is thought to provide the individual fish with multiple benefits including access to mates, efficient foraging and defense against predators. Three experiments were conducted, in which the zebrafish were allowed to explore the glass tank freely. In experiments 1 and 2, the arena size was kept proportional to the body length of the growing fish (for a particular age-group), but in experiment 3, two separate age-groups of fish (30 and 60 day old) were tested in six different arena sizes each.

Experiment 1: Longitudinal developmental analysis of shoaling

The purpose of this experiment was to investigate the trajectory of potential age-dependent changes of shoaling behavior in zebrafish. The same group of fish were followed throughout their development (a repeated measure design). All open field tanks employed for older age groups were scaled-up versions of the smallest tank (90*90*30 mm width*length*depth) used for the 3.2 mm long 5 dpf old fish. It was found that at the youngest age tested zebrafish appeared dispersed though they were attracted (slightly) to each other. The distance among shoal members decreases with age and reaches 5.81 body-lengths at 76 dpf.

Experiment 2: Cross-sectional analysis of age differences in shoaling

Since repeated handling induced elevation in fear could lead to enhanced shoaling, a non-repeated measure cross-sectional experiment was conducted. Shoaling behavior of seven different age groups of fish were analyzed. The fish were tested in the open field only once. The order of testing fish of different ages was randomized. This analysis confirmed that shoal density increased with age.

Experiment 3: Randomization of open field size

Two age groups of fish (30 dpf and 60 dpf) were exposed to different tank sizes but instead of increasing tank sizes over time, exposure to different arena sizes was randomized. It was found out that increasing tank sizes did not lead to decreasing average distance among shoal members in either age group studied [28].

CONCLUSION

Zebrafish model in neuro-behavior research serves as an ideal resource for scientists seeking valuable insight into the growing utility of zebrafish in neuroscience. The larval zebrafish is an excellent model for investigating locomotory kinetics as well as drugs with anxiolytic properties. High-speed video recordings of behavioral responses in this species are indeed very promising for high-throughput screening (which is not feasible in rodent models). In future studies some of the questions like how other neurotransmitter systems may be involved in shoaling in zebrafish and also importantly what neural circuits, and in general which brain areas, may be involved should be pondered upon. Zebrafish can be used for designing models to reverse drug seeking behavior such as punishment or reinforcement associated to withdrawal in order to weaken the brain reward systems. Also studies which show changes in brain because of low and high alcohol doses must be done. Future forward genetic analysis must be done to identify mutations that show altered sensitivity to ethanol that shall provide important insights into the genes involved in regulating the behavior.

CONFLICT OF INTEREST

There is no conflict of interest among authors.

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