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REVIEW ARTICLE

A Review on Application of Zebrafish Disease Models, Neurobehavioral Phenotyping, and Biochemical Markers in Preclinical Screening for Alzheimer's Disease

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Abstract: Preclinical evaluation of therapeutic candidates for Alzheimer's disease faces substantial attrition, largely arising due to high operational costs and translational limitations of traditional mammalian models. *Danio rerio* has gained prominence as a powerful alternative vertebrate system, offering high genetic homology, conserved neuroanatomy, transparent early development, and compatibility with high-throughput phenotypic screening. Diverse experimental approaches replicate distinct facets of neurodegeneration, encompassing pharmacological neurotoxins (scopolamine, aluminum chloride, okadaic acid, streptozotocin), synthetic amyloid-beta microinjections, and targeted genetic engineering (*psen1/2*, *app*, *MAPT*). Quantifiable behavioral paradigms, including spontaneous alternation assays, novel object recognition, inhibitory avoidance, and shoaling dynamics, reliably assess spatial navigation, declarative recall, associative learning, and social cohesion. Biochemical validation couples these functional outputs to neurochemical restoration by tracking acetylcholinesterase kinetics, reactive oxygen species accumulation, lipid peroxidation, endogenous antioxidant depletion, and pro-inflammatory signaling cascade suppression. Despite evolutionary distinctions in telencephalic lamination and teleost-specific gene duplications, integrating automated behavioral tracking with multi-omic and molecular endpoints establishes a robust translational bridge between *in vitro* assays and mammalian validation, accelerating the identification of disease-modifying neurotherapeutics.

Keywords: Alzheimer's disease; *Danio rerio*; Neurobehavioral screening; Cholinergic dysfunction; Tauopathy.

1. Introduction

Alzheimer's disease (AD) is the primary contributor to dementia on a global scale, presenting clinically with a progressive deterioration of cognitive faculties, short- and long-term memory retrieval, and behavioral stability [1]. Projections indicate that the global prevalence of dementia will exceed 150 million individuals by the middle of the twenty-first century, placing an immense socioeconomic burden on healthcare infrastructures worldwide [2, 3]. Clinical pharmacotherapy remains predominantly symptomatic, relying on acetylcholinesterase inhibitors such as donepezil, rivastigmine, and galantamine, alongside the *N*-methyl-D-aspartate receptor antagonist memantine [3]. The high attrition rate observed in late-stage clinical trials targeting individual pathological hallmarks has redirected therapeutic discovery toward multi-target neuroprotective compounds and refined preclinical evaluation pipelines [4, 5]. While mammalian rodent models have provided valuable mechanistic insights into the molecular etiology of amyloid-beta (Aβ) deposition and neurofibrillary tangle (NFT) formation, their high maintenance costs, lower experimental throughput, and variable predictive validity present ongoing challenges in drug discovery [6, 7]. *Danio rerio* (zebrafish) has emerged as a complementary vertebrate system for screening anti-Alzheimer candidate molecules [8].

Figure 1. An Adult Female Zebrafish

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The zebrafish reference genome demonstrates approximately 70% overall sequence conservation with humans, with over 75% of genes associated with human disease vulnerability possessing functional orthologs [9, 10]. Critical genes implicated in familial and sporadic AD, including *appa*, *appb*, *psen1*, *psen2*, *bace1*, *mapta*, and *maptb*, are actively expressed during early embryonic and larval stages [9, 10, 14–20]. Structurally, teleost neuroanatomy contains organized subdivisions that correspond to higher mammalian brain regions. The dorsal, lateral, and medial pallial divisions of the teleost telencephalon exhibit functional equivalencies to the mammalian isocortex, hippocampus, and amygdaloid complex, respectively [11, 12]. Neurochemical signaling is highly conserved; cholinergic, glutamatergic, GABAergic, dopaminergic, and serotonergic networks develop rapidly and operate through conserved enzymatic and receptor mechanisms [11, 13]. Table 1 summarizes the main genetic orthologs associated with Alzheimer's disease pathology shared between humans and zebrafish, highlighting sequence identity and conserved physiological roles.

Table 1. Genetic Homology and Functional Conservation of Alzheimer's Disease-Related Genes Between Humans and Zebrafish

Human Gene	Zebrafish Ortholog(s)	Sequence Identity (%)	Conserved Biological Function
APP	*appa*, *appb*	80 (*appa*), 71 (*appb*)	Amyloid precursor processing, neural development, and sleep regulation
BACE1	*bace1*	74	β-Secretase cleavage initiating the amyloidogenic cascade
PSEN1	*psen1*	73.9	Catalytic subunit of γ-secretase complex and Notch signaling mediator
PSEN2	*psen2*	74	Cleavage of amyloid precursor protein and mitochondrial homeostasis
MAPT	*mapta*, *maptb*	58 (*mapta*), 62 (*maptb*)	Microtubule assembly, axonal transport, and cytoskeleton stabilization
SORL1	*sorl1*	63.5	Endosomal trafficking and sorting receptor for APP degradation
APH1A/B	*aph1*	62	Assembly and structural stabilization of active γ-secretase complex
NCSTN	*ncstn*	56	Substrate recognition and presentation in γ-secretase cleavage
APOE	*apoea*, *apoeb*	22.4 (*apoea*), 25.5 (*apoeb*)	Lipid transport, cholesterol redistribution, and systemic lipoprotein metabolism

Zebrafish offer practical advantages that streamline phenotypic drug discovery. Adults are small (3–5 cm), exhibit high fecundity by producing hundreds of embryos per mating event, and reach sexual maturity within three months [21–23]. Embryonic development occurs externally, and larvae remain optically transparent through early life stages, permitting non-invasive *in vivo* imaging of neurodevelopmental processes, neural circuitry activation, and cellular apoptosis [21–23]. 5 days post-fertilization (dpf), larvae display fully integrated motor behaviors and intact blood-brain barrier functionality, allowing automated compound screening across multi-well plate formats where test molecules are introduced directly into the rearing water [21, 23]. Figure 2 shows the comparative biological, methodological, and economic advantages of the zebrafish model across the drug discovery pipeline relative to cell-based *in vitro* assays and rodent systems.

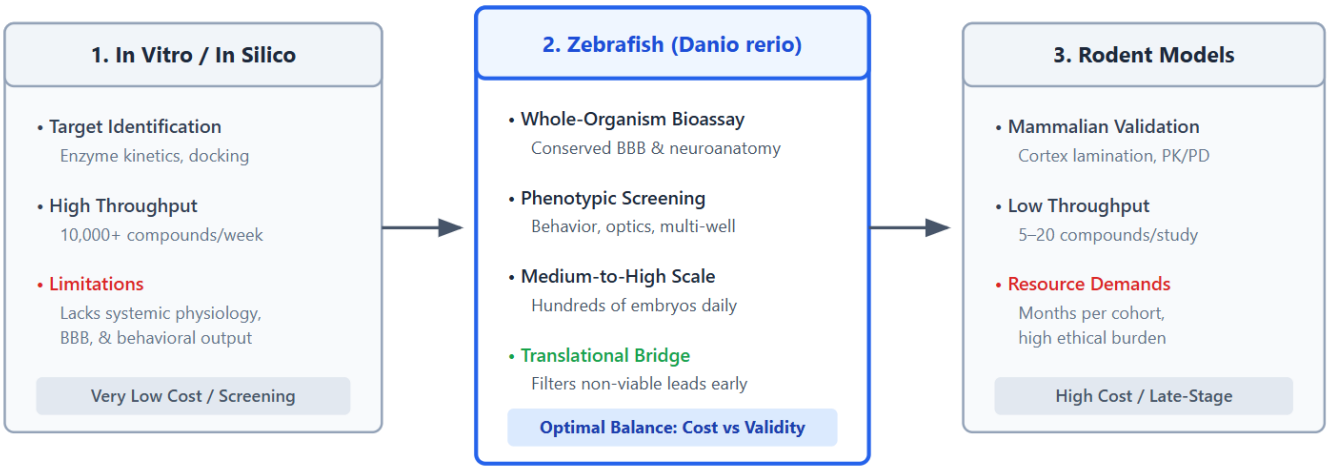

Figure 2. Applications of Zebrafish (*Danio rerio*) in Preclinical Drug Discovery

2. Pathological Models of Alzheimer's Disease in Zebrafish

2.1. Pharmacological and Chemical Induction Models

2.1.1. Scopolamine-Induced Cholinergic Deficit

Scopolamine, a non-selective muscarinic acetylcholine receptor antagonist, is widely utilized to simulate acute cognitive deficits in adult and larval zebrafish [26]. Exposure via immersion (typically 100–200 μM for 30–60 minutes) blocks central cholinergic transmission, leading to performance deficits in spontaneous alternation, spatial navigation, and novel object recognition tasks [27, 28]. At the molecular level, scopolamine exposure upregulates acetylcholinesterase (AChE) enzymatic activity, elevates reactive oxygen species (ROS) and malondialdehyde (MDA) concentrations, suppresses superoxide dismutase (SOD) activity, and triggers pro-inflammatory cytokine expression [27–30]. Investigational compounds evaluated in this model have demonstrated cognitive rescue through modulation of the PI3K/AKT/GSK3β signaling cascade, normalization of brain-derived neurotrophic factor (*bdnf*) expression, and restoration of endogenous redox homeostasis [31–35]. Table 2 lists out the established pharmacological and chemical neurotoxins utilized to model Alzheimer's disease features in zebrafish, detailing typical regimens, target mechanisms, and main phenotypic outcomes.

2.1.2. Aluminum Chloride ($AlCl_3$) Toxicity

Chronic immersion in $AlCl_3$ solutions (ranging from 0.04 mM in adult water tanks to 80 μM in larval culture media) models metal-induced neurotoxicity and progressive neurodegeneration [36–38]. Aluminum bioaccumulates within telencephalic structures, accelerating amyloidogenesis, elevating AChE activity, provoking lipid peroxidation, suppressing glutathione (GSH) production, and inducing chronic neuroinflammation [36–38]. This platform has served as a benchmark for screening diverse bioactive agents, including berberine chloride, plasmalogens, and arbutin, which counteract $AlCl_3$-mediated cognitive and biochemical impairments by reducing neuroinflammation and stabilizing cellular lipid homeostasis [39–42].

Table 2. Pharmacologically and Chemically Induced Alzheimer's Disease Models in Zebrafish

Model Toxicant	Developmental Stage	Standard Exposure	Pathological Target	Characteristic Phenotypic & Molecular Markers	Therapeutic Modifiers
Scopolamine	Larvae / Adult	100–200 μM (immersion for 30–60 min)	Non-selective muscarinic acetylcholine receptor blockade	Impaired Y-maze spontaneous alternation, reduced novel object preference, elevated AChE activity, and increased ROS/MDA	Donepezil, Galantamine, Rutin, Quercetin, Sweroside
Aluminum Chloride ($AlCl_3$)	Larvae / Adult	80 μM (larvae, 3–6 dpf) or 0.04 mM (adult, 21–30 days)	Telencephalic aluminum accumulation and lipid peroxidation	Memory retention deficits, telencephalic neuronal apoptosis, elevated AChE, decreased BDNF, and cytokine induction	Berberine chloride, Plasmalogens, Butyrolactone I, Arbutin
$AlCl_3$ + D-Galactose	Larvae	Combined immersion (80 μM $AlCl_3$ + 10–20 mM D-gal)	Synergistic oxidative stress and accelerated senescence	Elevated Aβ1-42 deposition, augmented senescence-associated β-galactosidase, telomerase decline, and high IL-1β expression	Free-radical scavengers, natural polyphenolic extracts
Okadaic Acid (OA)	Larvae / Adult	100 nM (immersion) or ICV injection	Selective inhibition of PP2A and hyperactivation of GSK3β	Tau hyperphosphorylation, loss of T-maze spatial memory, increased anxiety-like scototaxis, and neuronal death	TDZD-8 (GSK3β inhibitor), LKE, Carnosine, Photobiomodulation
Streptozotocin (STZ)	Adult	5 mg/kg daily for 7 days (ICV) or 350 mg/kg (IP) + 2.5 μL (ICV)	Central and peripheral insulin resistance; cerebrovascular breakdown	Reduced novel object recognition, blood-brain barrier leakage, elevated Evans blue extravasation, nitrite production, and MMP-13 activation	Astaxanthin, Donepezil, CL-82198 (MMP-13 inhibitor)

2.1.3. D-Galactose and Multi-Toxicant Combinations

D-galactose accelerates cellular senescence by triggering systemic oxidative stress, advanced glycation end-product formation, mitochondrial impairment, and telomere shortening [43]. Co-administration of $AlCl_3$ and D-galactose produces synergistic cognitive deficits in zebrafish larvae alongside elevated Aβ1-42 deposition, augmented senescence-associated β-galactosidase activity, and marked pro-inflammatory transcription, creating an integrated model of age-associated AD [44]. Similarly, dual exposure to $AlCl_3$ and lead acetate induces combined cholinergic dysfunction and lipid peroxidation, validating anxiolytic and promnesic drug effects in an environmentally relevant toxicological paradigm [45].

2.1.4. Streptozotocin-Induced Metabolic Impairment

Sporadic AD involves central insulin resistance and impaired cerebral glucose metabolism. Intracerebroventricular (ICV) administration of streptozotocin (STZ; 5 mg/kg daily) or combined intraperitoneal and intracerebral injections generate significant cognitive impairment in adult zebrafish, accompanied by altered peroxisome proliferator-activated receptor expression, blood-brain barrier permeability, nitrite elevation, and matrix metalloproteinase activation [46, 47]. Pharmacological treatments that restore insulin signaling pathways, such as astaxanthin, successfully attenuate these neuropathological and behavioral manifestations [47]. Figure 3 outlines the primary pathological 49odelling methodologies used in zebrafish, categorizing them into pharmacological agents, exogenous peptide microinjections, and transgenic manipulations alongside their corresponding target pathways.

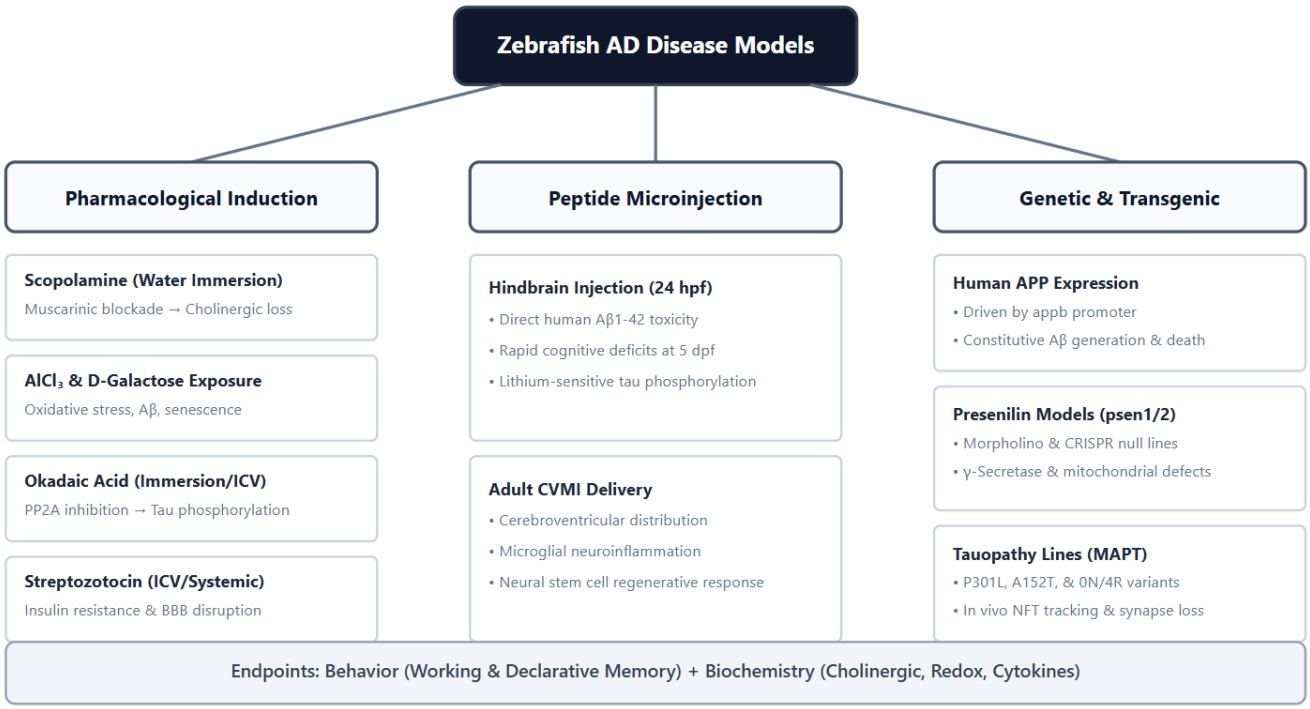

Figure 3. Classification of Alzheimer's Disease Pathological Models in *Danio rerio*

2.1.5. Okadaic Acid-Induced Tauopathy

Okadaic acid (OA) selectively inhibits serine/threonine protein phosphatases 1 and 2A (PP2A), stimulating glycogen synthase kinase-3 beta (GSK3β) activity and precipitating hyperphosphorylation of the microtubule-associated protein tau [48, 49]. Exposure to nanomolar concentrations of OA leads to memory deficits in maze paradigms, elevated anxiety-like scototaxis, and neuronal apoptosis [48–50]. Pharmacological interventions employing GSK3β inhibitors, lanthionine ketimine derivatives, carnosine, or photobiomodulation counter these OA-induced deficits by restoring PP2A activity, reducing tau phosphorylation, and sustaining Akt/CREB-mediated neuroprotective signaling [49–54].

2.2. Exogenous Peptide Microinjection Models

Direct microinjection of human Aβ1-42 peptides into the hindbrain ventricle of 24 hpf embryonic zebrafish or via cerebroventricular microinjection (CVMI) in adult specimens produces acute amyloid neurotoxicity [55, 56]. This intervention provokes widespread microglial recruitment, synaptic degeneration, tau hyperphosphorylation at GSK3β-dependent residues, and spatial memory impairment [55–57]. Uniquely, the adult zebrafish brain activates endogenous neural stem cell proliferation following Aβ42 insult,

providing a model for studying both neurodestructive cascades and regenerative mechanisms during therapeutic screening of anti-amyloid agents and nanomedicines [56–58].

2.3. Transgenic and Genetic Models

Genetic engineering in zebrafish enables stable modeling of early-onset familial AD mutations:

These genetic lines, particularly tauopathy models, facilitate real-time *in vivo* imaging of live neurodegenerative processes and have identified novel therapeutic targets, such as the epigenetic reader bromodomain-containing protein 4 (Brd4) [59–65]. Table 3 summarizes the primary genetic and peptide microinjection models used to evaluate targeted pathological pathways of Alzheimer's disease in zebrafish.

Table 3. Transgenic, Knockout, and Microinjection Techniques for Alzheimer's Disease Modeling in Zebrafish

Model	Specific Construct / Agent	Genetic / Technical Mechanism	Pathological Effects in Zebrafish	Experimental Utility
Synthetic Amyloid Microinjection	Synthetic human Aβ1-42 peptides	Hindbrain microinjection (24 hpf embryos) or adult cerebroventricular microinjection (CVMI)	Acute cognitive impairment, microglial recruitment, synaptic degeneration, and GSK3β-dependent tau phosphorylation	Rapid screening of direct anti-amyloid compounds, nanomedicines, and neural regeneration studies
APP Overexpression	Mutant human APP (*APPsw*)	Driven by zebrafish *appb* tissue-specific promoter	Elevated cerebral Aβ generation, vascular deposition, and progressive telencephalic neurodegeneration	Genetic platform for assessing secretase modulators and clearing agents
Presenilin Deficiency	*psen1* / *psen2* targeted disruption	Morpholino splice-blocking or stable CRISPR/Cas9 knockout	Disrupted Notch signaling, impaired γ-secretase activity, mitochondrial dysfunction, and loss of PSD-95 synaptic markers	Elucidating presenilin-mediated familial AD pathways and synaptic protection screens
Tauopathy Transgenic Lines	Mutant human *MAPT* (P301L, A152T) or wild-type 0N/4R tau	Neuron-specific Gal4/UAS or *HuC* promoter-driven expression	Tau hyperphosphorylation at disease epitopes, neurofibrillary tangle deposition, motor neuron loss, and microglial synaptic pruning	*In vivo* live optical imaging of neuronal apoptosis, autophagy enhancers, and Brd4/GSK3β inhibitors

3. Behavioral Phenotyping

3.1. Spontaneous Alternation and Spatial Navigation

Spatial working memory is frequently quantified using continuous spontaneous alternation tasks within symmetric Y-mazes or modified T-mazes [67, 68]. Exploiting the natural exploratory drive of zebrafish toward novel environments, animals alternate consecutive entries across the three maze arms. AD models exhibit disorganized navigation patterns and depressed alternation rates, which are restored toward baseline levels upon administration of standard cholinesterase inhibitors or antioxidant formulations [11, 67]. In conditioned T-maze protocols, acquisition and retention of spatial avoidance are measured by the latency to enter an arm paired with an aversive electrical stimulus [68].

3.2. Non-Spatial Recognition Memory

The Novel Object Recognition (NOR) paradigm evaluates declarative recognition memory without requiring physical stressors or food deprivation [69]. Following habituation and training with identical objects, one object is replaced by a visually distinct novel counterpart. Cognitively intact zebrafish spend significantly more time investigating the novel object, whereas neurotoxin-exposed or transgenic AD models exhibit loss of novelty discrimination, spending equal time between familiar and novel objects [69].

3.3. Inhibitory Conditioning and Social Dynamics

Passive avoidance assays take advantage of teleost scototaxis (the innate preference for dark environments over brightly illuminated areas) [70]. When entry into the preferred dark chamber is paired with an aversive stimulus, intact fish inhibit this entry during retention trials. Cholinergic antagonists like scopolamine shorten this latency, demonstrating memory retrieval deficits [70]. Shoaling

assays quantify social memory and affective state by measuring inter-fish distance, nearest neighbor proximity, and shoal polarization, providing sensitive indicators of neurotoxin-induced social disruption and dementia-like anxiety phenotypes [71–73]. Table 4 lists out the operational parameters, evaluated cognitive domains, and quantitative readout metrics across major neurobehavioral assays applied to zebrafish. Figure 4 shows the operational layouts of the four primary neurobehavioral apparatuses employed to quantify learning, memory, and affective responses in adult and larval zebrafish.

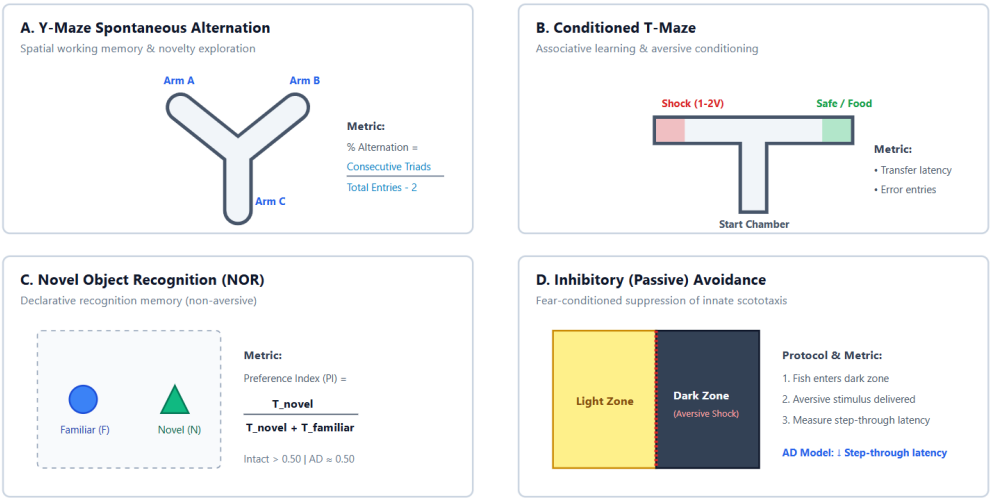

Figure 4. Methodological Schematics of Core Zebrafish Behavioral Assays

Table 4. Comparison of Neurobehavioral Assays for Zebrafish Cognitive Screening

Behavioral Assay	Evaluated Cognitive / Affective Domain	Apparatus & Setup	Quantitative Endpoints	AD Model Phenotype	Target Response
Y-Maze Spontaneous Alternation	Spatial working memory & exploratory drive	Symmetric 3-arm maze with 120° angles; free movement pattern	Spontaneous alternation percentage ($\% = \frac{\text{Consecutive 3-arm choices}}{\text{Total arm entries} - 2} \times 100$); total arm entries	Significant decrease in alternation rate; repetitive arm re-entry	Elevation of alternation percentage toward control baseline
Conditioned T-Maze	Spatial reference learning & associative recall	T-shaped tank with start area, decision point, and two distinct cue arms	Transfer latency to enter target arm; time spent in reward vs. punishment zone	Prolonged escape latency; inability to associate visual cue with safety/punishment	Significant reduction in transfer latency to reach rewarded compartment
Novel Object Recognition (NOR)	Non-spatial declarative / recognition memory	Open arena with distinct familiar and novel visual objects	Novelty preference index ($PI = \frac{T_{\text{Novel}}}{T_{\text{Novel}} + T_{\text{Familiar}}}$); exploration frequency within 2.5 cm zone	Preference index drops toward 50% (chance level discrimination)	Significant increase in exploration time devoted to the novel object
Inhibitory (Passive) Avoidance	Fear conditioning, scototaxis suppression, & long-term retention	Two-compartment light/dark tank paired with mild electric shock (1–2 V) in dark compartment	Step-through latency to enter dark compartment across acquisition and retention trials	Abrupt decrease in step-through latency (failure to inhibit preferred scototaxis)	Prolonged latency to enter the previously punished dark compartment
Shoaling Assays	Social cognition, group cohesion, & anxiogenic behaviors	Open tank housing groups of 5–10 fish with top-down video recording	Average inter-fish distance (IFD); nearest neighbor distance (NND); group polarization angle	Dissolution of cohesive schooling; increased IFD/NND; erratic polarization	Restoration of tight schooling geometry and baseline group polarization

4. Biochemical and Molecular Markers

Quantitative evaluation of neurochemical and molecular pathways is essential to substantiate behavioral outcomes:

4.1. Cholinergic Regulation

Quantifying AChE and choline acetyltransferase (ChAT) activities within homogenized telencephalic tissue remains a primary metric for assessing cholinergic stability [74, 75]. Pathological models often exhibit elevated AChE activity and diminished ChAT synthesis, accelerating synaptic acetylcholine clearance. Therapeutic candidate evaluation centers on the dose-dependent normalization of these enzymatic activities [75, 76].

4.2. Redox Equilibrium and Membrane Integrity

Cerebral oxidative damage is tracked by measuring radical accumulation using fluorescent indicators (e.g., $CM - H_2DCFDA$), alongside measuring tissue concentrations of MDA and protein carbonyls [77–80]. Enzymatic defenses, including SOD, catalase (CAT), and glutathione peroxidase (GPx), together with the reduced glutathione (GSH) pool, are monitored to determine whether test molecules can restore endogenous antioxidant defenses [80, 81].

4.3. Neuroinflammatory Cytokine Profiles

Chronic microglial activation triggers the transcriptional upregulation of pro-inflammatory cytokines via NF-κB signaling [82, 83]. Utilizing quantitative real-time PCR (qRT-PCR) and enzyme-linked immunosorbent assays (ELISA), investigators evaluate the expression of *tnfa*, *il1b*, and *il6*. The downregulation of these inflammatory mediators serves as an *in vivo* metric of neuroimmunomodulatory efficacy [83]. Table 5 summarizes the biochemical, enzymatic, and neuroinflammatory endpoints monitored in zebrafish brain homogenates to confirm the molecular efficacy of anti-Alzheimer drug candidates.

Table 5. Biochemical, Oxidative, and Inflammatory Endpoints in Preclinical Screening

Biochemical Category	Target Biomarker / Enzyme	Biological Role in Neural Tissue	Direction of Change in AD Phenotype	Assay Method
Cholinergic System	Acetylcholinesterase (AChE)	Terminates cholinergic transmission	Elevated / dysregulated	Ellman colorimetric spectrophotometry
	Choline Acetyltransferase (ChAT)	Synthesizes acetylcholine from choline and acetyl-CoA presynaptically	Depleted / decreased	Radiometric / colorimetric ELISA
Redox & Lipid Peroxidation	Malondialdehyde (MDA) & Protein Carbonyls	Byproducts of polyunsaturated fatty acid	Markedly elevated	Thiobarbituric acid reactive substances (TBARS) assay
	Superoxide Dismutase (SOD) & Catalase (CAT)	Dismutation of superoxide radicals and conversion of H_2O_2into H_2O	Exhausted / diminished	Enzymatic spectrophotometric rate assays
	Glutathione Peroxidase (GPx) & Reduced GSH	Scavenges peroxides and maintains intracellular thiol redox status	Severely depleted	DTNB (Ellman's reagent) spectrophotometric quantification
Neuroinflammation	Pro-inflammatory Cytokines (*tnfa*, *il1b*, *il6*)	Mediate microgliosis, astrocytic activation, and synaptic pruning	Highly upregulated	Quantitative real-time PCR (qRT-PCR) / Western blotting
	NF-κB Nuclear Translocation	Transcriptional regulator of inflammation	Activated / translocated	Immunofluorescence / Western blot nuclear fractionation

5. Conclusion

Danio rerio provides a versatile preclinical platform for evaluating anti-Alzheimer drug candidates, effectively integrating disease modeling, automated neurobehavioral tracking, and biochemical characterization. Despite its experimental strengths, the zebrafish model presents specific biological and technical considerations. A primary challenge involves the lack of unified experimental parameters across laboratories, where dosing regimens, delivery methods (immersion versus microinjection), and behavioral apparatus geometries vary widely. Genomically, the teleost-specific whole-genome duplication (3R event) has produced duplicated paralogs for several human disease genes (e.g., *appa* and *appb*, *mapta* and *maptb*), requiring coordinated multi-gene targeting strategies

during knockout studies. Neuroanatomically, teleosts lack a layered neocortex and reproduce human tau and amyloid pathology on different temporal scales. Nevertheless, automated tracking systems, standardized husbandry, and targeted genetic engineering position *Danio rerio* as a valuable, cost-efficient screening intermediary that bridges early *in vitro* discovery with downstream mammalian testing. Experimental refinement and validation will further enhance the reproducibility of zebrafish screens, accelerating the identification of potential disease-modifying therapies for neurodegenerative disorders.

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