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Review Article

Can zebrafish be used as animal model to study Alzheimer's disease?

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Abstract: Zebrafish is rapidly emerging as a promising model organism to study various central nervous system (CNS) disorders, including Alzheimer's disease (AD). AD is the main cause of dementia in the human population and there is an urgency to understand the causes of this neurodegenerative disease. In this respect, the development of new animal models to study the underlying neurodegenerative mechanisms of AD is an urgent need. In this review we analyze the current situation in the use of zebrafish as a model for AD, discussing the reasons to use this experimental paradigm in CNS investigation and analyzing the several strategies adopted to induce an AD-like pathology in zebrafish. We discuss the strategies of performing interventions to cause damage in the zebrafish brain by altering the major neurotransmitter systems (such as cholinergic, glutamatergic or GABAergic circuits). We also analyze the several transgenic zebrafish constructed for the AD study, discussing both the familial-AD models based on APP processing pathway (APP and presenilins) and in the TAU hyperphosphorylation, together with the genes involved in sporadic-AD, as apolipoprotein E. We conclude that zebrafish is in a preliminary stage of development in the AD field, and that the transgenic animals must be improved to use this fish as an optimal model for AD research. Furthermore, a deeper knowledge of the zebrafish brain and a better characterization of the injury caused by alterations in the major neurotransmitter systems are needed.

Keywords: zebrafish, Alzheimer's disease, neurotoxins, cholinergic neurotransmission, glutamatergic neurotransmission, GABAergic neurotransmission, apolipoprotein E, APP, presenilin, TAU

1. Alzheimer's disease (AD)

Alzheimer´s disease (AD) is the most common form of dementia characterized mainly by massive neuronal loss and impaired synaptic processes located in the cerebral cortex, particularly in the frontal and temporal lobes, and hippocampus [1]. For over a century, pathologic features have been used to define AD, whose phenotype is composed of two main hallmarks: (i) the deposition of senile plaques, which are extracellular amyloid-β (Aβ) peptide aggregates with axons and dendrites usually swollen or atrophic, and (ii) the neurofibrillary tangles composed of intracellular hyperphosphorylated TAU protein and aggregated into paired helical filaments [1].

Despite the intense research devoted to the elucidation of the mechanisms underlying AD, these are so far unknown. It is only well-documented the cause of a percentage less than 1% of cases, known as familial AD (FAD), which shows a pattern of autosomal dominant inheritance and early onset of symptoms. FAD is a monogenic disease associated with the presence of specific mutations mainly in three genes that increase the production of Aβ peptide of 42 amino acids [2]: amyloid-β precursor protein (*APP*), presenilin 1 (*PSEN1*), and to a lesser extent the presenilin 2 (*PSEN2*). The rest of the cases (>99%), called sporadic AD (SAD), presents a complex interaction between genetic susceptibility factors and environmental being the main risk factor for AD, the allele ε4 of the gene for apolipoprotein E (APOEε4) [3].

2. Zebrafish as a model for AD

Historically, rodent models have been used to

study AD. However, the causes of AD remain unknown, and more efforts to decipher the mechanisms underlying neurodegeneration are necessary. Despite the progress in this field, we still need a better understanding of AD, which supports the growing importance of further innovative research using experimental models of neurodegeneration. Therefore, additional animal models with complementary advantages must be used to analyze the basis of the neurodegeneration and, subsequently, to evaluate the effects of novel drugs, as a previous step to assays in rodents. In this respect, the zebrafish (*Danio rerio*) have recently become a focus of neurobehavioral studies since it displays neuropathological and behavioural phenotypes that are quantifiable [4, 5], and it has been recently proposed as a valid experimental paradigm to study AD [6]. Zebrafish is an ideal model for the study of human diseases because they present a number of features that make it unique as an animal model. For this reason, the zebrafish have experienced a dramatic rise in popularity as an experimental organism.

Zebrafish offer a reasonable compromise between physiological complexity and throughput, have a fully characterized genome, and display significant physiological homology to mammals, including humans [7]. Moreover, it presents a clear advantage in comparison to other animal models: the larva-adult duality. The availability of both forms is beneficial and enables the investigation of a wider spectrum of neurodegenerative-related phenomena throughout ontogenesis. The faster development and longer lifespan of zebrafish, compared to mice, makes them and ideal choice to model developmental trajectories of neurodegeneration. Moreover, as rodent models are expensive to maintain and more difficult to modify genetically, lower organisms emerge as useful species to increase the knowledge of the mechanisms underlying neurodegenerative processes. The zebrafish is a vertebrate more closely related to humans than invertebrate models such as yeast, worms or flies, which design and connectivity in the central nervous system (CNS) correlates with the human [8]. Although invertebrates can provide important insights into neurodegeneration, the absence of a complex nervous system limits their application in modelling intricate aspects of CNS disorders. Comparison between various species is also important for uncovering mechanisms associated to brain pathogenesis. While the complementary use of zebrafish could be an important strategy for AD research, it represents an integral part of a more global cross-species modelling (from fish to rodents) for uncovering evolutionary conserved mechanisms of neurodegeneration.

Furthermore, zebrafish has a short time of development to sexual maturity (3 months) and a high reproductive rate (hundreds of embryos per female per clutch and per week). Embryos are transparent and developed externally, facts that allow direct observation of embryogenesis and development of the CNS. Embryos are also easily amenable to methods for manipulating genes and protein activity such as injection of antisense oligonucleotides [9, 10], mRNAs or transgenes, and for screening of drug libraries being arrayed in microtitre plates [11]. Moreover, whereas the early zebrafish developmental stages are likely to be important to analyze CNS processes and abnormalities, adult fish with the full range of brain functions should have a significant advantage in the analysis of the complex brain functions characteristics of vertebrates [12], and in the studies of conductual phenotyping. Indeed, zebrafish exhibit many higher order behaviors including memory, conditioned responses, and social behaviors like schooling [13]. All these properties strengthen the zebrafish as an ideal model for studying human diseases, including CNS disorders [14].

3. The zebrafish brain organization

Zebrafish has been recently recognized as a reliable model for studies of behaviour, neural circuitry and neural disease [8], due to its phylogenetical proximity to the human. The comparison of the zebrafish brain structure with man shows a high conservation of basic brain organization [15], together with similar key neuroanatomical [16, 17] and neurochemical [18] pathways of relevance to human diseases. The organization of the zebrafish brain is similar to other vertebrates, despite the smaller cerebral hemispheres and the structure and function of the optic tectum, having similarly defined areas such as the hypothalamus and olfactory bulb, encompassing structures of the lateral pallium (located in the telencephalon), which appear to be homologous to the mammalian hippocampus. Indeed, zebrafish and mammalian encephalon share many structural properties,

such as the main organization (fore-, mid- and hind-brain, including diencephalon, telencephalon and cerebellum), or the principal neurotransmitter systems [18]. The major neurotransmitter circuits, such as the glutamatergic (excitatory) and the GABAergic (inhibitory), have been demonstrated to exist in the zebrafish brain [5]. In addition to the major excitatory and inhibitory neurotransmitters, muscarinic cholinergic receptors have been confirmed in brain extracts of adult zebrafish by radioligand binding methods [19]. Moreover, the neurotransmitters such as GABA, dopamine, serotonin, histamine, glutamate and acetylcholine are also found in zebrafish [12].

Additionally, zebrafish CNS also contains the main cellular types found in the mammalian brain, such as microglia [20], oligodendrocytes and myelin [21, 22], cerebellar Purkinje cells [23], motor neurons [24] or astrocytes [25].

This species also possess a tight junction-based blood-brain barrier, with substantial macromolecule permeability, and highly regulated [26], a fact essential to the search for novel neuroprotective compounds [27].

3.1. The zebrafish cholinergic neurotransmission

Acetylcholine (ACh) is an essential neurotransmitter in the central and peripheral nervous system (PNS) that developed very early in phylogenetical history. The cholinergic system performs a critical role in learning processes and memory functions, wherein their projections are severely affected in AD [28]. Indeed, AD is related to cholinergic system dysfunctions such as the loss of cholinergic neurons in the basal forebrain and hippocampus [29, 30], and in this respect, memantine (a glutamate receptor NMDA antagonist) has been currently used for the treatment of AD [31].

The presence of a cholinergic system in the zebrafish brain has been verified through electrophysiological, histological, and biochemical approaches. Electrophysiological studies demonstrated that carbachol, a muscarinic receptor agonist, modulated the electrically-induced neural activity in the isolated zebrafish brain [19]. Histologically, neurons containing acetylcholinesterase (AChE) were detected in most areas of the zebrafish CNS, including the olfactory bulb, telencephalon, cerebellum, medulla oblongata, and spinal cord [32]. In particular, the telencephalon, which is thought to be responsible for learning in teleost fish [33, 34], appeared to have AChE-positive neurons in various nuclei of the dorsal and ventral areas. The identification of cholinergic neurons in zebrafish CNS has been also previously reported by using specific antibodies against choline acetyltransferase (ChAT) [18, 32, 35]. In particular, the anatomical identification of ChAT immunoreactive neurons in adult zebrafish differs among the studies probably due to the distinct methodological approaches. In any case, ChAT appears to be present in the ventral and dorsal telencephalic areas [18], wherein the presence of ChAT immunoreactive axons indicates that the AChE-positive neurons are cholinoceptive. Finally, the main developmental pattern of ChAT positive neurons have been described for zebrafish [36].

Therefore, the cholinergic system of zebrafish has been shown to be widely distributed, according to the results of the immunocytochemical studies on ChAT and AChE in the zebrafish brain.

3.2. The zebrafish glutamatergic neurotransmission

Glutamate is the most widespread and important excitatory neurotransmitter in the CNS of vertebrates. However, an overstimulation of the glutamatergic system may lead to excitotoxicity, a phenomenon involved in many pathological conditions including AD [37, 38]

The neurotransmitter glutamate activates several classes of metabotropic receptor and three major types of ionotropic receptors: 2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl) propanoic acid (AMPA), kainate (KA) and N-methyl-D-aspartate (NMDA) [39]. The molecular characterization and embryonic expression of the family of NMDA receptor subunit genes has already been established in zebrafish [40]. Considering that long-term potentiation is the representative synaptic modification underlying the process of learning and memory, Nam and collaborators [41] studied the NMDA receptor-dependent long-term potentiation in the telencephalon of the zebrafish, which is analogous to hippocampus and amygdala of mammalian brain [33, 34]. In this context, a simple protocol of inhibitory avoidance task in adult zebrafish demonstrated

that the resulting memory is robust, long-lasting and sensitive to NMDA-receptor antagonist MK-801 given in the tank water immediately after the training session [42]. Furthermore, a biochemical study showed that MK-801 alter the Na^+, K^+ -ATPase activity and oxidative parameters in zebrafish brain [43].

Moreover Edwards and Michel [44] demonstrated the pharmacological characterization of ionotropic glutamatergic receptors in olfactory bulb in zebrafish. This group advanced the understanding of the glutamatergic system in teleosts by characterizing the distribution of functional NMDA and KA-stimulated neurons. Furthermore, Tabor and Friedrich [45] pharmacologically investigated the ionotropic glutamate receptor function in neuronal circuits of the zebrafish olfactory bulb.

Other via to neutralize the excess of extracellular glutamate is the high-affinity uptake mechanism executed by a family of excitatory amino acid transporters (EAATs) [37]. The presence of EAAT-related sequences in zebrafish CNS have been recently described [46]. Once identified these EAAT-related genes, some preliminary parameters of glutamate transporter activity were investigated through sodium-dependent glutamate uptake in distinct zebrafish brain structures [46].

3.3. The zebrafish GABAergic neurotransmission

The γ-aminobutyric acid (GABA) is a classical inhibitory neurotransmitter in the brain, playing essential roles in synaptic plasticity [47]. Defects in GABAergic neurotransmission are the cause of several CNS pathologies [48, 49]. In addition, given the hypothesis that the abusive stimulation of ionotropic glutamate receptors is in part responsible for the pathology of AD, it is important to note that disruptions or alterations in GABA-mediated neurotransmission may significantly impact the functional relationship between excitatory and inhibitory systems [50, 51]. In fact, the balance between excitatory and inhibitory neurotransmitters is involved in several events in CNS, with many neurodegenerative diseases being correlated with brain cells dysfunction, and, consequently, causing changes in these signaling systems.

GABA-containing neurons appear in the zebrafish olfactory bulb, telencephalon, tectum stratum, and in the hypothalamus [52]. In the cerebellar corpus and valves of the zebrafish, GABA receptors are present in the molecular layer, Purkinje cells, and groups of Golgi cells in the granular layer [53]. Furthermore, an electrophysiological study demonstrated the evidence of $GABA_A$ and $GABA_C$ receptors on zebrafish retinal bipolar cells [54]. Accordingly, immunohistochemical studies found expression of GABAergic cells during early embryonic stages in the zebrafish brain, and an increase in their number with developmental progression [55].

Glutamic acid decarboxylase (GAD) catalyzes the α-decarboxylation of glutamic acid to produce γ-aminobutyric acid. GAD is highly conserved and has been described in *Drosophila*, avian, and mammalian species [56, 57]. Martin and collaborators [58] analyzed the amino acid identities and similarities between zebrafish and human GAD65 and GAD67 cDNAs. Moreover, in situ hybridization revealed that both GAD65 and GAD67 were expressed early in the zebrafish embryo during the period of axonogenesis, suggesting a role for GABA prior to synapse formation. Both GAD genes were detected in the telencephalon, in the nucleus of the medial longitudinal fasciculus in the midbrain, and at the border regions of the rhombomeres in the rostral hindbrain.

Based on these data, the evaluation of parameters associated to GABAergic system emerges as a tempting strategy to assess neurochemical, behavioral, and toxicological phenotypes associated to AD-related models in both larval and adult zebrafish.

4. Pharmacological models for AD in zebrafish

4.1. Cholinergic neurotoxins

AD is characterized by the functional decreased of the cholinergic system, which is crucial to cognitive impairment. The early expression of AChE in diverse cell types suggests that it may have a developmental role and thus, could be a putative target for neurotoxicity in zebrafish [59]. The first study that used biochemical and molecular approaches demonstrated the enzyme activity and gene expression pattern for AChE in zebrafish brain [60]. Successive reports addressed the importance of this enzyme in regulating zebrafish brain functions. Afterwards, the effects of several drugs on cholinergic trans-

Table 1. Summary of recent studies of pharmacological models for AD utilizing larval and adult zebrafish (MOA: mechanism of action).

Neurotransmitter system	Neurotoxin	MOA	Model	Endpoint	Reference
Cholinergic	Scopolamine	Muscarinic receptor antagonist	Adults	Behavior	[63, 66]
	Carbachol	Muscarinic receptor agonist	Adults	Electrophysiology	[19]
	Pilocarpine	Muscarinic receptor agonist	Adults	Behavior	[72]
			Larvae	Locomotor activity	[71]
	Physostigmine	Acetylcholinesterase inhibitor	Adults	Behavior	[73]
Glutamatergic	MK-801	NMDA-receptor antagonist	Adults	Behavior	[77]
	Ketamine	NMDA-receptor antagonist	Adults	Behavior & Molecular biology	[88-90]
	APV	NMDA-receptor antagonist	Adults	Electrophysiology	[41]
	Memantine	NMDA-receptor antagonist	Larvae	Behavior	[84, 85]
	Kainate	KA receptor agonist	Adults	Behavior	[80]
	Domoate	KA receptor agonist	Larvae	Behavior	[81]
	CNQX	AMPA-receptor antagonist	Adults	Electrophysiology	[41]
GABAergic	Pentylentetrazole	GABA-receptor antagonsit	Larvae	Behavior, electrophysiology, molecular biology & biochemistry	[83, 93, 97]
			Adults	Behavior	[73, 95, 96]
	Picrotoxin	GABA-receptor antagonsit	Adults	Behavior	[95]
	Phenylpyrazole	GABA-receptor antagonist	Larvae	Development	[91]
	Valproate	GABA transaminase, voltage-gated sodium channels & T-type calcium channels inhibitor	Adults	Behavior, electrophysiology, molecular biology	[96]

mission have been evaluated. In this regard, the effects promoted by metals [61-63], typical and atypical antipsychotics [43], antiepileptics [64] and ethanol [65] on AChE activity have already been studied in zebrafish brain.

To demonstrate the activity of the cholinergic system, the effects induced by scopolamine (a cholinergic muscarinic receptor antagonist) have been analyzed in zebrafish. Similarly to what occurs on mammals, the results showed that this drug impairs both the acquisition of passive avoidance response and retention of the learned response, and that physostigmine (an acetylcholinesterase inhibitor that blocks the breakdown of the released ACh at the synaptic site) rescues the amnesic effects of scopolamine [66] (**Table 1**). In this regard, the use of both quercetin and rutin prevented scopolamine-induced memory impairment in zebrafish [63]. These results are very consistent with similar studies in rodents showing scopolamine-induced learning deficits in rats and mice [67-69].

In addition, saturation binding experiments showed relatively high receptor numbers and affinity for cholinergic agonists and antagonists including oxotremorine, carbachol, atropine, and pirenzepine [70]. In this respect, two of these muscarinic receptor agonists (carbachol and oxotremorine) suppressed the electrically evoked field potentials in the telencephalon of the adult zebrafish in a reversible and dose-dependent manner [19].

Another muscarinic receptor agonist, pilocarpine, is frequently used as neurotoxic drug inducing seizures. In fact, the anticonvulsant and/or proconvulsant effects of antidepressants against pilocarpine- and pentylenetetrazole-induced seizures in larval zebrafish and

mice has been determined [71]. Additionally, the cholinergic effects promoted by nicotine and pilocarpine on dopaminergic system have been verified in neurobehavioral parameters induced by chlorpyrifos exposure [72], and inhibitors of AChE such as soman and physostigmine also induce seizures, which further cause an excessive release of excitatory amino acids [73].

Altogether, these findings could facilitate the use of the zebrafish as a model for study of cholinergic mechanisms underlying learning and memory.

4.2. Glutamatergic neurotoxins

Glutamate-mediated neurotoxicity, also known as excitotoxicity, is associated to the AD pathogenesis, being caused by the over activation of the glutamate receptors, that ultimately leads to neuronal damage/death [74]. Given the early loss of glutamatergic neurons in AD in vulnerable pathways such as the medial temporal lobe/hippocampal network, the role of excitotoxic mechanisms has long been associated to this disease [75]. In this respect, memantine (a low potency NMDA receptor antagonist) is the main blockbuster drug for AD (together with cholinesterase inhibitors), although only a clinically poor symptomatic benefit has been demonstrated [76].

To date, the research in the field of glutamatergic transmission in adult zebrafish has been confined to studies analyzing the effects of MK-801 on their swimming behaviour, learning and memory [77] and to histological descriptions of ionotropic glutamate receptors [44] (**Table 1**). The presence of NMDA- and KA-sensitive ionotropic glutamate receptors was immunohistochemically detected in the zebrafish olfactory bulb [78]. Electrophysiologically, neural activity was blocked by CNQX, an AMPA receptor antagonist, and d-2-amino-5-phosphono-valerate (APV), an NMDA receptor antagonist, in the telencephalon [41]. In this respect, blockade of NMDA receptor activation that is crucial in the learning process in rats also blocks acquisition of passive avoidance learning in zebrafish [42]. Moreover, the synaptic plasticity induced in the medial division of zebrafish telencephalon was inhibited by CNQX [79].

More recently, we have demonstrate that KA receptors in zebrafish operate in a similar way to those in established models, wherein the KA-induced seizures can be specifically inhibited in a dose-dependent fashion by DNQX [80]. The inhibition of seizures by specific glutamate receptor antagonists makes the zebrafish a discriminatory model to study the excitotoxicity, even though a demonstration of a specific neuronal death after excitotoxicity in adult zebrafish is still remaining. These results are in line with previous reports using domoic acid (DA) in larval zebrafish [81]. DA, a structural relative to kainate and the neurotransmitter glutamate, activates AMPA and kainate subtypes of the glutamate receptor family resulting in excitotoxicity predominantly in brain tissues [82]. The microinjection of DA to fertilized eggs induced toxicity on zebrafish development [81]. These toxics effects include reduced hatching and uncontrolled pectoral fin motions and tonic-clonic like convulsions [81]. Moreover, *in ovo* exposure to DA increases the susceptibility of larval zebrafish to the seizure-inducing agent PTZ [83].

The effect of memantine has been also studied in the zebrafish model. Best and co-workers demonstrated that zebrafish larvae exhibit iterative reduction in a startle response to a series of acoustic stimuli, with memantine increasing this acoustic startle response and decreased habituation [84]. On the other hand, exposure to memantine did not alter the swim speed of zebrafish larvae [85].

Finally, ketamine, a non-competitive antagonist of NMDA receptor, has been used to induce sedation and analgesia [86, 87]. Sub-anesthetic doses of ketamine produced a variety of abnormal behaviors, such as altered gill movement, stress responses and aberrant circling behavior in adult zebrafish [88]. Acute exposure of ketamine reduced anxiety, impaired intra-session habituation and evoked circular swimming of adult zebrafish. Additionally, ketamine reduced whole-body cortisol levels and elevated brain c-fos expression in zebrafish [89]. Finally, Kanungo and collaborators demonstrated that ketamine adversely affected motor neuron axon length and decreased cranial and motor neuron populations [90].

Considering the involvement of the glutamatergic system in the AD pathogenesis, and that the results obtained with glutamatergic neurotoxins in zebrafish and rodents are qualitatively comparable [81, 85], it becomes important to con-

sider the zebrafish as a model for future developments in AD.

4.3. GABAergic neurotoxins

The investigation of environmental toxins (e.g.,organic compounds and heavy metals) has been used to understand the zebrafish GABAergic neurotransmission. The use of phenylpyrazole insecticide (fipronil) on embryos showed that, although this insecticide acts as an inhibitor of GABA receptors, it may also inhibit a structurally related glycine receptor subtype expressed during the development of spinal locomotor pathways [91] (**Table 1**).

On the other hand, pentylenetetrazole (PTZ) acts via blockade of the GABAergic system, in particular by inhibiting the $GABA_A$ receptor-mediated inhibitory postsynaptic potentials [92]. In zebrafish, exposure to PTZ induced a concentration-dependent sequence of behavioral changes culminating in clonus-like convulsions [93], that can be inhibited by common antiepileptic drugs, such as valproate or diazepam, and different agents that block synaptic transmission, like tetrodotoxin (to block Na^+-dependent action potentials), kynurenate (a non -specific blocker of postsynaptic glutamate receptors) and a cocktail containing non-NMDA and NMDA glutamate receptors blockers [93]. Additionally, PTZ-treated adult zebrafish showed deficits in the acquisition and maintenance of passive avoidance response [73]. Furthermore, there is evidence about epilepsy and a disorder of GABA metabolism through succinic semialdehyde dehidrogenase deficiency [94]. Moreover, it has been described that PTZ and picrotoxin evoke seizure-like states were evoked in adult zebrafish [95].

These impairments in the learning of passive avoidance responses after PTZ treatment are suppressed with a valproate pretreatment [96]. More recently, it has been reported that low doses of PTZ can reverse the normal behavioral response to alternating periods of light and dark in zebrafish larvae [97].

These results, together, indicate that the GABAergic system can be impaired in zebrafish, being a relevant target for the study of the balance between neuronal circuits, and for the evaluation of antiepileptic drugs and neuroprotectants.

4.4. AD neurotoxins

The classical neurotoxins used in rodents to mimic the AD pathology have not yet been developed in zebrafish. Although the pharmacological disruption of Aβ clearance has been proposed as of great interest in AD, only one study of Aβ administration in larval zebrafish has been documented [98]. The administration of Aβ40 induced premature senescence in the vascular endothelium, impairing angiogenesis [98]. Moreover, although the effects of exogenously applied Aβ have been widely examined in rodents [99], in adult zebrafish no effect of Aβ administration has been developed or investigated to date.

Because hyperphosphorylated and aggregated forms of TAU protein are present in AD brain and cerebrospinal fluid, much recent attention has been devoted to investigating TAU in animal models [100, 101]. Experimentally, TAU hyperphosphorylation has been mimicked by okadaic acid (OA) in culture cells [102] and in animals [103]. OA is a potent inhibitor of phosphatases 1 and 2A, that induces characteristics that resemble AD-like pathology such as memory impairment accompanied by remarkable neuropathological changes including hippocampal neurodegeneration, a paired helical filament-like phosphorylation of tau protein, formation of β-amyloid containing plaque-like structures, oxidative stress and specific astroglial alterations [103, 104]. To the best of our knowledge, OA has not been evaluated in the zebrafish.

5. Transgenic zebrafish for AD

One important advantage of the zebrafish is the genome organization and the genetic pathways controlling signal transduction and development, because they are highly conserved between zebrafish and vertebrates [105]. Indeed, the zebrafish genes share as well as 50-80% homology with most human sequences and, in this respect, several genome databases similar to mouse have been generated: the Zebrafish Informartion Network (ZFIN) [106] that contains mounting genetic and phenotypic data, or the recently developed Zebrafish Neurophenome Project (ZNP) database [107] that collects the repository of neurobehavioral and related physiological phenotypes in zebrafish. For these reasons, transgenic zebrafish are continuously increasing in number and being used to model

Table 2. Genes involved in sporadic and familial AD, the homologous genes in zebrafish and the percentage of protein sequence homology.

Human Gene	Zebrafish gene	Protein homology	Reference
APP	*appa*	70%	[111]
	appb	70%	
PSEN1	*psen1*	74%	[118]
PSEN2	*psen2*	74%	[119]
Pen2	*Pen2*	74%	[129]
Aph1A&B	*Aph1*	62%	[129]
Nicastrin	*Nicastrin*	56%	[128]
TAU	*mapta/maptb*	Unidentified	[135]
APOE	*APOE*	28%	[143]

human diseases.

5.1. APP pathway

5.1.1. APP

There are two main routes of APP protein processing; (i) the predominant one is the non-amyloidogenic route and involves the enzyme called α-secretase, that excludes the generation of β-amyloid peptide [108]; (ii) the other is the amyloidogenic pathway, in which Aβ peptide is generated by the sequential action of two proteases known as β- and γ-secretase [109]. This route mainly generates two species of peptide Aβ: Aβ40 (of 40 amino acids) and Aβ42 (of 42). Although the most abundant isoform is Aβ40 (approximately 90%) the insoluble Aβ42 peptide has a greater tendency to aggregate into fibrils and therefore is more cytotoxic than the Aβ40 peptide [110].

Zebrafish have two genes homologues of human *APP* gene, *appa* and *appb* [111] (**Table 2**). The gene *appa* encodes a protein that is about 70% identical to the one encoded by *appb.* The human APP_{695} and zebrafish *appa* have approximately 70% amino acid identity, with 80% identity in the Aβ42 region, 95% identity within the transmembrane domain and 94% identity in the cytoplasmic region. The Aβ region within appb protein is 71% identical to human Aβ, 94% identical in the cytoplasmic region and 100% identical in the transmembrane region [111]. Appb more closely resembles human APP_{695} that is expressed exclusively in the mammalian brain, while the presence of the Kunitz Protease inhibiotr (KPI) domain makes appa more similar to the human APP_{770} isoform, which is expressed ubiquitously [112]. Expression of *appa* and *appb* is widespread throughout different developmental stages. These two genes show differences in their expression patterns during the segmentation period, whereas at 24 hours post-fertilization (hpf), appa and appb shared anterior expression in the telencephalon, the ventral diencephalon, the trigeminal ganglia and the posterior lateral line ganglia [111].

Regarding to APP zebrafish models, Lee and co-workers used the zebrafish *appb* promoter to express GFP in transgenic animals [113] (**Table 3**). This model revealed that appb is mainly expressed in sub regions of brain, spinal cord and the developing vasculature of the zebrafish embryos. Moreover, in adult transgenic zebrafish, appb was abundantly expressed in the brain (telencephalon, optic tectum, thalamus and cerebellum) [113]. The demonstration that zebrafish *appb* gene regulatory elements can be used to drive GFP expression in both embryos and adults will be important for future studies that focus on expressing mutant human APP in zebrafish. In this study, the authors predicted that zebrafish expressing mutant human APP will generate Aβ plaques, although did not demonstrate their presence.

In other study, Joshi and co-workers knocked down the zebrafish *appa* and *appb* function by morpholino (MO) injection resulting in a shortened body axis, a short, curly tail, mild synophthalmia and defective convergent-extension movements during gastrulation [114]. Injecting appa-MO showed no or mild defects in developing embryos whereas appb-MO caused phenotypic changes similar to those observed when both MOs were injected together. These defects can be rescued by human APP_{695} mRNA, partially rescued by human APPsα mRNA, but can-

Table 3. Zebrafish transgenic/knockdown models for AD (CNS: Central Nervous System. MO: morpholino).

Gene	Genetic characteristics	Phenotype	Reference
APP	appb promoter- GFP	appb expression in larvae and adults	[113]
	appa and appb MO	Reduced body weight & defective movements	[114]
PSEN1	psen1 MO	Somite defects & defective brain development	[120, 121]
PSEN2	psen2 MO	Altered gene expression & CNS defects	[123, 124]
TAU	Transient overexpression of 4R-Tau-GFP	TAU phosphorylation & tangle-like structures	[137]
	Human 4R/0N tau	TAU accumulation resembling tangles	[136]
	Human TAU-P301L	TAU hyperphosphorylation, tangles & neuronal death	[138]

not be rescued by APP bearing the Swedish mutation (APP_{swe}) [114]. These data suggest that APP processing plays an important role in APP function and that APP_{swe} is functionally deficient and the possibility that APP_{swe} may not only contribute to AD pathogenesis during aging but also may exert changes during embryonic development.

5.1.2. Presenilins

Presenilins (PSENs) are the most well-known proteins belonging to the catalytic subunit of the proteolytic γ-secretase complex, that cleave Notch and APP [115]. Two separate *PSEN* genes have been identified in humans, *PSEN1* and *PSEN2*, which show a high sequence homology. PSEN1 and PSEN2 are membrane spanning proteins, which are endoproteolytically cleaved into two stable fragments, one N-terminal and other C-terminal. Models of familial AD mutations have demonstrated an enhancement of APP proteolytic processing through aberrations of the γ-secretase complex [116]. These alterations generally increase the production of Aβ42 or decrease the production of Aβ40, which results in changes to the Aβ40/Aβ42 ratio [117].

Orthologues of human presenilin genes, *psen1* [118] and *psen2* [119] have been identified in zebrafish, which show a high degree of sequence identity to the human *PSEN1* and *PSEN2*, respectively (**Table 2**). Sequence comparison to human PSEN1 demonstrates that the protein encoded by the zebrafish *psen1* is 74% identical to PSEN1. Expression of *psen1* was detected in blastomeres before the onset of zygotic transcription, demonstrating maternal inheritance. Similar to human PSEN1, zebrafish presenilin-1 requires two critical aspartate residues for its function, and overexpression of zebrafish presenilin-1 in human cells promotes increased Aβ production [118].

In zebrafish, reduction of psen1 activity by MO injection leads to disruption of somite formation consistent with partial loss of Notch signaling in the presomitic mesoderm [120] (**Table 3**). Moreover, embryos injected with the psen1 MO have a shorter tail, and a smaller head. Reduction of psen1 results in the loss of Notch target gene *her6* (orthologue of human *HES1*) expression and increased expression of neurogenin 1 (ngn1) mRNA, a marker of neural progenitor cells, further indicative of reduced Notch signaling [121].

The zebrafish gene *psen2* encodes a protein that is about 74% identical to the one encoded by human *PSEN2* and 70% amino acids identity to the zebrafish psen1 [122] (**Table 2**). Alignment of the zebrafish psen2 and human PSEN2 protein sequences shows that the primary structure is highly conserved between them with only the N-terminal and the C-terminal fragments of the cytoplasmic loop domain being highly variable. [119]. Zebrafish *psen2* is ubiquitously expressed during early embryogenesis, but becomes more restricted by 24 hpf. Psen2 protein expression is initiated between 6 and 12 hpf, suggesting strict regulation of *psen2* translation. At 24 hpf, high levels of *psen2* transcript could be detected in the anterior developing CNS and eye, in the midline of the developing spinal cord, and also in cells derived from the neural crest [119].

In zebrafish, reducing psen2 expression by MO injection give rise a phenotype resembling loss of psen1 activity, included hydrocephalus and reduced pigmentation [123, 124] (**Table 3**).

Moreover, blockade of translation of zebrafish psen2 transcripts produces significant changes in embryo development including expansion of brain ventricles at 48 hpf and decreased trunk neural crest formation with increased numbers of interneurons and dorsal sensory neurons at 24 hpf [124]. A combined decrease of psen1 and psen2 activity partially reverses the effect of psen2 MO, suggesting an opposing effect of psen1 and psen2, pointing a non-redundant roles for both presenilins in zebrafish development [124].

5.1.3. Other genes of the APP pathway

Besides the PSENs, other important proteins of the γ-secretase complex are nicastrin, anterior pharynx defective 1 (APH-1), and presenilin enhancer 2 (PEN-2) [125]. Nicastrin is a glycosylated integral membrane protein that binds to both the N- and C-terminal fragments of PSENs. Suppressing nicastrin expression using siRNA results in decreased levels of PSENs, indicating that nicastrin is one of the stabilizing factors of the PSENs fragments [126]. These proteins stabilize each other upon assembly and allow for the correct trafficking of the mature complex to the cell surface and to endocytic compartments where γ-secretase exerts its activity [127].

APH-1, PEN-2 and nicastrin have been identified in zebrafish. The gene *nicastrin* has been identified and has sequenced in zebrafish by several groups, showing a 56% homology to human nicastrin [128] (**Table 2**). Zebrafish pen-2 has 74% homology to human PEN-2, and zebrafish aph-1 has 62% residues that are identical to both human homologues of APH-1 (APH-1A and APH-1B) [129]. Both zebrafish genes, *Pen-2* and *Aph-1*, are present from the fertilization, being ubiquitously expressed in the embryo at 12 hpf and, predominantly in the brain at 24 hpf [121].

However, despite conservation of the Aβ domain and of the secretases between zebrafish and humans, a zebrafish Aβ peptide has not yet been found and it is not known if the above post-translational modifications that occur in human APP processing also occur in zebrafish.

5.2. TAU

Microtubule-associated protein TAU (MAPT) plays a large role in the outgrowth of neuronal processes and the development of neuronal polarity. TAU promotes microtubule assembly, stabilizes microtubules, and affects the dynamics of microtubules in neurons [130], being abundantly present in the CNS and predominantly expressed in neuronal axons [131]. The phosphorylation of TAU regulates microtubule binding and assembly [132]. In contrast, pathological TAU becomes hyperphosphorylated, which destabilizes microtubules by decreased binding to microtubules, resulting in the aggregation of hyperphosphorylated TAU. TAU dysfunction is a major histological characteristic of AD and is common factor in a number of other neurodegenerative disorders included frontotemporal dementia with Parkinson linked to chromosome 17 (FTDP-17), corticobasal degeneration (CBD) and progressive supranuclear palsy (PSP) [133].

Recent developments strengthen the zebrafish as a model for the study of tauopathies (reviewed by [134]). In this regard, two paralogues of MAPT in zebrafish, called *mapta* and *maptb* has been identified (**Table 2**). The two proteins encoded by the zebrafish genes have not yet been detected, but both *mapta* and *maptb* mRNAs were expressed in the developing CNS [135]. A complex pattern of alternative splicing of the *mapta* and *maptb* transcripts suggests that, like human TAU, zebrafish tau isoforms with different numbers of microtubule-binding repeats are expressed in the CNS, and larger forms of tau are expressed in the PNS [135]. Moreover, the zebrafish genome contains highly conserved orthologues of each of the kinases implicated in tau phosphorylation [136].

Regarding the TAU zebrafish models, Tomasiewicz and collaborators developed a fish transiently expressing mutant human TAU fused to GFP under the control of the zebrafish neural-specific GATA-2 promoter [137] (**Table 3**). GFP-positive neurons were found in the brain, retina and spinal cord. This mutant form of human TAU expressed in zebrafish neurons produced a cytoskeletal disruption that closely resembled the neurofibrillary tangles in human disease. Further, the human TAU-GFP fusion was phosphorylated in the zebrafish brain demonstrating that human TAU could be a substrate for kinases expressed in the larval zebrafish brain.

Afterwards, Bai and co-workers developed a stable transgenic zebrafish line expressing the

4-repeat isoform of the human TAU under transcriptional control of the *eno2* promoter [136]. This stable transgenic Tg(*eno2:Tau*) zebrafish showed widespread CNS neuronal expression of TAU, at high levels compared with normal human brain. Human 4R-Tau was found within axons, neuropil and in ectopic accumulations in neuronal cell bodies of Tg(*eno2:Tau*) zebrafish.

More recently, a transgenic zebrafish line expressing the human protein TAU-P301L (a mutation genetically linked to frontotemporal dementia) in neurons by the Gal4-upstream activating-based (Gal4/UAS-based) vector system has been generated [138]. This model allows monitoring the early pathology, including disease-specific hyperphosphorylation and conformational changes of TAU, neuronal and behavioral abnormalities. Finally, the use of inhibitors of human GSK3β reduced TAU phosphorylation in this transgenic zebrafish, suggesting that this model can be used for the search of drugs that inhibit human TAU phosphorylation.

5.3. APOE

To date, the main genetic risk factor associated with the sporadic form of AD is the allele 4 of the apolipoprotein E gene (APOE-ε4) [3, 139]. Presence of one copy of the APOE-ε4 allele increases the risk to suffer AD by about three times, while two copies increase by about 12 times [139]. Furthermore, the presence of one or two copies of APOE-ε4 lead to an earlier age of onset by about 10-20 years compared with non-carriers [3]. However, the apoE allele-ε4 is neither necessary nor sufficient for disease development [140]. For this reason, although there have been numerous studies attempting to elucidate how the APOE influences AD, the underlying mechanisms of this association have not yet been deciphered [141].

A zebrafish gene homologous to mammalian *APOE* has been previously identified [142] (**Table 2**). However, deduced amino acid sequences of apoE shows only a 28% of identity with human apoE sequence [143], and a different genomic organization. The zebrafish gene consists of five exons (an additional intron that splits the last exon in two exons) instead of four in the mammalian gene. Although the homology between zebrafish apoE and human APOE is low, a region in zebrafish apoE enriched in basic amino acids is similar to the lipoprotein receptor binding domain of human apoE. ApoE is expressed in the zebrafish eyes, and some cells of the mesencephalic, telencephalic, and rhombencephalic brain areas, suggesting that it may play a significant function in the CNS [143]. Moreover, a complex *APOE* expression pattern has been demonstrated during embryonic and larval development of zebrafish [143, 144]. Functionally apoE seems to be important for zebrafish nutrition since it is expressed in the yolk syncytial layer [143], suggesting that zebrafish can be used as a simple and useful model for studying the role of apoE in brain morphogenesis and regeneration. However, to the best of our knowledge, APOE zebrafish transgenic models have not been developed to date.

6. Conclusions

In the present review, we highlight the main advances that zebrafish offers in order to understand the pathological mechanisms of Alzheimer's disease (AD). Here, we reinforce the idea that this vertebrate is an interesting tool that can be strategically incorporated into the analysis of the neurodegeneration cascade, covering the existing gap between the drug discovery in cellular models and the preclinical assays in rodents. In any case, zebrafish should be used in large scale before pharmacological validation in rodent models, especially because of its larva-adult duality. In fact, over the last years, zebrafish has emerged as an attractive model for AD-related research.

Although we have provided diverse examples to demonstrate the scope for zebrafish to model AD, several pieces of the puzzle are still lacking. For example, a better understanding of the comparative brain anatomy and physiology of adult zebrafish will be required, and more transgenic zebrafish to model an AD-like pathology are a current need. Indeed, the most important gene to build a valid AD zebrafish model is the APOE, because of this apolipoprotein is the main risk factor for AD, after aging.

Moreover, few pharmacological models causing specific neurotoxicity have been developed to date. In the future, we will need fast models of neuronal damage, in a similar way than the mouse models based in the neurotoxin injections have been historically developed. In this sense, Aβ and TAU modulation experimental

systems will be needed to decipher the mechanisms of AD pathology in zebrafish. In any case, to date, and in a similar way than the wild-type mouse models, Aβ deposits have not been found in the zebrafish brain.

In addition, a higher knowledge of the specific neuronanatomical circuits participating in the neurodegeneration must be elucidated, especially those highly related with the mammalian hippocampus, such as the zebrafish telencephalon.

We concluded that a more effort must be done to definitely optimize the zebrafish as a valid model for AD. Afterwards, the complementary use of several animal models including zebrafish, with distinct pros and contras, will help us to understand the molecular basis of the AD, and to develop novel strategies to prevent the neurodegenerative diseases.

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