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Towards zebrafish model applications in drug discovery targeting central nervous system diseases and neurotoxicity assessment

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ABSTRACT

Zebrafish have emerged as a pivotal model for drug discovery targeting central nervous system (CNS) diseases and neurotoxicity assessment, leveraging their genetic, anatomical, and functional similarities to humans. Despite their growing utilization, comprehensive reviews integrating both applications are scarce. Critical challenges persist in standardizing models and improving the accuracy and reliability of experimental outcomes. This review compares the human and zebrafish CNS, highlighting sex-specific difference, examines behavioural assays for disease modeling and neurotoxicity studies. It focuses on zebrafish application in drug discovery for stroke and Alzheimer's disease (AD), and neurotoxicity assessment, highlighting their preclinical value. To address ongoing challenges, further efforts should prioritize standardized guidelines, improved reproducibility and advanced genetic engineering to optimize zebrafish models. These advancements will solidify zebrafish as an indispensable tool for accelerating CNS drug development and neurotoxicity evaluation, addressing critical global health challenges.

1. Introduction

Zebrafish, a vertebrate species, exhibit high genetic and physiological homology to humans, possessing over 26000 protein-coding genes, approximately 70 % of which are orthologous to human genes and 82 % associated with human diseases (Howe et al., 2013). Their central nervous system (CNS) closely mirrors the human CNS in macro-organization, cellular morphology, major neurotransmitter systems and functional neuroendocrine systems. Notably, cortisol serve as the primary stress hormone in both zebrafish and humans, displaying comparable potency at glucocorticoid receptors (Alsop and Vijayan, 2009; Faught and Vijayan, 2022; Toso et al., 2023). Functionally, the zebrafish CNS supports classical sensory (vision, olfaction, taste, tactile, balance and hearing), motor, learning and memory modalities. Key pharmacokinetic parameters and brain penetration profiles of drugs like irinotecan and lorcaserin in zebrafish strongly correlate with human data (Kulkarni et al., 2017). As a first-step model, zebrafish offers a cost-effective and high-throughput screening platform for drug screening and toxicity assessment, reducing reliance on rodents and aligning with the 3R principles (replacement, refinement, and reduction). Additional advantages for neuroscience research include conserved cell types, organs and physiological systems; sufficient physiological complexity and high physiological and genetic similarities to humans; ease of genetic manipulation with 1000 transgenic and mutant strains available; quick and abundant reproduction; rapid development and transparent embryos; and external development. As a result, a growing number of experts advocate for zebrafish as excellent organisms for advancing CNS drug discovery and neurotoxicity evaluation.

However, limitations also exist. Delivery of non-water-soluble compounds pose significant challenges, often requiring organic solvents like dimethyl sulfoxide (DMSO). Although DMSO concentration below 1 % are generally considered non-toxic (Hoyberghs et al., 2021), synergistic toxicity can occur at lower concentration(e.g., 0.1 %) when combined

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with chemicals like vanadium (Kim and Lee, 2021). Inadequate solubility may lead to particles formation, potentially clogging zebrafish bills, causing hypoxia and confounding toxicity assessments. Other limitations include less-developed brain areas (e.g., the cortex) compared to mammals, differences in the blood–brain barrier, and limited development of complex behaviours (Kalueff et al., 2014). Therefore, findings derived from zebrafish models require validation in higher-order vertebrate models to ensure translational relevance. Despite these challenges, their numerous advantages have driven their widespread use in CNS-related studies. This review provides a comprehensive overview of zebrafish applications in CNS drug discovery and neurotoxicity assessment.

2. Zebrafish CNS: biology and applications

2.1. Comparison of CNSs between zebrafish and mammals

Zebrafish CNS development follows a well-documented cascade of events occurs (Kelsh and Raible, 2002). It begins with an ectodermal epithelium on the dorsal side of the embryo neural plate. By approximately 10 h post-fertilization (hpf) after gastrulation, a dorsomedial thickening forms the neural plate. Within the next 1–2 h, two lateral thickenings appear, fusing the lateral and medial thickenings into a neural keel after morphogenetic convergence and further transforming into a neural rod by approximately 16 hpf. Cavitation begins at approximately 17 hpf and establishes the central canal for the hollow neural tube that forms the brain anteriorly and the spinal cord posteriorly, containing both neurons and glia (Fig. 1) (Wilson et al., 2002). The zebrafish brain is further divided into the telencephalon, diencephalon, mesencephalon, and rhombencephalon, which have overarching cognitive, endocrine, homeostatic and motor functions. The inverted telencephalon is homologous to the mammalian amygdala and hippocampus (Jurisch-Yaksi et al., 2020). The brain architecture and developmental process of zebrafish exhibit significant similarities to those of mammals, although key differences in neural tube folding exist. In zebrafish, the neuroepithelium reorganizes into the neural keel whereas in mammals it folds around ventricles. Moreover, the telencephalon undergoes eversion in zebrafish but evagination in mammals. Environmental and genetic factors asymmetrically modulate the activity of the left and right brain hemispheres-a phenomenon also documented in humans(Duboc et al., 2015). An exploration of developmental gene expression in the brain revealed similarities between 1- to 2-year-old zebrafish and 40- to 59-year-old humans(Nakajima et al., 2021). Studies of brain morphology defects in 16 mutant zebrafish have already identified genes essential for zebrafish brain morphogenesis and corresponding functions (Lowery et al., 2009).

At the cellular level, the first glial cells that emerge at 10 hpf are radial glial cells, capable of self-renew and sequentially differentiation into neurons, oligodendrocyte progenitor cells and astrocytes(Neely and Lyons, 2021). Despite zebrafish lack star-shaped glial cells, radial glial cells perform homeostatic roles at both neural circuits and brain barriers, similar to astroglia and radial glial cells in mammals (Jurisch-Yaksi et al., 2020). The most well-known mammalian excitatory and inhibitory neurotransmitters, including dopamine, serotonin, acetylcholine, purine nucleotides, nucleosides, histamine, nitric oxide, glutamate, glycine and GABA, are gradually found in the zebrafish brain from 1 day post-fertilization (dpf) (Rico et al., 2011). Critical mammalian signalling pathways, including bone morphogenetic protein signalling, Wnt signalling, fibroblast growth factor signalling, and Notch signalling pathways, are also conserved and active in zebrafish embryo brain and other organs development (Stuhlmiller and Garcia-Castro, 2012). Unlike mammals, zebrafish possess neural stem cells of constitutively generating or regenerating new neurons throughout their whole life, a marked difference from the limited neurogenesis observed in adult mammals (Kizil et al., 2012). The blood-brain barrier is also highly conserved between zebrafish and mammals in terms of structure and function, anatomical features, tight junctions, drug efflux transporters and metabolizing enzymes (Li et al., 2017). With the development of single-cell RNA sequencing, the zebrafish brain development atlas have been well constructed which defines the cell types from embryo (12 hpf) to adult (5 dpf). A minimal group of 2 or 3 enriched gene markers were uniquely identified which enables the reconstruction of cellular trajectories and lineages(Raj et al., 2020). Despite minor differences, conserved vertebrate brain morphology resulted in striking similarities in neuroanatomical features, neuronal cell morphology and function, and signalling pathways.

2.2. Sex differences in the zebrafish CNS

Sex-specific differences have been observed in the prevalence and symptoms of most psychiatric and neurological disorders in humans, as well as in the outcomes of neurobiological assays and therapeutic interventions (Pawluski et al., 2020). Accumulating evidence indicates significant sex-dependent variations in zebrafish responses to a range of substances including alcohol, mianserin, chlorpromazine, ibuprofen, 17β-oestradiol, 11-ketotestosterone, 4-methylbenzylidene camphor and

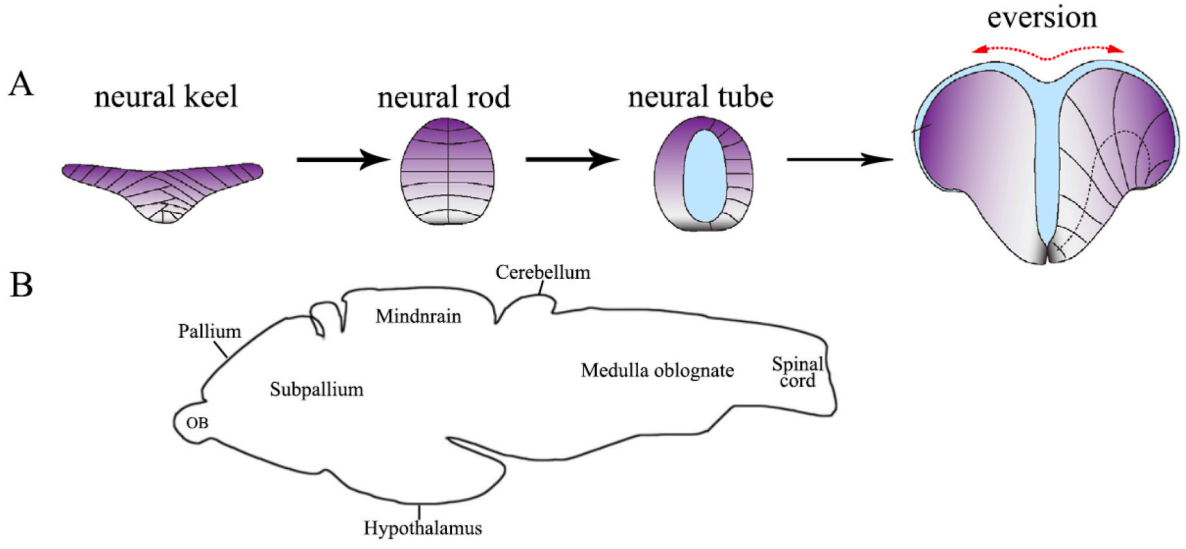

Fig. 1. The CNS of zebrafish. A. The development process. B. The schematic sagittal section.

nano-plastics (Genario et al., 2020; Xian et al., 2024). while the fundamental architecture of zebrafish CNS is substantially conserved across sexes, significant sex-based variations exist at cellular, neurotransmitter, and behavioural levels (Table 1). It is crucial to recognize that these sex differences are not isolated phenomena; rather, they are interconnected and may exhibit causal relationships. For instance, variation observed at the cellular and neurotransmitter levels can subsequently induce distinct behavioural manifestations. Despite these differences, many studies preferentially use males to minimize data variability or analyse data from mixed-sex populations collectively. This neglect of sex differences compromises the validity, reproducibility and replicability of research findings(de Abreu et al., 2022b). Given the increasing prominence of zebrafish in neuroscience research, including both females and males in studies is essential to identify additional sex-specific differences and elucidate the underlying mechanisms.

2.3. Zebrafish behaviours and assay

Zebrafish behavioural responses are evolutionarily conserved and highly similar to those of humans. For example, when visual stimuli evoke a complex behavioural response, the retina of a larva encodes behavior-associated visual information related to the neural activity distributed across feature-selective ganglion cells so that signals representing distinct stimulus properties arrive in different areas or layers of the brain(Bollmann, 2019). Neurobehaviors are the integration of neurofunctions such as sensation, motor control, attention and motivation. The first movements of zebrafish larvae, which are spontaneous contractions of the tail, appear at 17 hpf. Within the next 3–4 h, activity patterns convert from uncorrelated bursting patterns to coordinated side-to-side alternations. Touch-evoked responses appear at 21 h, and until 1–2 days, zebrafish swim in a coordinated manner and respond exactly to stimuli at different locations. By 4 dpf, the brain continues to grow, major brain structures and axon tracts containing different clusters of neuro-modulatory neurons are formed, and the larvae start to feed (Orger and de Polavieja, 2017). By 5–10 dpf, robust spontaneous and sensory-evoked behaviours are present. In conclusion, zebrafish behaviours originate from spontaneous coiling or swimming, gradually develop into responses to sensory stimuli and finally evolve into complex or learned behaviours(Nelson and Granato, 2022). A comprehensive catalogue detailing robust behavioural phenotypes in zebrafish was published in 2013 (Kalueff et al., 2013). This catalogue encompasses not only well-studied simple early-stage behaviours such as escape, startle, illumination, phototaxis, optic flow responses, hunting and feeding, but also complex adult zebrafish behaviours including social interactions, learning, memory, shoaling, schooling, decisions in groups, aggressive encounters and mating. Among them, learning and memory performance, critical for Alzheimer's disease (AD) drug discovery, are commonly assessed through tests of inhibitory avoidance, appetitive conditioning, light-dark, Y/T-maze and novel object recognition (Tan et al., 2022). Detailed experimental procedures of these zebrafish behavioural testing are well-documented (Legradi et al., 2015; Dasgupta et al., 2022).

Accurate behavioural assays relies heavily on reliable monitoring and analysis systems. Video tracking offers a versatile and effective approach for the remote monitoring of behavior. It enables the capture and recording of both simple and complex movements from a single perspective. The resulting behavioural data can be converted into quantitative metrics for subsequent analysis (Orger and de Polavieja, 2017). Computational and automated methodologies mitigate human bias, enhancing objectivity. Currently, high-throughput behavioural assays and platforms have been gradually developed to investigate seizures, toxicity and social behaviours (Green et al., 2012; Fuller et al., 2018; Tantry et al., 2022; Dasgupta et al., 2024; Locubiche et al., 2024). For example, a fully automated high-throughput behavioural assay system with automated image analysis has enabled the precise quantification of rheotaxis behavior by detecting individual zebrafish and

Table 1
Sex-specific differences in the zebrafish CNS across three levels.

		Differences between male and female zebrafish
Cellular level	Telencephalon	In the lateral zone of dorsal telencephalon, females displayed higher α2 adrenoceptors' expression (Ampatzis and Dermon, 2016). In telencephalon, genes *tph1a, tph2,sst3, slc6a3* were uniquely expressed in aggressive females (Filby et al., 2010). In the medial zone of dorsal telencephalic area, the ventral part of periventricular pretectal nucleus and the periventricular nucleus of posterior tuberculum, adult female zebrafish exhibited higher levels of cycling cells, while in dorsal zone of periventricular hypothalamus, males displayed higher density of mitotically active cells (Ampatzis et al., 2012)
	Hypothalamus	Genes *thp1b, sstr1, drd2c* were uniquely expressed in aggressive male, whereas *tph1, sst3, slc6a3, slc6a4a, nos1, mao, cyp19a1b, esr1* were uniquely expressed in female (Filby et al., 2010) Female exhibited higher glucose utilization than male in response to novelty (Ampatzis and Dermon, 2016).
	Olfactory bulb	In female, more GnRH3 neurons were observed with tac1processes (Ogawa et al., 2020).
	Cerebellum	Male displayed higher densities of β adrenoceptors' expression (Ampatzis and Dermon, 2016).
Neurotransmitter level	Dopamine	Following agonistic interaction, females displayed higher concentration of dopamine than males in forebrain (Dahlbom et al., 2012).
	Serotonin	Following agonistic interactions, male exhibited higher 5-hydroxyindolacetic/ 5 hydroxytryptamine ratios than female in forebrain (Dahlbom et al., 2012).
Behavioural level	Social interaction	After social buffering, males showed a greater reduction in freezing behavior than females. but in social contagion, males responded more than females by freezing at a high intensity (Akinrinade et al., 2023). Aggressive males are more likely to choose shoals of males over females and more likely to swim alone, whereas no correlations were found between aggressive behaviors and social choice in females (Snekser and Diestler, 2023).
	Aggressive behavior	Male are generally more aggressive than female, explore more in novel environments (Genario et al., 2020).
	Stress response	When exposed to unpredictable chronic stress, males displayed more aggressive behavior and increased whole-body cortisol levels than females (Rambo et al., 2017).
	Anxiety behavior	In anxiety assay of novel tank diving task and the light-dark test, females showed higher levels of anxiety-like behavior compared to males (Fontana et al., 2020).
	Reward-related behavior	The cocaine withdrawal was associated with an anxiety-like state which develops earlier in females but more robust and persistent in males (Lopez Patino et al., 2008).

computing their orientation within a test population (Todd et al., 2017). Continued advances in high-content and high-throughput technologies would further expanded the applicability of zebrafish models.

2.4. Zebrafish CNS development for disease modeling and neurotoxicity

Understanding zebrafish CNS across development across stages is pivotal for advancing disease modeling and targeting gene screening, particularly for neurodevelopmental and neurodegenerative disorders. In the embryonic stage (12–24 hpf), neurogenesis establishes basic CNS structure (forebrain, midbrain, hindbrain), with transparent embryos enabling real-time visualization of neural development(Wilson et al., 2002; Howe et al., 2013). This facilitates studies of genes like *SH3 and multiple ankyrin repeat domains,* implicated in autism spectrum disorder, through CRISPR/Cas9 (Rea and Van Raay, 2020). By the larval stage (5 dpf), functional neural circuits support behaviors such as photomotor response and locomotion, making zebrafish ideal for modeling epilepsy or attention-deficit/hyperactivity disorder (Kalueff et al., 2013). Single-cell RNA sequencing reveals diverse neuronal cell type (e.g., glutamatergic), enhancing insight into neurodevelopmental mechanism (Lange et al., 2020). In adult, mature CNS circuits enable modeling of neurodegenerative disorders, such as Parkinson's disease (PD) and AD, with high-throughput behavioural and imaging assays identifying disease phenotypes (Babin et al., 2014; Vaz et al., 2019). Zebrafish's genetic manipulability supports rapid screening of target genes associated with these disorders(Howe et al., 2013). In neurotoxicity studies, stage-specific CNS complexity informs the differential effects of toxicants (e.g., pesticides), which may disrupt neuronal development and exacerbate toxicity (Ton et al., 2006). Despite progress, gaps remain in understanding adult brain circuitry and complex behaviors, underscoring the need for further research to enhance translational relevance (Soussi-Yanicostas, 2022), These insights position zebrafish as powerful first-step model for neuroscience research, bridge in vitro and mammalian studies.

3. Zebrafish models in drug discovery targeting CNS diseases

As populations age, CNS disorders are increasingly significant contributors to global disability and mortality, accounting for the highest disability-adjusted life-years (DALYs) and the second-highest death in 2016. Stroke (42.3 %), migraine (16.3 %), Alzheimer's disease and other dementias (10.4 %), and meningitis (7.9 %) were the leading DALY drivers, with dementia cases projected to increase over 300 % in developing countries like India and China by 2040 (Ferri et al., 2005; GBD 2016 Neurology Collaborators, 2019). However, CNS drug development faces significant challenges, the clinical success rates (1995–2007) for CNS drugs (6.2 %) are lower than non-CNS drugs (13.3 %) (Lindsley, 2014), and the approval period for CNS drugs is prolonged, averaging 19.3 months, a 31 % increase compared to non-CNS drugs. From 2020 to 2022, only 24 new neurological drugs were approved comprising 16.9 % of total new drug approvals, including 5 first-in-class drugs (8.9 % of total first-in-class approvals), 18 next-in-class drugs (23.38 % of total next-in-class approvals), and 1 first-in-indication drug (11.1 % of total first-in-indication approvals) (Kayki-Mutlu and Michel, 2021; Kayki-Mutlu et al., 2022, 2023). These challenges, including lower success rate and prolonged approvals, have led to reduced investment in CNS programs by pharmaceutical companies. Zebrafish offer a cost-effective and high-throughput preclinical platform, as discussed in section 2. Beyond CNS disease (Khan et al., 2017), zebrafish are utilized in cancer biology and precision therapy (Fazio et al., 2020), liver disease (Katoch and Patial, 2021), heart disease (Le et al., 2022), hearing loss (Ou et al., 2010), and rotavirus infection (Song et al., 2022). For CNS diseases, zebrafish support drug discovery for cerebral ischaemia (Matsumoto et al., 2021), stroke (Crilly et al., 2022), seizure (Ibhazehiebo et al., 2020), neurodegenerative diseases (Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis) (Wang et al., 2021b; Nadiga and Suman, Krishna, 2024), cognitive enhancers (Kolesnikova et al., 2024), and neuropsychiatric disorders (anxiety, depression, addiction, schizophrenia) (Kalueff et al., 2014). They are also used to efficacy assessment (Chaoul et al., 2023), drug repurposing (Del Rosario Hernandez et al., 2024) and testing traditional Chinese and American medicines (Wang et al., 2021a; de Abreu et al., 2022a). Subsection 3.1-3.3 details validated zebrafish model for CNS disease, f focusing on stroke and AD, prioritized to their significant DALYs burden and therapeutic needs.

3.1. Validated zebrafish models for CNS disease

Zebrafish CNS disease models have evolved from early non-genetic, phenotype-driven approaches to precise, gene-targeted manipulation, each with distinct advantages and limitations (Table 2). Fig. 2 illustrates the schematic workflow of zebrafish CNS disease model development.

Table 2
validated zebrafish CNS disease models.

Disease	Model method	Gene/Inducer	Key phenotypic features
Epilepsy	acute chemical exposure	PTZ	seizure-like behavior (Baraban et al., 2005)
	targeted mutation	GABRA1	spontaneous seizure (Samarut et al., 2018)
ASD	embryonic chemical exposure	VPA	social deficits, repetitive behaviors(Chen et al., 2018)
ADHD	knockout	LPHN3	hyperactivity, impulsivity, reduction and misplacement of dopaminergic neuron(Lange et al., 2012)
Depression	environmental paradigm	chronic unpredictable stress	reduced locomotion, reduced exploratory behavior(Fulcher et al., 2017)
PD	acute chemical exposure	MPTP	motor deficits, dopaminergic neuron loss(Lal et al., 2024)
	CRISPR/Cas 9 knockout	PINK1, PARK2	motor deficits, mitochondrial dysfunction, dopaminergic neuron loss (Yushko et al., 2023)
Dravet syndrome	targeted mutation	SCN1A	spontaneous seizure, neural excitability (Yushko et al., 2023)
Leigh syndrome	knock-in mutation	COA6	locomotor defects, mitochondrial dysfunction (Yushko et al., 2023)
Peripheral neuropathy	CRISPR/Cas 9 knockout	MFN2, KIF5A	sensory/motor impairment, axonal degeneration (Yushko et al., 2023)
Costeff syndrome	gene disruption	OPA3	visual dysfunction, optic nerve atrophy (Yushko et al., 2023)
Congenital myasthenic syndrome	targeted mutation	SLC25A1	Neuromuscular deficits (Yushko et al., 2023)
Huntington's disease	targeted mutation	HTT	motor deficits, neuronal aggregation(Kumar et al., 2021)
Stroke (ischemic)	hypoxia induction	cerebral vessel occlusion	reduced blood flow, neurodegeneration (Xinge Yu, 2011)
AD	Chronic chemical exposure	$AlCl_3$	learning and memory deficits(Kaur et al., 2022)
	CRISPR/Cas 9, Knock-in, transgenic	APP, PESN1/2, MAPT, APOE4	cognitive deficits, amyloid plaques, Tau pathology(Pu et al., 2017)

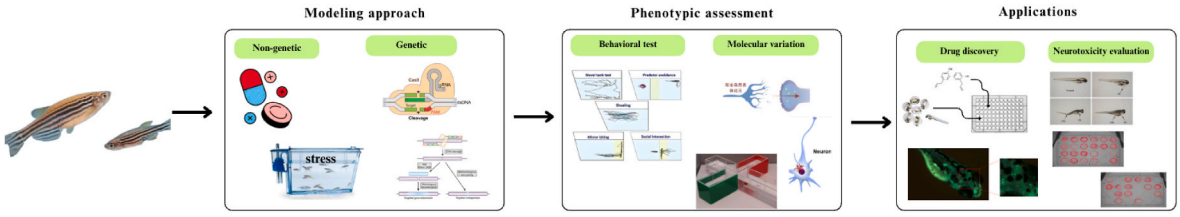

Fig. 2. Schematic workflow of zebrafish CNS disease model development.

Early non-genetic models used chemical induction, environmental stress or physical injury to replicate diseases-relevant phenotypes. Examples include pentylenetetrazol (PTZ) induced seizure-like activity for epilepsy (Baraban et al., 2005), valproic acid (VPA) embryonic exposure to mimic autism spectrum disorder (ASD)-like behavioural deficits(Chen et al., 2018), aluminum chloride ($AlCl_3$) administration for Alzheimer's-like memory deficits (Kaur et al., 2022), chronic stress paradigms (e.g., unpredicted stress) for depression-like behavior (Fulcher et al., 2017), and hypoxia for ischemic stroke (Xinge Yu, 2011). These models are cost-effective, rapid to implement, and ideal for therapeutic compound screening. However, their specificity is often limited, and underlying mechanism may only partially recapitulate human pathophysiology. In contrast, genetic models offer high disease specificity through targeted manipulation of orthologous human disease gene using technologies of zinc finger nucleases(ZFNs), transcription activator effector-like nucleases(TALENs), and clustered regularly interspaced short palindromic repeats associated protein 9(CRISPR/Cas 9) (Yushko et al., 2024). Well-established models include PINK1 or PARK2 knockout for PD, SCN1A mutation models for Dravet syndrome, OPA3 mutation for Costeff syndrome, MFN2 and KIF5A knockout for Peripheral neuropathy and dominant optic atrophy, COA6 mutation for Leigh syndrome, SLC25A1 mutation for congenital myasthenic syndrome or severe combined DL-2 hydroxyglutaric aciduria (Yushko et al., 2023). These models accurately reproduce molecular and cellular phenotypes, such as dopaminergic neuron loss and Aβ accumulation. But Their development is time-consuming development and phenotypic penetrance can vary. Together, these models provide robust platforms for drug discovery, advancing therapeutic development.

3.2. Stroke

Animal stroke models have proven to be versatile tools in the investigation of stroke pathophysiology and the discovery of novel therapeutic agents. Since late 1970s, models have been broadly categorized into ischaemic stroke and intracerebral haemorrhage models. Ischaemic stroke models are further subdivided into global and focal ischaemia models(Li and Zhang, 2021). While rodents, particularly mice and rats, have been the predominant, zebrafish have recently emerged as a unique and powerful alternative, due to their ability to model both ischaemic and haemorrhagic stroke models. Ischaemic stroke models in zebrafish models are commonly induced by exposing larvae or adults to hypoxic conditions via low-oxygen chambers or chemical agents, or by using photosensitive dye like Rose Bengal to cause cerebrovascular injury. Haemorrhagic stroke models are successfully generated using biochemical treatment, such as atorvastatin or Aβ peptides, or by using the bubblehead mutant(Crilly et al., 2022). Post model establishment, comprehensive assessment are conducted using behavioural tests (locomotor activities, advanced cognitive function), and in vivo live imaging techniques to visualize brain vasculature, microtubule structures, neutrophil myeloperoxidase, calcium, redox and synaptic structures (Chen et al., 2021). The integration of genetic manipulation further enhances the utility of these zebrafish stroke models, facilitating efficient therapeutic agents screening and mechanistic studies.

Zebrafish ischaemic stroke models successfully mimic human pathophysiology. For instance, ponatinib exposure in 2 dpf larvae induces cerebral vascular endothelial injury, thrombosis, reduced blood flow, inflammation, apoptosis and reduced motility. Treatment with six known human therapeutics (aspirin, clopidogrel, Naoxintong capsules, edaravone, Xinnaojing injection, Shuxuening injection and urokinase) significantly reduced stroke incidence and cerebral thrombosis in model zebrafish(Zhu et al., 2020). The ponatinib-induced ischaemic stroke model also confirmed the anti-stroke effects of traditional Chinese medicines *Salvia miltiorrhiza Bunge* (Danshen) and *Ligusticum chuanxiong Hort.* (Chuanxiong), consistent with findings in mouse middle cerebral artery occlusion/reperfusion models (Pang et al., 2024). Furthermore, multiphenotypic screening of Guhong injection identified baicalein and rutin as prominent antithrombotic substances, and chlorogenic acid and gallic acid as active compounds mitigating locomotor dysfunction, .with mechanisms involving expression downregulation of coagulation factors F7 and F2, NF-κB and proinflammatory cytokines(Wang et al., 2022). In a photothrombotic zebrafish telencephalon ischaemic stroke model, 6S-5-methyltetrahydrofolate-calcium, an alternative to folic acid, exhibited neuroprotective effects on neurocytes, effectively alleviating motor dysfunction and neuro-behavioural defects by increasing the activities of GSH-Px and SOD and decreasing the level of MDA (Bin et al., 2024). Collectively, these studies highlight the reliability of zebrafish ischaemic stroke models for rapidly discovery.

Zebrafish haemorrhagic models are Increasingly recognized for their applicability in drug discovery duo to spontaneous hemorrhages in larvae, and the ease of evaluating efficacy through non-invasive visualization haematoma size measurement (Crilly et al., 2022). In 2019, the Journal of Visualized Experiments (JOVE) endorsed zebrafish with genetically or chemically (atorvastatin) disrupted cerebrovascular development as a valuable in vivo system for preclinical intracerebral haemorrhage studies, recapitulating human brain damage, neuroinflammation, and locomotor activity (Crilly et al., 2019). Consequently, this intracerebral haemorrhagic stroke model has seen increasing application in stroke-related studies. Using the atorvastatin-induced intracerebral haemorrhage model, neuroprotective mechanisms of clinically relevant stroke therapeutic drugs like rutin, tanshinone I and IIA were elucidated. Rutin reduced haematoma size, ROS production, apoptosis and the genomic expression of inflammatory genes (*il6, tnfa, il10 and irf2a)* by suppressing oxidative stress and inflammation(Rana et al., 2022). Tanshinone I and IIA protected endothelial integrity by stabilizing cell−cell adhesion junctions via inhibition of Src-mediated VE-cadherin and actin-myosin contractility(Huang et al., 2018; Zhou et al., 2018).

3.3. Alzheimer's disease

Zebrafish AD model, induced by neurotoxins(scopolamine, aluminum chloride, okadaic acid, amyloid β (Aβ) injection and pentylenetetrazole) or genetic manipulation, have been validated for drug screening and mechanism studies. Okadaic acid, an protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) inhibitor, is most commonly used to mimic AD pathological hallmarks, including tau hyperphosphorylation, amyloidogenesis and synaptic deficits. PPA2 dysfunction influences the activities of several Ser/Thr protein kinases (Sontag and Sontag, 2014).Okadaic acid also induces oxidative stress, neuroinflammation, glial activation, cholinergic dysfunction, glutamate

excitotoxicity and mitochondrial dysfunction, which are potential AD pathogenic mechanisms (Thawkar and Kaur, 2021). This model identified potential neuroprotective compounds like lanthionine ketimine-5-ethyl ester and 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3, 5-dione (a glycogen synthase kinase 3β (GSK3β) inhibitor) (Koehler et al., 2018, 2019). Scopolamine and pentylenetetrazole, by inhibiting muscarinic acetylcholine and N-methyl D-aspartate (NMDA) receptors, replicated basal forebrain cholinergic neurons loss, contributing to memory and attention deficits(Ferreira-Vieira, T.h. et al., 2016). Excessive NMDA receptor activity induces excitotoxicity and promotes nerve cell death, which were considered as potential AD aetiologies (Wang and Reddy, 2017). Current approved AD treatment mainly target cholinergic and NMDA systems, containing three acetylcholinesterase inhibitors (donepezil, rivastigmine and galantamine) and one NMDA receptor antagonist (memantine). Clinical trials indicated that these drugs offer partial relief of cognitive or behavioural symptoms and slow mild-to-moderate AD progression but lack disease-modifying potency (Tan et al., 2014). Identifying disease-modifying agents remains challenging due to negative clinical outcomes and undesirable side effects. While animal experimental and clinical studies continue, determining the exact efficacy of new drugs is still difficult (Mangialasche et al., 2010). In a scopolamine-induced AD model, α-bisabolol β-D-fucopyranoside ameliorated memory deficits, inhibited cholinesterase activity, and attenuated oxidative stress (Jeyakumar et al., 2023). Toxic Aβ42 accumulation, the key cause of AD, is modelled in zebrafish via cerebroventricular synthetic Aβ42 peptides injection, eliciting synaptic degeneration, inflammation, neuronal death and cognitive deficits. A detailed protocol for Aβ42 peptide and injection mixture preparation, anaesthesia, cerebroventricular microinjection, recovery, tissue processing, and immunohistochemistry was published in JOVE(Bhattarai et al., 2017). enabling successful AD model establishment in embryos, young and aged adult zebrafish using different forms of Aβ-42 (oligomeric form) (Nery et al., 2014; Patta et al., 2022). These models support not only Aβ pathogenesis studies but also new potential therapeutics screening, identifying candidates like casein-coated gold nanoparticles and chrysin-loaded chitosan nanoparticles (Javed et al., 2019; Kiper and Freeman, 2022; Saleem et al., 2022).

Key human AD related genes, including presenilin 1 (*psen1*), presenilin 2 (*psen2*), Aβ precursor protein (*app*), apolipoprotein E (*apoe*), microtubule-associated protein tau (*mapt*), beta secretase 1 (*bace1*), and beta secretase 2 (*bace2*) (Kiper and Freeman, 2022; Shenoy et al., 2022), are conserved in the zebrafish. Transgenic zebrafish AD models have been established by manipulating these genes. For example, knockdown of human APP gene under the zebrafish *appb* promoter successfully induced Aβ precursor protein expression in the brain, eyes, heart and vasculature, causing AD-like learning and memory impairment (Pu et al., 2017). Similarly, microinjection of parkinsonism gene linked to chromosome 17 (FTDP-17) constructs encoding human tau protein and the neural-specific GATA-2 promoter into embryos at the 1–2 cell stage indued human tau protein and neurofibrillary tangles, recapitulating AD cytoskeletal pathologies (Henry G. Tomasiewicz et al., 2002). Furthermore, transgenic zebrafish expressing DsRed fluorescently labelled tau protein, generated using a Gla4/USA-based bidirectional expression system, rapidly recapitulated key pathological features of tauopathies, including tau protein phosphorylation and conformational changes, neuronal tangle formation, behavioural disturbances and cell death. The active tau kinase GSK3β inhibitor AR-534 effectively reduced tau phosphorylation in this zebrafish model (Paquet et al., 2009). Exogenous BDNF AND the GSK3β inhibitor LiCl similarly alleviated tau toxicity, suggesting novel efficient therapeutic targets (Barbereau et al., 2020). Moreover, surfen and oxalyl surfen also significantly improved spinal motoneuron axon-branching defects and stereotypical touch-evoked escape response by inhibiting tau hyperphosphorylation and mitigating neuron deficits (Alavi Naini et al., 2018).

4. Neurotoxicity assessment using zebrafish models

The CNS is recognized as the most frequently involved organ for toxicity, with over 450 compounds identified as potential human neurotoxins(van Thriel, 2019). The developing brain in particularly sensitive to chemical toxicants compared to the adult brain. Epidemiological evidence suggests strong relationships between early-life exposure to industrial chemicals and developmental neurotoxicity occurrence. To date, twelve industrial chemicals, including lead, methylmercury, polychlorinated biphenyls, arsenic, toluene, manganese, fluoride, chlorpyrifos, dichlorodiphenyltrichloroethane, tetrachloroethylene and polybrominated diphenyl ethers, are recognized as inducers of neurodevelopmental disorders and subclinical dysfunction (Grandjean and Landrigan, 2006, 2014). Moreover, a significant proportion of childhood cancer survivors, ranging from one-third of general cancer survivors to 40 %–100 % of brain tumour survivors, experience cancer-related cognitive deficits in memory, attention, visual-motor integration and executive function, significantly impacting survivors' well-being and quality of life. Traditional chemotherapeutic agents, immune checkpoint inhibitors, monoclonal antibodies, and targeted therapies contribute to neurotoxic effects and potential neurocognitive impairments (Phillips et al., 2021). Assessing developmental neurotoxicity of chemicals pose significant challenges, especially for subtle effects from low concentrations exposures. It require large scale rodent studies which are costly, time-consuming, and ethically contentious. Zebrafish offer a compelling first-step alternative due to their advantageous characteristics However, limitations include challenges with toxicity assessment of non-water-soluble drugs and the necessity of validating findings in high-order animals or humans.

PubMed data showed a steady increase in zebrafish-related publications on toxicity, neurotoxicity and developmental neurotoxicity from 2000 to 2024 (Fig. 3). In 2024, 250 neurotoxicity-related publications accounted for 5.04 % of all zebrafish-related and 15.7 % of zebrafish toxicity-related publications, with 40.8 % focusing on developmental neurotoxicity. Over the past decade, h neurotoxicity and developmental neurotoxicity publications have increased approximately fivefold. This surge highlight the expanding utility and inherent advantages of zebrafish as a model organism for neurotoxicity evaluation. Comprehensive endpoints, including gene expression patterns, neural morphogenesis, and neurobehavioral profiling, enable in-depth evaluation of chemical toxicity on t zebrafish CNS, leveraging their functional and structural similarities to humans (d'Amora and Giordani, 2018). Standardized neurobehavioral paradigms have been developed and successfully applied to assess neurotoxicity related to visual impairment, olfactory toxicity, ototoxicity, locomotor activity, learning and memory (de Esch et al., 2012). For instance, Gaballah et al. (2020) detailed a procedure for evaluating per- and polyfluoroalkyl substances (PFASs) neurotoxicity in zebrafish embryos. Embryos exposed to PFASs from 0 to 5 dpf to measure locomotor activity at 6 dpf. The detailed measurement process was as follows: 1) Zebrafish were acclimated for a minimum of 120 min in a dark, temperature-controlled behavior testing room (26 °C); 2) Locomotion was recorded for 60 min using a Noldus tracking apparatus, consisting of a 20 min dark acclimation period (0 lux) followed by a 40 min testing period(20 min light period [5 lux] and a subsequent 20 min dark period [0 lux]); 3) Locomotor activity during the 40 min test period was analysed using EthoVision software. PFASs induced developmental neurotoxicity including body axis malformation, swim blade defects and hyperactivity (Gaballah et al., 2020). Similarly, our previous studies first reported the developmental neurotoxicity of honokiol in zebrafish, evidenced by significant impairments in locomotor activity and the induction of apoptosis in brain (Li et al., 2019, 2022). Sulukan et al. reported that global warming and glyphosate exposure simultaneously affected neurobehaviors in adult and offspring zebrafish (Sulukan et al., 2023a, 2023b). The high concordance between zebrafish and mammalian studies is a notable feature of zebrafish toxicity evaluation. For example, 57 out of the 60

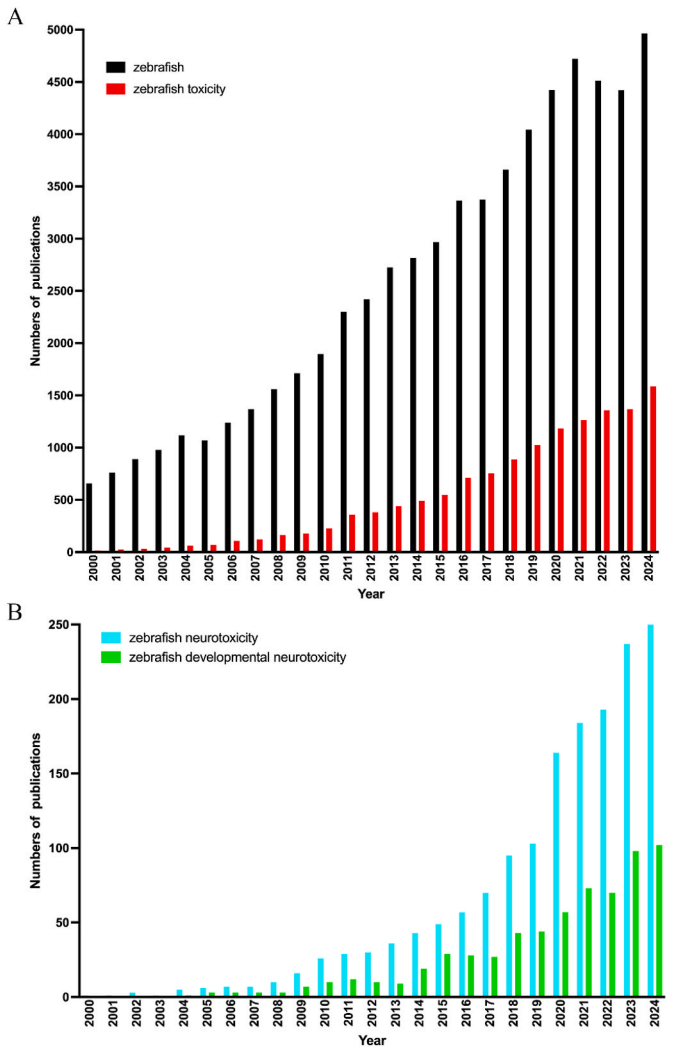

Fig. 3. Publication trends from 2000 to 2024. A. Number of publications related to zebrafish and zebrafish toxicity. B. Number of publications related to zebrafish neurotoxicity and zebrafish developmental neurotoxicity.

water-soluble toxic compounds affected zebrafish behaviours through suppression, stimulation or a combination of both(Ali et al., 2012). In developmental neurotoxicity assessment of 10 compounds, Selderslaghs et al. (2013) demonstrated a 90 % concordance between zebrafish and mammalian data (Selderslaghs et al., 2013). For 214 ToxCast chemicals, zebrafish concordance with rats (52 %) and rabbits (47 %) was comparable to rat-rabbit concordance (58 %). Ocfentanil and 2-furanylfentanyl similarly reduced basal locomotor activity in zebrafish larvae and mice (Bilel et al., 2023). It is important to note that the concordance between zebrafish and mammalian models maybe influenced by study design, as well as the nature and number of compounds tested (Sipes et al., 2011).

Zebrafish are also ideal model organisms for studying the intricate interplay between chemical exposures, microorganisms and host neurotoxicological outcomes (Bertotto et al., 2020; Lin et al., 2023), as well as for elucidating underlying toxicological mechanisms (Qin et al., 2024; Wang et al., 2024; Zhang et al., 2024). For example, certain neurotoxins have been shown to affect dopamine levels and the number of detectable tyrosine hydroxylase immunoreactive neurons in specific brain nuclei (Panula et al., 2010). Cadmium exposure impairs locomotion and diminished environmental signals reactivity by reducing neuronal density and altering microglial morphology through apoptosis and neuroinflammatory signalling pathways (Xu et al., 2022).

5. Further perspectives

As zebrafish model applications expand, standardizing protocols to ensure validity, reproducibility and replicability is a critical challenge. Experts have emphasized that parameters such as zebrafish strain, housing conditions, developmental stage, experimental design, test procedure, data acquisition and interpretation of results significantly influence outcomes of various behavioural tests (e.g., photomotor response, locomotor, light/dark transition and miscellaneous) (Widrick et al., 2023). Ambient temperature also profoundly influences neurotoxicity and CNS drug discovery outcomes in zebrafish models by modulating drug penetration and pharmacodynamic responses, neurobehavioral patterns (Abozaid et al., 2020), brain protein expression (Nonnis et al., 2021). Elevated temperatures can lead to the accumulation of misfolded proteins in notochord sheath cells, altering cell type proportions, gene expression programs, and developmental timing, causing structural defects and neurotoxic effects (Dorrity et al., 2023). These effects can synergistically amplify neurotoxicity of chemical substances such as heavy metals (Toni et al., 2023). Meta-analysis publications on larval zebrafish behavioural assays, further highlight the necessity of standardized reporting of experimental variables (Hill et al., 2023). Analogous to fish embryo acute toxicity tests, standardized guidelines for test procedures, data handling, automated video tracking systems, and study design should be established for drug screening and neurotoxicity evaluation (Legradi et al., 2015). Although the Organization for Economic Cooperation and Development issued test guideline 426 for chemical developmental neurotoxicity testing in 2007, this guideline recommends rats as the preferred species, and the detailed procedures are specific to rats. A retrospective performance assessment of developmental neurotoxicity studies highlighted the relevance and reliability of data generated under existing guideline, which is currently regarded as the best available scientific framework for assessing developmental neurotoxicity in the context of human health risk assessment (Makris et al., 2009). The timely establishment of such protocols is anticipated to enhance the consistency, interpretability, and regulatory acceptance of zebrafish-based neurotoxicity data.

In addition to the development of standardized guidelines, innovations in assessment methodologies and experimental tools offer valuable enhancements to zebrafish-based research. A novel paradigm based on brain activity patterns has been introduced for neurological drugs screening. By analysing local field potentials from many zebrafish larvae concurrently over extended periods, this approach enables more accurate prediction of drug efficacy and potential side effects compared to traditional behavioural assays (Eimon et al., 2018). Novel bioassay methods have been increasingly applied in zebrafish research. For example, bioassay-guided micro-fractionation has identified several novel antiseizure molecules from *Solanum torvum* leaves, *Skeletonema marinoi* and *Pharbitis nil* seeds (Challal et al., 2014; Brillatz et al., 2018; Liu et al., 2019). Similarly, a compound exhibiting enhanced acetylcholinesterase affinity and inhibitory efficacy was isolated from *Tephrosia purpurea* (L.) using zebrafish brain-based bioassay (Pitchai et al., 2018). Expansion microscopy, enabling 3D nanoscale resolution imaging, facilitates visualization of cellular processes and subsynaptic proteins distribution, high-resolution compositional synapses mapping, and intranuclear invaginations, advancing zebrafish neuroscience and developmental biology research (Freifeld et al., 2017).

Leveraging advantages of easy egg accessibility and relatively short generation time, genetic manipulation in zebrafish has evolved from antisense oligonucleotide knockout to efficient transgene integration and genome editing. The rapid development of CRISPR–Cas9 systems has significantly propelled loss-of-function studies, with detailed protocols for generating gene knockout zebrafish published (Medishetti et al., 2022). CRISPR–Cas9-mediated *grna* knockout zebrafish lines exhibit neurodevelopmental defects and motor behavior changes (Zhu et al., 2021). Moreover, CRISPR–Cas13d efficiently depletes specific mRNA transcripts in embryos (Kushawah et al., 2020). To date, diverse

CRISPR–Cas screening platforms are predominantly applied in cancer research and drug discovery (Chen et al., 2019; Katti et al., 2022). The authoritative zebrafish information network (http://zfin.org) reports 4759 CNS mutants, knockdowns, and transgenics. Among these, CRISPR–Cas9-generated models of PD and Huntington disease are widely used and more accurately recapitulated human phenotypes compared to transgenic animals expressing mutant genes under exogenous promoters(Yang et al., 2016). These tools provide a high-throughput platform for CNS disease research and therapeutic development.

Despite significant progress and increasing utilization of zebrafish models, critical challenges remain in standardizing establishment of zebrafish models and enhancing accuracy and reliability of findings. Further research should focus on establishing zebrafish-specific guidelines to enhance standardization and reproducibility, and leveraging genetic engineering advancements to optimize models, making zebrafish indispensable for CNS drug development and neurotoxicity assessment.

6. Conclusion

Zebrafish has solidified their position as a transformative model in neuroscience, offering unparalled advantages for CNS drug discovery and neurotoxicity assessment. Their striking genetic, anatomical, and functional similarities to human CNS, combined with cost-effective, high-throughput capabilities, position them as a critical bridge between in vitro studies and mammalian models. The observed sex-specific differences in zebrafish CNS underscore the importance of inclusive study design to enhance translational relevance. By enabling rapid screening for stroke and AD therapeutics and providing sensitive endpoints for neurotoxicity evaluation, zebrafish are accelerating the development of novel treatments and improving our understanding of chemical-induced neurological risk. To fully realized their potential, the field must prioritize the establishment of standardized protocols to ensure reproducibility and reliability, alongside the adoption of cutting-edge genetic engineering and innovative assessment technologies. With these advancements, zebrafish will continue to drive breakthroughs in CNS research, offering hope for addressing the growing global burden of neurological disorders and enhancing human health outcomes.

CRediT authorship contribution statement

Hui Li: Writing – review & editing, Writing – original draft, Investigation, Conceptualization. **Aiqin Li:** Investigation. **Hongtao Jin:** Writing – review & editing, Conceptualization. **Xiaolan Bian:** Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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