



Zebrafish (*Danio rerio*): An Alternative Model Organism for Central Nervous System Disorders Screening

Abhishek Nadiga¹, K. L. Krishna^{1*}, Seema Mehdi¹, Sahyadri. M¹, Suman.P²

¹. Department of Pharmacology, JSS College of Pharmacy, JSS Academy of Higher Education & Research, Mysore-570 015. India

². Department of Dravyaguna, Govt. Ayurvedic Medical College & Hospital, New Sayyajirao Road, Mysuru-570 001

*Corresponding Author: klkrishna@jssuni.edu.in

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ABSTRACT

Search for alternatives to animal models in biomedical and allied research is a recent trend in basic drug research communities; Zebrafish (*Danio rerio*) (ZF) would be an alternative animal model for biomedical research. Various animal model(s) have been used to study the cause and mechanism behind central nervous system disorder. However, still there is no clear evidence that triggers the cause of disease in these models. Currently, both larval and adult ZF emerged in scientific research as an alternative to *in-vivo* vertebrate species for CNS disorders due to their physiological and genetic similarity with humans. ZF research has characterized the various genes involved in neurodegenerative diseases and the same are found in rodents models. The use of ZF models is in line with the 3Rs principle of CPCSEA. In the present review, we summarize the ZF as an alternative to the animal model in CNS disorders and also highlight the advantage of ZF in basic biomedical research.

INTRODUCTION

Experimental animal models have a major role in bridging the gap between preclinical and clinical research. The pathophysiological phenomenon observed in the *in-vivo* animal disease models is used to understand the disease pathogenesis in humans and eventually can be translated into treatment approaches. Even though many animal models have been replaced by an alternative method for drug development, legitimacy and reliability of science, still animal models have an important role in scientific evaluation. Considering the animal model in biomedical research, probably the most common used are rats, mice and rabbits. However, in the past decade, researchers across the globe are shown interest in the use of small freshwater Zebra Fish (ZF) for basic biomedical research. In the

United State, NIH (National Institute of Health) has recently established the world biggest ZF research Centre (**Koroshetz *et al.* 2018**).

It is reported that limited research has been conducted in the field of CNS disorders modeling, including modulations in brain circuits, neuro-immune interactions and neurotransmitter systems, which is lacking newly approved drugs to treat CNS disorders (**Piirainen *et al.* 2017**). Rodent models of CNS disorder have been examined extensively and have given ample opportunities for disease model. However, still, there is no clear evidence that triggers the cause of neurodegeneration. Furthermore creating CNS disease models to mimic multiple symptoms becomes important for studying brain disorders (**Kalueff *et al.* 2013**). Researchers using ZF have identified the functions of various genes which are responsible for neurodegenerative disorders, which found strenuous to examine in other animal models (**Wang *et al.* 2010**). In this context, ZF has emerged in scientific research as an alternative in vivo vertebrate model organism for CNS disorder.

Zebrafish (*Danio rerio*), is a freshwater *teleostei*, comes under the family *Cyprinidae* and order *Cypriniformes* (**Biran *et al.* 2018**). ZF is native to tropical freshwater, originally found in South Asia including India, Pakistan, Bhutan, Bangladesh, Myanmar and Nepal (**Biran *et al.* 2018**). ZF is small in size, where adult fish measure up to 4-5 cm in length, cylindrical body with distinct color patterns having alternative dark and light stripes, exhibit sexual dimorphism. Females are thicker with silvery in the ventricle region and males are more slender with golden color in the ventricle region (**Mustafa *et al.* 2019**). The expansion of ZF in the field of neurology has repeatedly increased due to its easy access to all stages of development; in addition, it is a vertebrate model with physiological, morphological, and genetic similarity to humans especially with CNS (**Biran *et al.* 2018**). Small size enables the high number of housing in limited infrastructure, a short life cycle with rapid and external development and a high fecundity rate. Optical transparency of the larva helps in real-time imaging; the availability of multiple strains enables to study differences among them in neurobehavioral and neurobiochemical response (**Mustafa *et al.* 2019**). Furthermore, the use of ZF comes under 3Rs (Reduction, Replacement and Refinement) principles of preclinical evaluation as essential guidelines from the international and national regulatory bodies like CPCSEA (Committee for the purpose of Control and Supervision of Experiments on Animals). In addition to this, ZF helps in experimental time reduction and consumption of limited resources when compared to other rodent models and also provides additional information with predictive results when compared to *in-vitro* results (**MacRae and Peterson, 2015**). As a result, the ZF model can be used to minimize or replace the usage of other animal models in biological research. This model is also used as a supplementary model to compare the last existing results obtained, thus it also has the potential to refine the results (**Bailone *et al.* 2019**). In the current paper we reviewed the use of ZF as an alternative animal model for the evaluation of CNS disorders and their advantages.

1. Zebrafish Model

The potential characteristics of ZF model allow researchers to use it in different disciplines including pharmacology, neurology, embryology, behavior genetics and drug discovery (Choi and Eastman, 1995), (Ab. Aziz *et al.* 2021). However, from the last decade ZF model is used to study the pathogenesis of many human diseases and this is rapidly increasing (Nag *et al.* 2020). The main reasons are high genetic homology, sharing (80-85%) of the human disease genes, as well as the similarity of physiology & morphology with humans and other rodents models (Howe *et al.* 2013). Short growth period, embryos hatch within 72 hour post-fertilization (hpf) and become mature in 90 days, *ex-utero* development of the embryo which takes place outside the mother body, and high reproductive capacity (200-400 embryos per each spawning) (Fig. 1). Besides these, its small size enables housing a large storage in limited space (100 adults per 12 L of the tank), which do not require a large infrastructure facility as in the case of other laboratory animals. It is a cost-effective species compared to other models; the cost of the rearing mice model is thrice larger than the ZF (Sieber *et al.* 2019).

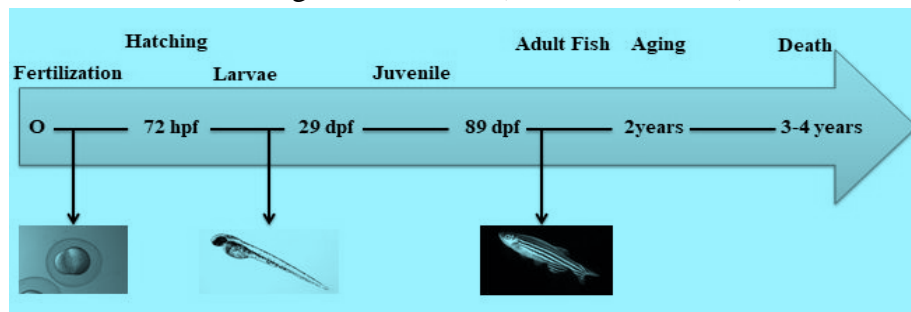


Fig.1. Life cycle of the Zebrafish

ZF has more protein-coding genes (26,206) than any other previously sequenced vertebrate (Collins *et al.* 2012). In comparison to humans, mice and chickens, ZF contain more species-specific genes in their genome. A minimum of one ZF orthologous can be found in almost 71.4 percent of human genes. In contrast, 69% of ZF genes share at least one direct human gene. Further, 47% of the genes of humans share a one-to-one relationship with the ZF orthologue (Table 1) (Howe *et al.* 2013). The second large orthologous class contains human genes that are related to multiple ZF genes ("one human to many ZF" class), with an average of 2.28 ZF genes for each human gene, possibly reflecting Target Site Duplication (TSD). The above advantages of ZF have gained more popularity in the field of biomedical research and are also supportive to implement the 3R principles of a regulatory body like Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) in India.

2. Zebrafish in Neuroscience Research

The application of ZF in the field of neuroscience research has markedly increased in the last decade; Reasons include being a vertebrate organism, possessing similar physiological and genetic functions to humans & other vertebrate species and similarities in the morphology of CNS (Kalueff *et al.* 2013). Even though the size difference

between ZF and humans is obvious and highly significant, their nervous systems are anatomically similar. In both organisms, the nervous system is divided into peripheral nervous and enteric components making up the PNS. The CNS is made up of the brain and spinal cord, which are classified into: forebrain, midbrain, and hindbrain. Furthermore, the cranial nerves and the fundamental structure of specialized sense organs such the olfactory system, eyes, and ear are similar between humans and ZF (Nag *et al.* 2020). ZF shares molecular and structural homology with many brain regions relevant to human disease. In general, gene expression patterns in large-scale areas of CNS, such as the forebrain, midbrain, hindbrain, and spinal cord, are often conserved in ZF. These regions exhibit regional connectivity of sub-structures such as the optic rectum, hypothalamic regulatory nuclei, thalamus, cerebellum, medulla oblongata, and spinal cord (Fig. 2) (Piirainen *et al.* 2017). Further, they also possess neurochemical identities such as neurotransmitters and receptors (Jones and Norton, 2015).

Table 1: Similarity of human and zebrafish orthology relationships and their protein-coding genes.

Type of relationship	Human	Zebrafish	Core relationship	Ratio
1 to 1	-	-	9,528	-
1 to many	3,105	7,078	-	1:2.28
Many to 1	1,247	489	-	2.55:1
Many to many	743	934	233	1:1.26
Orthologous total	14,623	18,029	13,355	1:1.28
Unique	5,856	8,177	-	-
Coding-gene total	20,479	26,206	-	-

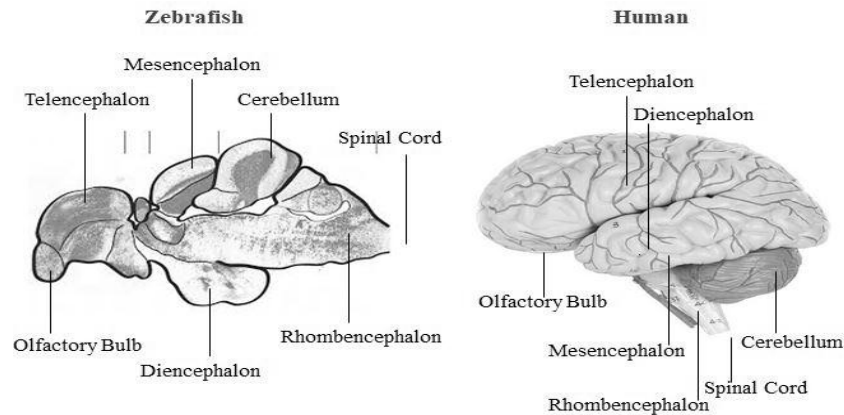


Fig.2. Conserved brain regions between Zebrafish and human.

3. CNS Disorder

CNS disorders may occur due to gradual neurodegeneration leading to loss of brain functions which include behavior, cognitive (memory, learning and thinking) as well as motor functions, and genetic polymorphisms being the major risk factor for these disorders (**Keykhaei et al. 2021**). Most common neurodegenerative diseases are Alzheimer's, Parkinson and Huntington's. Researchers have identified and characterized the genes linked with all those neurodegenerative diseases, but it is still clearly unknown that how the mutation of such genes causes neurodegeneration. This could be a major challenge for future research in the area of neuroscience.

3.1 ZF models for Alzheimer's disease

Due to greater probability of failure in the drug development process, medication research and development for Alzheimer's disease (AD) is extremely difficult and complex. The excessive production and deposition of A β plaques in the elderly and the formation of intracellular neurofibrillary tangles (NFT) are recognized diagnostic features of AD. In preclinical research, many genetically engineered Alzheimer's disease models have cost and time restrictions.

ZF has become an excellent complementary model for AD research because of its simpler structure with robust and clearly visible patterns of behavior. ZF has cholinergic and glutamine pathways which are responsible for learning and memory. AD manifests in the hippocampus region of the human brain, and the dorsal lateral pallium of ZF is the homolog to it. In addition to this, the blood brain barrier (BBB) of ZF exhibits similar structure and function to the human BBB allowing the assessment of novel neuro-drugs for their permeability. Genes identified in ZF that play an important role in AD pathogenesis are clear orthologous to the human genes. The gene PSEN 1 and PSEN 2 of ZF are orthologous to human PSEN 1 and PSEN 2 genes respectively **Hin (2020)**. The genes APP a and APP b of ZF are human APP co-orthologous (**Barthelson et al. 2021**). In addition to this, the genome of ZF contains orthologous to genes which are components of gamma-secretase complex PSENEN (psenen) (**Jiang et al. 2018**), APH1b (aph1b), NCTN (nctn) and beta-secretase complex BACE1 (bace1) (**Moussavi et al. 2007**), and BACE2 (bace2) (**Van Bebber et al. 2013**). The human microtubule-associated protein tau MAPT, APOE and SORL 1 are co-orthologous to mapt a and mapt b (**Chen et al. 2009**), apoe a and apoe b (**Pitchai et al. 2019**), and sorl 1 (**Lee et al. 2017**) are also present in the genome of ZF respectively. ZF also possess the neurotransmitter system including dopaminergic, glutamanergic, serotonergic, cholinergic, glycinergic, and γ -aminobutyric acid system. Various cognitive paradigms such as light and dark test, T-maze test, a three-chamber spatial alternation, conditioned placed preference and tap-elicited startle reflex response are validated to test the memory and learning ability of ZF (**Cleal et al. 2021**). Many of the same behavioral tests that are used on rodents may also

be used on ZF. Various neurotoxic agents such as scopolamine, okadaic acid, cigarette smoke extract and various metals are used in the ZF model to induced AD-like symptoms which can be further used in the process of drug discovery for therapeutics against AD (**Thawkar and Kaur, 2021**).

Based on the cognitive and behavioral responses of AD, researchers have examined the non – associative learning in the ZF larva. Introduction of A β ₁₋₄₂ into the hindbrain of 1dpf ZF embryos leads to significant memory impairment with elevated Tau phosphorylation (**Nery *et al.* 2014**). Most recently, another group of researchers developed a pharmacologic model of AD by using okadaic acid, where increasing concentrations of okadaic acid were found to increase both A β plaques and Tau phosphorylation in adult ZF (**Nada *et al.* 2016**). Furthermore, the Administration of *H. erinaceus* extracts, cotinine and 6-Hydroxy-L-Nicotine potentially improved the cognitive function and brain oxidative stress in ZF in scopolamine induced AD which was evaluated by various behavioral and biochemical tests (**Valu *et al.* 2021**) (**Muthuraman *et al.* 2019**).

It is possible that the zebrafish model has emerged as an intriguing tool for the strategic study of AD. The research using this model system can effectively bridge the gap between drug discovery based on cellular models and pre-clinical assays. The zebrafish is the best candidate for high-throughput pharmacological screening of drugs before they are validated in rodent models. A great deal of research has already been done to expand on the scope of zebrafish as a model to understand AD. The current requirement is for a good AD transgenic model that expresses both A β and Tau pathologies. This will aid in the completion of the puzzle of understanding AD, some pieces of which are still missing.

3.2 Parkinson Disease

Movement disorders are primarily classified into hyperkinetic and hypokinetic. Parkinson's disease (PD) is associated with hypokinetic syndromes. Common risk factors identified to trigger the PD are age, gender, environmental toxins and genetic factors. PD is characterized by dopaminergic neuron loss and presence of intra cytoplasmic inclusions called Lewy bodies (**Fanning *et al.* 2020**). Finding effective treatments for these diseases is difficult and involves the development and characterization of accurate animal models. ZF can be used as alternative animal model for investigating PD. The ZF's compact size, optical translucency, rapid development and high throughput screening allow several chemicals to be studied simultaneously (**Kalueff *et al.* 2013**). Most of the related molecular mechanisms are substantially conserved and the brain pathways involved in ZF movement have been thoroughly defined. ZF dopaminergic neuron system is well defined in both embryo and adult stages. A functional blood-brain barrier was identified in the ZF on the 3rd postnatal day (**Razali *et al.* 2021**). Six genes have been identified which are associated with PD, they are Parkin, α -Synuclein, PINK1, UCHL-1, DJ-1 and LRRK2 (**Abeliovich and Flint Beal. 2006**). The human gene PARK2

is co-orthologous to the park2 gene of ZF which lies on chromosome 13 and encodes 458 amino acids out of 465 in humans (**Flinn et al. 2009**). In addition to that 54% similarity orthologous was identified with PINK1 (pink1) (**Anichtchik et al. 2008**). Various models including neurotoxins and genetics commonly used to develop PD in rat models are being used on the ZF model and have shown similar results. Most widely used neurotoxins to induced PD are rotenone, 6-Hydroxydopamine (6-OHDA), MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), paraquat and maneb (**Razali et al. 2021**). Various disease models of PD, their pathological hallmarks, motor Phenotype and observations in ZF were mentioned in **Table 2**.

An earlier study reported that exposure of adult ZF to leads to 28 days of rotenone (2 µg/L) leads to motor and non-motor PD like symptoms such as decrease in dopamine level, less distance travelled, anxiety and depression like behavior in the light and dark test (**Wang et al. 2010**). Another group of researchers found that induction of 50 µmol/L MPTP to ZF embryo, exhibited dopaminergic neurons loss, PD like locomotor behavior and abnormal expression of alpha-synuclein (α -syn), PTEN-induced putative kinase 1 (pink1), tyrosine hydroxylase (TH) and E3 ubiquitin-protein ligase (parkin) PD related genes (**Dong et al. 2021**). Furthermore, isotope labeling techniques and mass spectrometers were used to analyze changes in the peptide profile of ZF brains induced by 6-OHDA in Parkinson's disease conditions. These analyzes enabled the identification and quantitation of 118 peptides, 9 of which showed significant changes. Further, fragments of intracellular and extracellular proteins related with lipid metabolism and the dynamic cytoskeleton had the most diverse configurations. These findings open up new avenues in the function of peptides in PD (**Fiametti et al. 2021**).

3.3 Huntington Disease

Huntington's disease (HD) is an autosomal dominant inherited, monogenic neurodegenerative disorder. Progression of HD includes movement, cognitive and psychiatric symptoms. Huntington (HTT) a mutant protein arises in an expanded polyglutamine (polyq) repeat in the amino-terminal region. The insufficiency and gain of function of the IT15 (HD gene), including caspase activation, protein aggregation and mitochondrial dysfunction are responsible for the development of this disorder (**Mwaffo et al. 2015**). But still, no specific drugs apart from symptomatic treatment are available to slow down the progression of HD. In addition to that, the exact mechanism behind the HD is poorly understood.

Researchers have identified a ZF orthologous of HTT (**Karlovich et al. 1998**), the role of molecular chaperones in suppressing the protein aggregation and neuronal cell death caused by miss expression of ploy-q expanded HTT fragment (**Schiffer et al. 2007**) and anti-prion compound in inhibiting the formation of poly-q aggregation in ZF (**Miller et al. 2010**). Only four glutamines are encoded by the ZF ortholog of the human HTT, compared to up to 35 glutamines in humans (**Vaz and Silvestre, 2020**). Various functions

of the ZF HTT protein are associated with the regulation of brain development, iron, lipoprotein, cholesterol and energy metabolism. Changes in the phenotypes are seen in its knockdowns, such as decreased expression of BDNF, neurophysiologic abnormalities, neuronal apoptosis in the midbrain and hindbrain, decreased formation of neural tubes, cell adhesion, increased activity of metalloproteinase (ADAM10 and N-cadherin), haemoglobuline deficiency and severe reduction in cartilage biogenesis (**Vaz and Silvestre, 2020**).

Table 2: Zebrafish models of Parkinson disease.

Zebrafish Model	Pathological Hallmarks	Motor Phenotype	Observations
Chemical			
1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)	Degradation of Dopaminergic neurons	Evoked swimming response deficits. Increased immobility time.	Neuronal and motor impairments
6-Hydroxydopamine (6-OHDA)	Dopaminergic neurons degradation	Less distance covered.	Levodopa + Carbidopa rescue motor impairments
Paraquat	Dopamine level decreases	Less distance covered.	ND
Rotenone	Dopamine level decreases	Less distance covered.	ND
Titanium dioxide nanoparticles	Dopaminergic neurons degradation	Less distance covered.	ND
Ziram	Dopamine level decreases	Less distance covered.	Apomorphine rescues motor impairments
Genetic			
β - or γ 1-Synucleins knockdown	Dopamine level decreases. Dopaminergic neuron development is delayed.	Less distance covered.	ND
1-Synuclein overexpression	Aggregation of Alpha-synuclein	ND	ND
Human α -synuclein overexpression	Aggregation of Alpha-synuclein	ND	ND
Pink1 knockdown	Dopamine level decreases.	Evoked swimming response defects'.	ND
Parkin knockdown	Dopaminergic neurons degradation	Less distance covered.	ND
J-1 knockdown	Dopaminergic neurons degradation	ND	ND
LRRK2 knockdown	Aggregation of Alpha-synuclein. Degradation of Dopaminergic neurons	ND	ND
ATP13A2 knockdown	ND	Less distance	ND

covered.

Similarly, patients with HD reveal variation in iron homeostasis, BDNF expression, energy metabolism and metalloproteinase activity (**Zuccato et al. 2008**). Complete abolition of HTT expression leads to embryonic lethality in ZF (**Diekmann et al. 2009**). Similarly, HD models in the mouse lack a clear phenotype or exhibit premature death. As a result, the ZF HTT morphant is a potential alternative model for studying HTT cell activity and its role in HD pathogenesis. Exposure of quinolinic acid (QA) into the striatum of adult rodents has been used to induce HD-mimicking brain damage (**Stepanova et al. 2020**). Similarly, QA causes cell death and microglial infiltration in the CNS when it is introduced into adult ZF. Furthermore, it promotes cell proliferation and neurogenesis, resulting in the complete healing of the injury (**Skaggs et al. 2014**). From this research it was evident that the ZF model is a powerful tool for studying neuronal regeneration and for testing potential disease modifying therapies. Collectively, the HTT of ZF shares several important features with the mammalian orthologue. Hence, ZF lines overexpressing the HTT mutant are useful for HD modeling and evaluate the responsiveness to drug therapies.

3.4. Epilepsy

Epilepsy is a chronic neurological disorder associated with alteration in the neurobiological and neurobehavioral patterns characterized by recurrent spontaneous epileptic seizures (**Karoly et al. 2021**). Along with the neurobiological alteration, impairment in the neurobehavioral patterns including cognitive dysfunction was more frequently seen in epileptic patients (**Phuong et al. 2021**). The major problems associated with epilepsy research are the side effects of AEDs and the lack of sensitive in vivo models. There is no model that can properly depict the human epileptic state by meeting all characteristics. However, the chemically induced epilepsy ZF model has good quality in identifying causal mechanisms, phenotypic characteristics and response to epilepsy treatment (**Yaksi et al. 2021**). ZF is considered a beneficial animal that simulates seizures by characterizing their response to chemical reagents (**Cassar et al. 2019**). Replacing the ZF model can overcome this limitation by using different parameters and techniques to better understand the molecular and behavioral mechanisms. A multifaceted nervous system with the simple behavior of the ZF is suited to evaluate seizure or epilepsy. Larval and adult ZF are now widely used for epileptic behavior and brain function studies (**Choo and Shaikh, 2021**). The genes KCNQ2 and KCNQ3 are highly expressed in ZF which provides more support to use this model in studies related to epilepsy (**Desmond et al. 2012**). Chemo-convulsants including, glutamatergic receptor agonists (Quinolinic acid and Kainic acid) and GABA receptor antagonists (Bicuculline, Picrotoxin and Pentylene tetrazol), cholinesterase inhibitor (Physostigmine) and the cholinergic receptor agonist (Pilocarpine) had their convulsive actions in rodent model found to work similarly in ZF (**Da Silva et al. 2020**). Furthermore, methotrexate, domoic acid, 4-

aminopyridine (4AP), an agonist of glutamatergic receptors, a K_p channel blocker have been used as chemo-convulsants in ZF (**Da Silva *et al.* 2020**). At any developmental stage of ZF, the phenotypic characteristics of seizures are easy to recognize and resemble those of rodents. Nevertheless, PTZ and KA leads to seizure-like symptoms in both larvae and adult ZF, probably due to alteration in the glutamatergic system (**Cassar *et al.* 2017**). Another group of researchers also found that the behavioral response of PTZ was affected by the body weight and gender of adult ZF (**Menezes and Da Silva, 2017**). Various chemo-convulsants used in the ZF and there behavioral, electrophysiological, molecular and cellular outcomes were mentioned in the **Table 3**.

3.4.1 Genetic and non-chemical method to induce seizures in ZF.

Intracranial infection, trauma, acute electrolyte imbalance, hypoxia and hyperthermia are also used to induce seizure in ZF (**Dadas and Janigro, 2019**). Febrile seizures are the most prevalent type of seizure in children (**Leung *et al.* 2013**). ZF is the finest model to explore the mechanism of febrile seizures because increasing the temperature of the larval ZF water bath will lead to abnormal activity of electrographic seizures (**Yaksi *et al.* 2021**). The mechanism behind the increased activity of the ZF electroencephalogram (EEG) is related to the TRPV4 channel, which is a non-selective cation channel with high permeability to Ca²⁺. MK-801 blocks glutamate receptors by activating these channels, which enhances glutamate release (**Menezes and Da Silva, 2017**).

3.5 Stress and anxiety

ZF emerged as a novel model for anxiety research, the behavioral strain difference, sensitivity to various drug treatments, strong cortisol stress response; strong correlations exist between zebrafish neuroendocrine responses and behavioral endpoints. Additionally, these fish are extremely vulnerable to a variety of environmental stresses, such as novelty stress, predator exposure, alarm pheromone, anxiogenic drugs, and drug withdrawal. Additionally, different strains of zebrafish exhibit varying degrees of baseline anxiety, all these characteristics that make them a novel model for anxiety research.

In addition to these, various physiological phenotypes of the ZF contribute markedly to anxiety research (**Collier *et al.* 2017**) (**Nadig *et al.* 2020**). The ZF hypothalamus-pituitary-inter renal (HPI) axis is analogous to humans' hypothalamus-pituitary-adrenal (HPA) axis, with cortisol serving as the primary stress hormone in both (**Faught and Vijayan, 2018**). After animals are exposed to stress, the hypothalamus secretes corticotrophin-releasing hormone (CRH), which activates the pituitary and sends a signal to the pituitary to release adrenocorticotrophic hormone (ACTH) (**Dedovic *et al.* 2009**). Under stimulation with ACTH, interrenal of ZF glands or adrenal of mammals synthesize glucocorticoids from cholesterol precursors. Elevated glucocorticoid levels initiate

metabolism and regulate responses to stress, including anti-inflammatory effects, gluconeogenesis and suppression of the immune system (**Dedovic et al. 2009**).

Table 3: Effects of several chemo convulsants used in Zebrafish.

Chemo convulsants	Behavioral effects	Electrophysiological effects	Molecular and cellular outcomes
Pentylentetrazol Antagonist at the Picrotoxin site of the gamma amino butyric acid type A (GABA A) receptor	Stage I: Dramatic increase in fish swimming activity. Stage II: Circling like behavior Stage III: Loss of posture.	Epileptiform discharges	Increased c-fos expression, Decline in BDNF expression and neurogenesis.
Pilocarpine Agonist of cholinergic receptor	Lurching/head banging, Increased mouth Movements, Tremor, Body contortions, Stiffening Loss of posture	Epileptiform discharges	Increased c-fos expression, Decline in BDNF expression and slc1a2b expression.
Physostigmine Reversible cholinesterase inhibitor	Rapid and abrupt movements	Epileptiform discharges	Reduced neurogenesis
Kainic acid Agonist of ionotropic glutamatergic receptors	Stage I: Immobility. Stage II: Circling behavior Stage III: Burst swimming left to right. Stage IV: Convulsions that resemble clonus. Stage VI: Bottom dwelling. Stage VII: Death	Epileptiform discharges	Decline in GFAP cells. Reduced S100 β levels and glutamate uptake.
Domoic acid Agonist of ionotropic glutamatergic receptors	Uncontrolled pectoral fin motion. Tonic- clonic type convulsions	N.D	Elevated expression of transcription factors and molecules of signal transduction
Bicuculline Competitive antagonist at GABAA receptors	Burst swimming pattern	Increased electrically evoked neural response	N.D
4-Aminopyridine (4AP) Blocker of K β channel	Burst swimming pattern	Intense bursts of neural activity	N.D

Negative feedback on the hypothalamus and pituitary gland inhibits the release of CRH and ACTH; thereby it reduces the impact of the stress response (**Tsigos and Chrousos, 2002**). ZF is a useful model for investigating cortisol-mediated stress responses due of its evolutionarily conserved stress response (**Alderman and Vijayan, 2012**). Analyzing the

ZF neuroendocrine response to stress is a valuable tool for complementary behavioral research (**Fig. 3**). The ZF cortisol test is simple and inexpensive, easy to use in the lab and easily comparable to anxiety behavior (**Egan *et al.* 2009**).

ZF anxiety research is much more challenged pharmacologically. To demonstrate the pharmacogenetic anxiety and the susceptibility of ZF to various anisotropic drugs, ZF is usually treated with tranylcypromine (TCP) which blocks serotonin degradation (**Jie *et al.* 2009**). Dizocilpine (MK-801) is a potent hallucinogen that works on numerous serotonin receptors, while lysergic acid diethylamide (LSD) is an antagonist of the N-methyl-D-aspartate receptor (NMDA) (**Backstrom *et al.* 1999**). Monoamine oxidase inhibitors (MAOIs) are most commonly used in clinical practice in the treatment of both acute and chronic anxiety. Similar to rodents, TCP and MAOI induced acute and chronic anxiety in the ZF novel tank test (**Stewart *et al.* 2012**). LSD has been frequently utilized in rat models and it produces a biphasic effect, with anxiety-like hypoactivity followed by hyperactivity. Similarly, LSD activity was recently demonstrated in ZF, exhibiting anxiolytic-like effect for both chronic (elevated top duration time in novel tank test) and acute administration (e.g., Elevated top duration time in the novel tank test and more number of entries to the center area in the open field test) (**Grossman *et al.* 2010**). The antagonist MK-801 of the NMDA receptor evokes reduced predator avoidance, increased locomotor and exploratory activity in rodents. Similarly, MK-801 in ZF evokes the circling-like and hyper locomotor activity. Another group of researchers examined the behavioral and endocrine effects of MK-801 which revealed the anxiolytic behavior such as decreased latency to the top area, increased top duration time in novel tank test. In addition to these, they all noticed lower cortisol levels as well as increased erratic movements (**Egan *et al.* 2009**). Exposure to net stress and caffeine leads to an increase in the concentration of cortisol levels in ZF and often occur concurrently with stress-related behaviors (**Aponte and Petrunich-Rutherford, 2019**). Another study showed that dead ZF leads to an increased level of cortisol and defensive responses throughout the living ZF, which is part of the anticipatory stress response to the coming threat. Further, ZF exposed to restraint stress exhibit elevated average swimming speed, elevated locomotor activity, elevated body cortisol and late CRH reduction (**Egan *et al.* 2009**).

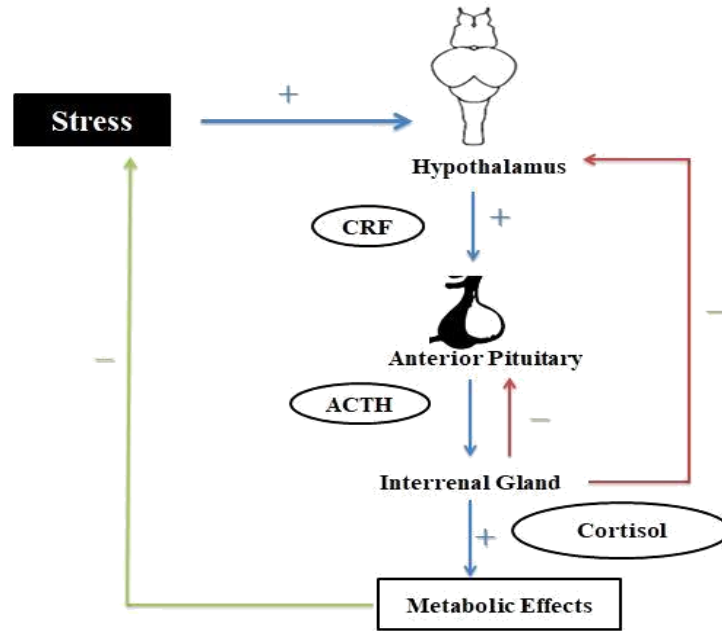


Fig. 3. Endocrine stress axis in zebrafish.

ACTH: Adrenocorticotrophic hormone, CRH: Corticotrophin-releasing hormone,

Activation or inhibition of activity is indicated by the “+” or “-” marks.

3.6 Depression

Depression is another serious stress-related mental illness that affects millions of people around the world. It is typically characterized by mood alternation, social isolation, anhedonia and other severe debilitating mental symptoms (Escobar *et al.* 2018). ZF has become an important research model organism for studying the pathogenesis of depression and its possible treatments (Bühler and Carl, 2021). Similar to humans, common phenotypes that may reflect the depression like state are associated with increased anxiety level, increased basal cortisol level, neuro-motor retardation, decreased shoaling and anhedonia can be observed in ZF (Fonseka *et al.* 2016). In addition, the ZF neurotransmitter system is very similar in structure to the mammalian system. The Comparative neurophysiology of depression-related neurotransmitter systems in mammals and zebrafish are shown in **Table 4**.

ZF exposed for chronic unpredictable mild stress (CUMS) showed a decreased exploration and increased responsiveness to dark light transition which indicates the anxiety-like behavior, which akin to those observed in rodent models (Zhang *et al.* 2021). Clinical depression is strongly correlated to environmental stress, genetic factors and neurochemical disturbances which seem to have similar phenotypic functions in ZF (Beck and Alford, 2009). For example, the *grs357* gene of ZF, with mutated glucocorticoid receptors displaces an increased glucocorticoid level and abnormal

behaviors including a reduction in locomotor activity and impaired habituation. These resemble the same phenotypic characteristics recorded with human clinical depression (Ziv *et al.* 2013). Adult ZF with a mutation in the gene *grs357* showed the disturbed glucocorticoid-mediated negative feedback, which was demonstrated by increased behavioral stress markers such as decreased exploratory behavior, increased immobility with response to mild stress, suppression of cortisol level, increased CRH, ACTH and precursors of these. Whereas diazepam and fluoxetine treatment restores the normal behavior (Sireeni *et al.* 2020). Furthermore, administration of sub anesthetic doses of anesthetic agent ketamine is also meant to reduce systemic cortisol and stress-related behaviors in ZF (Zakhary *et al.* 2011). Following the application of acute restriction stress, the spectrum of oxidative stress in the ZF brain also increases, as evidenced by a significant decrease in catalase activity and an increase in levels of lipid peroxides and non-protein thiols (Dal Santo *et al.* 2014).

Table 4: Comparative neurophysiology of depression-related neurotransmitter systems in mammals and zebrafish.

Neurotransmitter system	Human receptor class	Zebrafish receptor class
MAO	MAO-A, MAO-B	zMAO
Norepinephrine	a1AAR, a1BAR, a1DAR, a2AAR, a2BAR, a2CAR, b1AR, b2AR, b3AR	Adrenergic receptors such as <i>adra2a-c</i> , <i>2db</i> , <i>2da</i> . Adrenergic receptors such as <i>adrb1</i> , <i>2a</i> , <i>3a</i> , <i>2b</i> , <i>3b</i> .
Dopamine	D1 receptor such as: DRD1, DRD5. D2 receptor such as: DRD2–4	<i>drd1</i> . <i>drd2a-c</i> , <i>4a-c</i> .
5HT	5HT1A,B,D–F. 5HT2A–C.5HT3A,B. 5HT4. 5HT5A,B. 5HT6. 5HT7.	<i>htr1aa</i> , <i>-ab</i> , <i>-bd</i>

4. ZF neurological imaging

The simple nervous system of ZF allows imaging of neurons easily and helps to visualize the specific proteins of interest (Anichtchik *et al.* 2006). The minute size and optical transparency of the ZF larvae are highly suitable for real-time imaging in confocal microscopy (Ritter *et al.* 2001). In addition, pathological features and signs will appear earlier in ZF when compared to rodent models. Thus real-time imaging using ZF provides

an opportunity to understand the neurobiological and neurodegeneration process in depth. Researchers could detect a neural circuitry imaging of ZF (**Leung et al. 2013**). Including brain function under natural behavior conditions, widespread of nervous system control in the brain and examination of behavior and modulatory neurotransmitter system. The imaging timing of cell division in ZF embryo (**Kozawa et al. 2016**), Tau induced neuronal degeneration, fragmentation of mitochondria upon apoptosis induced by drugs like staurosporine and valinomycin were also recorded (**Paquet et al. 2009**).

4.1 Ca²⁺ Imaging in ZF.

A microscopic technique optically measures the status of calcium present in an isolated cell, tissue or medium. This technique has made it easy to study the calcium signaling in different varieties of cells. Electrical activity in nerve cells is always carried out by Ca²⁺ influxes; hence Ca²⁺ imaging is a tool to monitor the electrical activity of neurons and glial cells within neuronal circuits. With the use of this technique, researchers have identified the neuronal development of larval and adult ZF which aids in a better understanding of the basic principles underlying vertebrate development and metamorphosis **Lovett (2021)**. Another group of researchers identified the reticulospinal neurons in the ZF and the effect of calcium green dextran on mauthner cells in the live ZF (**Takahashi et al. 2002**). In addition to that this technique also monitors the cellular events of axonal dystrophy arising in neurodegeneration (**Varga et al. 2014**).

5. Behavioral Neuroscience

The behavior of animals determines how they cooperate with their environment. ZF, a promising vertebrate model provides a platform to study the interconnection among brain and behavior. ZF as a similar structural organization and cellular morphology with other vertebrate's models like rat, mice and chicken (**Kalueff et al. 2013**). Structural organs of ZF including the cerebellum, spinal cord, retina and olfactory bulb are having similar architecture with other vertebrates (**Friedrich et al. 2010**). In addition, the ZF brain is very much similar to other vertebrates with respect to neurochemistry (**Piirainen et al. 2017**). Several neurochemical pathways which are involved in the behavioral modulation of adult and larval ZF are well characterized (**Filippi et al. 2010**). The optical clarity of the ZF larvae helps in visualizing the neural circuits during their behavior performance and the brain size of larval ZF at 5 days post fertilization (dpf) is very compact at 500um in thickness and 1.5 mm in length. Thus at this phase, all the nerve cells are easily accessible to *in-vivo* imaging which helps to examine the work of brain function during normal and abnormal behavior (**Chow et al. 2015**). Researchers have developed a ZF behavioral catalog (ZBC) (**Kalueff et al. 2013**). Various neurophenotypic behavioral patterns are found in ZF are similar to those found in rodent models like motor behavior (locomotion, speed, velocity, and distance traveled), cognitive behavior (spatial memory,

reward selection and learning), emotional behavior (anxiety and fear) and social behavior (schooling, aggressiveness and social preference).

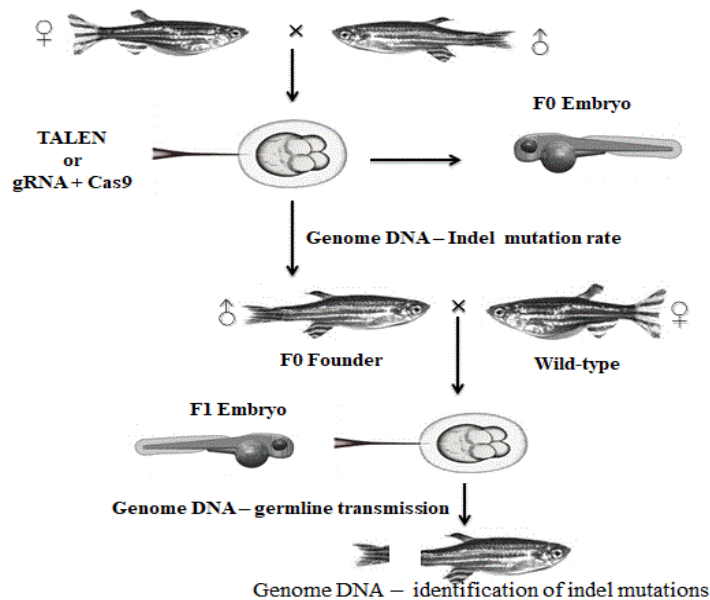
6. Zebrafish gene editing technology

The advanced genetic technology in use today makes ZF a most suitable model for the research in neurological diseases including AD, PD and HD (**Doğanli *et al.* 2013**). Microinjection of morpholinos antisense oligonucleotides is widely used method for the transient gene silencing in ZF. Morpholinos are meant to halt the translation of the target mRNA by hybridizing with the ATG start codon or combining with the exon insertion signal. Morpholinos are injected into single-cell embryos immediately after fertilization to prevent the translation of particular mRNA during the early stages of development. This leads to a high-throughput gene silencing, but this effect will soon be eliminated. Morpholinos was partially effective 3 days after fertilization and completely disappeared after 5 days after fertilization (**Bandmann and Burton, 2010**).

Besides, there are some common problems. Potentially morpholinos microinjection is a very effective method. Microinjection of morpholinos can trigger the p53-dependent cell death pathway. The morphine toxicity phenotype caused by p53 leads to abnormal development of body and notochord and craniofacial diseases in ZF embryo. Due to the limitations of the morpholinos method, researchers are interested in different strategies that lead to stable silent lines. Alkylating chemicals like N-ethyl nitro (ENU) can result in random mutations across the genome. TILLING (genome-directed local damage) was applied first to Arabidopsis and then to ZF. The TILLING method provides a stable mutant line with time-consuming and low efficiency (**Bandmann and Burton, 2010**). Zinc finger nuclease (ZFN), a DNA-binding protein that provides targeted regulation of the genome by creating a double-stranded break in certain genes (**Miller and Gerlai, 2007**). ZFNs are injected into unicellular ZF embryos to induce ZFN-induced mutations. ZFN has three genetic fingers, allowing the gene of interest to be combined from a specific point to two strands of a specific target sequence. Fok1 is a restriction endonuclease which is the head of the ZFN. The activation of Fok1 leads to double-strand breaks in the target gene (**Foley *et al.* 2009**). By stimulating cell repair pathways, non-homologous ends (NHEJ) and homologous recombination (HR) combine to repair nicks in the formed DNA. While the silence is repaired with NHEJ, partial integration of the genome is achieved through the HR mechanism (**Akbudak and Kontbay, 2017**). Even though methods have been established to facilitate ZFN design, the inability to target the desired sequence is a limiting factor for all ZFN-based methods. Transcriptional activator like effector nucleases (TALENs) is the other member of the sequence-specific nuclease family which has emerged as an alternative to the ZFN system. A pair of TALENs is required to connect to the upper and lower strands to cut the desired DNA region. The linked TALEN allows the Fok1 endonuclease to cut the target DNA sequence. Then activate the DNA repair mechanism (**Akbudak and Kontbay, 2017**). Even though the

specificity is high, it is reported that using TALENs non-target mutations can occur in genome regulation (Clasen *et al.* 2016). In ZF, the periodically divided palindromic cluster/CRISPR-associated nuclease 9 system (CRISPR/Cas9) can achieve extremely high gene silencing levels (Irion *et al.* 2014). In the CPISPER technique, a targeted RNA sequence and Cas9 endonuclease are sufficient to cut. Whereas by creating a Cas9RNA (SgRNA) DNA complex, a double-stranded cleavage occurs in the target area. Condition for the realization of the cut is the presence of the sequence NGG which is also called Protoarque adjacent motif (PAM) which is at end of the target region 30 (Akbuldak and Kontbay, 2017). Strategies to establish targeted gene knockouts were shown in Fig 4.

Fig.4. Methods for creating targeted gene knockouts in Zebrafish.



Adult ZF or embryos are injected with TALEN or gRNA/Cas9. Genome editing activity was measured using the rate of indel mutations in the genomic DNA of embryos injected with TALEN or gRNA/Cas9. Adult F0 founders are crossed with wild-type offspring and genomic DNA is taken from F1 embryos. The hetero duplex mobility assay is used to investigate germ line transmission in F1 embryos and to detect indel mutations in F1 fish that are developing.

7. Limitation of using Zebrafish

Out of various advantages of ZF as an alternative to an animal model, a few drawbacks are there with this model in studying CNS disorder. Pharmacokinetic fluctuations may happen easily with ZF model since the test drug/chemical will be added into water, but it is difficult to predict the quantification of chemicals or drugs entered into the fish. Because the whole body of the fish is being exposed to aqua media and the drug/chemicals can randomly enter through the gills and skin. As with other models, certain areas of the ZF brain are not well developed (e.g. cortex region); CNS and well-characterized behavior develop over time (e.g. lack of social behavior in larval ZF). Sometimes ZF possesses a duplication of the genome and lack of well-characterized

strain; this could be other limitations of the ZF model (Kalueff *et al.* 2013). Due to species variation with humans and rodents, it may lead to complications if the same amount of dosage is given to ZF (Cachat *et al.* 2011).

CONCLUSION

The rationale of using the animal model in scientific research is to better understand the cause and mechanism of a particular disease. Although *in vivo* animal models and *in vitro* cell cultures are extensively used in neurobehavioral research, sometimes the results are not comparable and expensive in terms of money and time. Hurdles by the regulatory agency in approving the required number of animals for the desired study and lack of expertise in animal handling are also some of the limitations. Hence there is a need for alternative models and methods to overcome the limitation. In this regard, it is now accepted that ZF has great potential as an alternative *in vivo* animal model for CNS disorder due to its genetic attributes and physiological similarity to humans. Also has the advantage of being small in size, rapid development, cost-effective, space-efficient and further, it adheres to the 3R principle of CPCSEA. These advantages put ZF as an alternative to the mammalian model for neuropharmacological research and still, further studies are required to make it a reliable model for neuroscience research in forthcoming years.

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