REVIEW

Utilizing zebrafish and okadaic acid to study Alzheimer's disease

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Abstract

Despite the many years of extensive research using rodent models to study Alzheimer's disease (AD), no cure or disease halting drug exists. An increasing number of people are suffering from the disease and a therapeutic intervention is needed. Therefore, it is necessary to have complementary models to aid in the drug discovery. The zebrafish animal model is emerging as a valuable model for the investigation of AD and neurodegenerative drug discovery. The main genes involved in human AD have homologous counterparts in zebrafish and have conserved function. The basic brain structure of the zebrafish is also conserved when compared to the mammalian brain. Recently an AD model was established by administering okadaic acid to zebrafish. It was used to test the efficacy of a novel drug, lanthionine ketimine-5-ethyl ester, and to elucidate its mechanism of action. This demonstrated the ability of the okadaic acid-induced AD zebrafish model to be implemented in the drug discovery process for therapeutics against AD.

Key Words: *Alzheimer's disease; Zebrafish; okadaic acid; protein phosphatase 2A; learning and memory; lanthionine ketimine-5-ethyl ester*

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Introduction

The social and economic epidemic of Alzheimer's disease (AD) is upon us. It is estimated that by the year 2050 14–15 million people in the United States alone will suffer from AD and that it will cost the United States $1.1 trillion dollars in healthcare expenses. Currently, it is the 6th leading cause of death in the United States, and the only cause of death (on the top 10 list) that cannot be prevented, treated or cured. Even with it being a debilitating disease that has extreme economic and social effects, there are only five approved drugs on the market for AD. These five drugs exist in three classes: acetylcholine esterase inhibitors, a blocker of the N-methyl-d-aspartate receptor, and a combined drug of the two aforementioned classes (Alzheimer's Association, 2018).

There are hundreds of animal models that exist in aiding the study of neurodegenerative diseases and AD specifically. However, a number of these models are hindered by only exhibiting fractions of the AD pathology. Now, take into consideration the current animal models that exhibit all the molecular and behavioral pathologies. These models suffer from their limitations of cost-effectiveness and time constraints. This is where the zebrafish (*Danio rerio*) has emerged as a promising tool to study neurodegenerative diseases. Its conserved genetics enable us to establish effective, efficient, and powerful neurodegenerative models using the zebrafish. Several transgenic zebrafish models mimicking some of the pathologies seen in AD exist, but they only simulate a part of the AD pathology while memory deficiencies are seldom exhibited (Van Dam and De Deyn, 2011; Xi et al., 2011; Santana et al., 2012). A pharmacological model using adult zebrafish and the administration of okadaic acid (OKA) has recently been developed in our lab. This model involves the main pathophysiological hallmarks and behavioral conditions observed in AD while cutting down on time and cost constraints. This makes it a unique, robust, and highly effective *in vivo* AD drug screening tool.

Pathology of AD

AD is a chronic neurodegenerative disease associated with progressive cognitive decline. The two main neuropathological hallmarks associated with AD are the formation of insoluble extracellular amyloid-beta (Aβ) plaques and intracellular neurofibrillary tangles (NFTs). Neurofibrillary tangles are mainly comprised of hyperphosphorylated tau (Selkoe, 2001). The Aβ plaques are comprised of Aβ fragments Aβ39, Aβ40, Aβ42, and Aβ43 (Hamley, 2012). However in the AD brain, the plaques are mainly comprised of Aβ42 (Selkoe, 2001; Hamley, 2012). In addition to these traditionally mentioned pathological hallmarks, AD is characterized by glial activation, cerebral amyloid angiopathy, and ultimately neuronal and synaptic loss (Kumar et al., 2015).

AD is classified as being familial AD (FAD) or sporadic AD (SAD). FAD, which accounts for less than 10% of AD cases, occurs before the age of 65, and SAD occurs after the age of 65. Most cases of FAD can be attributed to genetic mutations in three genes: amyloid precursor protein (APP), presenilin 1 (PSEN1), and presenilin 2 (PSEN2). These mutations increase the ratio of Aβ42/Aβ40, therefore causing the formation of amyloid plaques (Hamley, 2012). The biggest risk factor, besides old age, in developing SAD is the possession of the ε4 allele variant

of apolipoprotein E (APOE). This causes a decrease in the clearance of Aβ fragments (Wildsmith et al., 2013).

AD commences in the entorhinal cortex which is considered to be a "gateway" to the hippocampus (Khan et al., 2014). Whenever the entorhinal cortex is affected so is the hippocampus, and it is confirmed by imaging techniques that severe atrophy in the hippocampus is suffered by patients with AD. The hippocampus plays a key role in the formation of long-term memories from short-term memories, long-term potentiation, and in spatial memory (Lindberg et al., 2012). As the disease timeline progresses, so does the outward spread of the disease into the cerebral cortex causing further problems including enhanced memory loss, decreased ability to process sound, a decrease in language comprehension, personality changes, and impairments in their executive functioning which leads to a complete loss of independence.

Use of OKA in Neurodegenerative Research

OKA has been used as a tool to study neurodegeneration across various cell types and rodent models. OKA is a selective inhibitor of protein phosphatases 1 (PP1) and protein phosphatase 2A (PP2A) (Medina et al., 2013). PP2A, specifically, is a major dephosphorylating enzyme of tau protein (Goedert et al., 1995). In AD, a decrease in PP2A expression and function is observed and evidence shows that this decrease directly attributes to the pathophysiology of AD (Rudrabhatla and Pant, 2011). When tau becomes phosphorylated it dissociates from the microtubules thereby destabilizing the microtubules. When an abnormal amount of phosphorylated tau is present in the central nervous system (CNS), they aggregate together and form toxic NFTs. Therefore, the dephosphorylation of tau by PP2A can inhibit its microtubule dissociation and eventual formation of NFTs. Many studies show that OKA generates an increase in the major tau phosphorylating kinase glycogen synthase 3beta (GSK3β), tau hyperphosphorylation, deposition of Aβ, neurodegeneration, and cognitive impairments. Other studies display that OKA also induces oxidative stress, neuroinflammation, glial activation, cholinergic dysfunction, glutamate excitotoxicity, and mitochondrial dysfunction (Kamat et al., 2013, 2014).

Modeling AD in Zebrafish

Overall, the nucleotide sequence of zebrafish genes shows about 70% homology with that of human genes, and 84% of genes that are known to be associated with human disease have a zebrafish counterpart (Kalueff et al., 2014). This supports the translational value of zebrafish models. The basic zebrafish brain structure has a high conservation when compared to the mammalian brain. The mammalian and zebrafish brain are both organized into a hindbrain, midbrain, and forebrain while further organizing them into the divisions of telencephalon, mesencephalon, metencephalon, and myelencephalon. Within these specified regions are many similarly defined areas including but not limited to the olfactory bulbs, hypothalamus, and cerebellum (Wullimann et al., 1996; Santana et al., 2012; Kalueff et al., 2014). Since AD manifests in the hippocampus of the human brain, it is important to note that the dorsal lateral pallium of the zebrafish is its homolog. The zebrafish blood-brain-barrier (BBB) is also similar in structure and function to the mammalian BBB allowing for novel neuro-drugs to be assessed for their permeability of the BBB. Zebrafish also possess the main genes involved in AD which are microtubule-associated protein tau (MAPT), APOE, APP, PSEN1, and PSEN2. The major neurotransmitter systems, such as the dopaminergic, serotoninergic, cholinergic, glutamatergic, glycinergic, and γ-aminobutyric acid (GABA)ergic systems are all present in the zebrafish brain (Santana et al., 2012). Various cognitive paradigms are also in place to test the learning and memory ability of the zebrafish. These include but are not limited to a two-chamber spatial alternation task, a three-chamber spatial alternation task, conditioned placed preference, associated learning in a plus maze, and tap-elicited startle reflex response (Williams et al., 2002; Eddins et al., 2010; Levin et al., 2011; Collier and Echevarria, 2013). In fact, many of the same behavioral tests done with rodents can be conducted on the zebrafish (Kalueff et al., 2014).

Utilizing OKA in Zebrafish

Our lab was able to develop a novel and robust AD model utilizing adult zebrafish and OKA. The advantages that this model provides are its ease of implementation and that it provides all the major molecular hallmarks of AD while maintaining cost effectiveness and efficiency. The initial development of the model comprised of testing various doses ranging from 10 nM to 1 μM OKA (with the inclusion of a control group), evaluating the expression changes of ptau, tau, Aβ fragments, pGSK3α/β, and the formation of senile plaques while also subjecting the various groups to a spatial alternation learning and memory test. The OKA is administered to the adult zebrafish by dissolving the OKA in solution and then adding OKA directly into the fish water. When compared to the controls, each of the different dosing groups had significant increases in the aforementioned tested molecular hallmarks of AD indicating that OKA does induce AD-like pathology in the zebrafish. The results of the learning and memory also demonstrated that OKA induces a cognitive decline in zebrafish. The learning and memory para-

digm is a two-choice design, and therefore 50% correct is mathematically deemed as random chance. Animals who demonstrate functional learning and memory ability in this paradigm would progress from around 50% to around 75–90% correct towards the end of the testing period. In this study, the control zebrafish were able to correctly execute the task 75% or more of the time towards the end of the testing period. Each group treated with OKA was not able to perform above 50% indicating that learning and memory were not evident. The initial testing proved that, at 100 nM OKA, the maximum number of fish survived while still inducing the major molecular hallmarks of AD (Nada et al., 2016).

An interesting observation was made during the initial dosing study. The fish that did not survive died of hemorrhaging in the brain region. As mentioned previously, it is reported that about 80% of AD patients also suffer from some form of cerebral amyloid angiopathy (CAA). CAA is characterized by the buildup of amyloid deposits in the blood vessels of the brain, and at its most severe stage, causes intracerebral hemorrhaging. It needs to be further explored if this bleeding was indeed caused by amyloid deposition in the vasculature of the zebrafish central nervous system (Nada et al., 2016).

Stemming from the initial development of the zebrafish model of OKA-induced AD, we started to utilize the model as a drug screening tool. Our first drug screening study had fish divided into 3 groups containing 8 fish per group. Group #1: Negative control, Group #2: 100 nM OKA, Group #3: 100 nM OKA + 500 μM lanthionine ketimine-5-ethyl ester (LKE). Group #3 was concomitantly treated with 100 nM OKA and 500 μM LKE. LKE is a derivative of a naturally occurring brain sulfur metabolite. LKE has exhibited neuroprotective and neurotrophic properties in murine models of neurodegeneration, but its complete mechanism of action remains to be clarified. In our study, LKE proved neuroprotective against OKA by augmenting the levels of the anti-apoptotic kinase pAkt (Ser473), brain derived neurotrophic factor (BDNF), and the transcription factor phospho-cAMP response element-binding protein (pCREB) (Ser133). This was observed in conjunction with a decrease in apoptosis in the dorsal lateral pallium. When compared to the control and 100 nM OKA groups, LKE increased the expression of BDNF, pAkt, and pCREB. A TdT-mediated dUTP nick-end labeling (TUNEL) assay analysis demonstrated that there was no difference in the number of cells undergoing apoptosis in the LKE + OKA and control groups whilst the OKA group had a significant increase in the number of cells undergoing apoptosis. When concomitantly exposed to OKA and LKE, the zebrafish were able to successfully perform the learning and memory paradigm. However, when exposed to OKA only, the fish were not able to demonstrate learning and memory. LKE is able to inhibit the cognitive impairment induced by OKA (Koehler et al., 2018).

Limitations

Outcomes discerned from zebrafish drug discovery studies are recommended to be duplicated in "higher" models such as rodents. This is because rodents are more related to humans than zebrafish. Also, even though studies have established basic conservation of brain structure and functions between zebrafish and mammals, it is important to continue to better understand the detailed brain anatomy and physiology of adult zebrafish in comparison to the mammalian brain (Santana et al., 2012; Newman et al., 2014). Also the zebrafish has the ability to regenerate neurons at a greater rate than observed in mammals (Kizil et al., 2012). This profound neurogenesis ability has raised the question if the zebrafish is a good model to study late-onset AD. However, the quick and continuous insult that OKA provides, along with the previously shown increase of the number of apoptotic cells in the zebrafish brain after administration of OKA, makes using OKA to induce neurodegeneration in the zebrafish a formidable model. There is an inherent limitation with the use of OKA to induce Alzheimer's like pathology; OKA is a PP2A inhibitor and PP2A is found in abundance throughout the body. Therefore, using OKA might induce undesirable side-effects in addition to neurotoxicity. A strength of this model is its quick turnaround time of inducing AD-like pathology, but this also provides a limitation. The pathological changes in AD manifest many years before clinical symptoms appear (Sperling et al., 2011). Therefore, the disconnect between the human timeline and the timeline of this model suggests that the exact disease progression might vary.

Conclusion and Future Directions

Given the advantages that this model provides, it promotes itself as a formidable higher throughput drug screening tool when compared to other existing models. To date, we have only published on work that involved concomitant exposure of a treatment and OKA. We are currently working on testing various drugs after the fish have already been exposed to OKA for a specific period of time. This experimental design enhances the real-life application of the drugs, as most people have already been suffering from AD before ever receiving treatment.

Also, further known AD pathologies ought to be explored in this model including but not limited to glial activation, oxidative stress, neuroinflammation, cholinergic dysfunction, and mitochondrial dysfunction. Exploring these pathologies would only further the robustness of this zebrafish model since AD has a multifaceted pathology.

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