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The GSK3β inhibitor, TDZD-8, rescues cognition in a zebrafish model of okadaic acid-induced Alzheimer's disease

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ABSTRACT

Currently, no treatments exist that are able to directly treat against Alzheimer's disease (AD), and we are facing an inevitable increase in the near future of the amount of patients who will suffer from AD. Most animal models of AD are limited by not being able to recapitulate the entire pathology of AD. Recently an AD model in zebrafish was established by using the protein phosphatase 2A inhibitor, okadaic acid (OKA). Administering OKA to zebrafish was able to recapitulate most of the neuropathology associated with AD. Therefore, providing a drug discovery model for AD that is also time and cost efficient. This study was designed to investigate the effects of GSK3β inhibition by 4-benzyl-2-methyl-1, 2, 4-thiadiazolidine-3, 5-dione (TDZD-8) on this newly developed AD model. Fish were divided into 4 groups and each group received a different treatment. The fish were divided into a control group, a group treated with 1 μM TDZD-8 only, a group treated with 1 μM TDZD-8 + 100 nM OKA, and a group treated with 100 nM OKA only. Administering the GSK3β inhibitor to zebrafish concomitantly with OKA proved to be protective. TDZD-8 treatment reduced the mortality rate, the ratio of active: inactive GSK3β, pTau (Ser199), and restored PP2A activity. This further corroborates the use of GSKβ inhibitors in the treatment against AD and bolsters the use of the OKA-induced AD-like zebrafish model for drug discovery.

1. Introduction

Alzheimer's disease (AD) is a chronic neurodegenerative disease with the two main neuropathological hallmarks being amyloid-β (Aβ) aggregates and neurofibrillary tangles (NFTs) (De-Paula et al., 2012; Villemagne et al., 2018). According to the 2018 Alzheimer's Association Report, 5.7 million Americans are suffering from Alzheimer's disease (AD) and this is expected to grow to 13.8 million Americans by the year 2050 (Association, 2018). To date, the therapeutic standards of care for AD do not modify disease progression. They merely mask the cognitive symptoms of AD and this is poorly accomplished, as the average delay of symptom progression is only 6–12 months (Massoud and Gauthier, 2010). In fact, of the top 10 causes of death in the United States, AD is the only one that cannot be prevented, cured, nor delayed.

For the near future, due to the growing number of people suffering from AD and the lack of treatment, it is apparent that novel medical interventions against AD are dire and our current methodologies for the discovery of those interventions are not sufficient. Current commonly used animal models used do not provide us with quick or cost-effective *in vivo* research designs in screening for potential drug candidates. This is where the zebrafish has been a promising emerging *in vivo* tool to model and combat AD. Zebrafish have highly conserved genetics and behavioral traits when compared to rodents. They also exhibit sensitivity to all major neurotropic drugs and respond to them in a similar fashion as humans (Kalueff et al., 2014). This marks them as an organism with high utilization in neuropharmacological research while having significantly reduced costs associated with their care. Several AD models including pharmacological (effecting cholinergic, glutamatergic, and GABAergic systems) and transgenic models (APP, PSEN1, PSEN2, and TAU) have been utilized, but none of them are able to recreate most of the molecular hallmarks and behavioral hallmarks of AD (Santana et al., 2012; Xi et al., 2011). Recently, a pharmacological model was established that was able to recapitulate both the traditional pathological hallmarks of Alzheimer's disease and cognitive decline.

Abbreviations: ANOVA, Analysis of Variance; AD, Alzheimer's disease; APP, Amyloid Precursor Protein; PSEN1, Presenilin 1; PSEN2, Presenilin 2; Aβ, Amyloid-beta; NFT, Neurofibrillary tangles; OKA, Okadaic Acid; TDZD-8, 4-benzyl-2-methyl-1, 2, 4-thiadiazolidine-3, 5-dione; PP1, Protein Phosphatase 2; PP2A, Protein Phosphatase 2A; GSK3β, Glycogen Synthase Kinase 3β; pNPP, p-Nitrophenyl Phosphate

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Exposing zebrafish to the protein phosphatase 1 (PP1) and 2A (PP2A) inhibitor, okadaic acid (OKA), resulted in memory impairments, Aβ fragment deposition, senile plaque induction, hyperphosphorylated tau protein, and cell loss (Koehler et al., 2018; Nada et al., 2016). Furthermore, this model was used to test the potential neuroprotective role of an experimental drug, lanthionine ketimine-5-ethyl ester (LKE), demonstrating this model's worth in as a screening tool in neurodegenerative drug discovery (Koehler et al., 2018).

However, this model needs to be further implemented in order to gain acceptance as a commonly used AD model. In this report, it was demonstrated that a commercially available compound, 4-benzyl-2-methyl-1, 2, 4-thiadiazolidine-3, 5-dione (TDZD-8), was effective in ameliorating the AD-like pathology that OKA induces in zebrafish. Further demonstrating the potential of this newly developed OKA-induced Alzheimer's disease model in zebrafish. Glycogen synthase kinase 3 β (GSK3β) has been a strongly studied drug target in the CNS to combat AD (Bhat et al., 2004; Churcher, 2006; Llorens-Martin et al., 2014). Utilizing GSK3β inhibitors as potential therapeutic against AD is supported by the protective effects displayed when TDZD-8 is administered to the OKA acid exposed zebrafish.

2. Materials and methods

2.1. Animals

All animal experiments were approved by the University of Toledo Health Science Campus Institutional Animal Care and Use Committee. AB zebrafish (*Danio rerio*) used in the various experiments were between the ages of twelve to fifteen months. The fish were divided into 4 groups, and each group contained a total of 12 fish at pre-treatment (6 male and 6 female). They were housed at 26–28 °C with a 14:10 h light/dark cycle with feeding twice a day. The fish were purchased from the Zebrafish International Resource Center (Eugene, OR) (Catalog ID: ZL1).

2.2. Drug treatment

Okadaic acid (OKA) sodium salt (product # O-5857) ≥98% pure was purchased from LC Laboratories (Woburn, MA, USA). The OKA was dissolved in 95% ethanol and further diluted in fish water to a concentration of 100 nM 2-methyl-4-(phenylmethyl)-1,2,4-thiadizolidine-3,5-dione (TDZD-8) (item # 16287) was purchased from Cayman Chemical (Ann Arbor, MI, USA). TDZD-8 was dissolved in 95% ethanol, and diluted in fish water to a final concentration of 1 μM. The fish were divided into 4 treatment groups; a control group, a group treated with 1 μM TDZD-8 only, a group treated with 1 μM TDZD-8 + 100 nM OKA, and a group treated with 100 nM OKA only. For the negative control group, an ethanol volume equivalent to that used to dissolve the OKA and TDZD-8 was added to the water. The final concentration of ethanol each group was exposed to was 0.014%. The exposure period lasted for 9 days and the water along with the various treatments were refreshed every other day as described previously (Massoud and Gauthier, 2010). Before and after the treatments were conducted, the fish were subject to a learning & memory function test. After the final learning and memory test, the fish were euthanized by being immersed into ice cold water (0–4 °C). The telencephalon region of the zebrafish forebrains were removed and snap frozen and stored at −80 °C until use.

2.3. Learning and memory test

Pre-treatment (learning) and post-treatment (memory) tests were performed as described previously (Williams et al., 2002). Briefly, the fish were placed into individual 10 L aquariums (N = 1 per aquarium) and assigned random numbers to assure that testing personnel were blinded. Each 10 L aquarium was filled with 26–28 °C DI/RO water with 60 mg/L of Instant Ocean® sea salt (Instant Ocean, Blacksburg, VA). Each aquarium was divided into two equal sections by a central opaque divider that allows for adequate space for the fish to swim from one side to the other side of the aquarium. One end of the aquarium is colored red as a means for visually distinguishing the two sections of the aquarium as zebrafish have the ability to see red (Nada et al., 2016). Before the test is to begin, the fish have their diet restricted for 48–72 h and are introduced to their respective aquariums for at least 48 h prior to testing. Trials were initiated with a light tap (discriminative stimulus) at the center of the aquarium. After the light tap, there was a 5 s delay followed by food presentation. To avoid satiation and to keep the fish positively motivated, only a small amount of food (approximately 5 brine shrimp nauplii) was dispensed per trial. In 20 min intervals, food presentation continued on alternating sides for a total of 28 trials (14 trials per side). A response was analyzed as correct if the fish was physically present on the side of food presentation within 5 s of the discriminative stimulus. Zebrafish are usually deemed to have learned the task when 75% of the responses are correct (Koehler et al., 2018).

2.4. PP2A activity assay

For analysis of PP2A activity, brain tissue was lysed in RIPA buffer plus 1x protease inhibitor cocktail (ThermoFisher, cat # 88266) and incubated for 30 min on ice. The samples were then centrifuged at 14000 rpm (4 °C) for 10 min, and the supernatant was mixed with phosphate buffer ((40 mM Tris–HCl, pH 8.4, 34 mM MgCl2, 4 mM EDTA and 4 mM DTT) and assayed for PP2A activity by the p-Nitrophenyl Phosphate colorimetric assay (pNPP) (Lorenz, 2011). Absorbance readings were taken at 405 nM.

2.5. Western blotting

For Western blot analysis, brain tissue was lysed in tissue extraction reagent (ThermoFisher, cat # FNN0071) plus with a 1x protease inhibitor cocktail (ThermoFisher, cat # 88266) and incubated for 30 min on ice. The samples were then centrifuged at 14000 rpm (4 °C) for 10 min, and the supernatant was assayed for protein concentration by the Bradford method (Bradford, 1976). Equal amounts of protein were mixed with reducing sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate [SDS], 10% glycerol, 0.002% bromophenol blue, and 5% β-mercaptoethanol) and boiled for 5 min at 98 °C. Proteins were subject to electrophoresis across 10–15% SDS-polyacrylamide gels and transferred to polyvinylidine difluoride (PVDF) membranes (pore size 0.45 μM, and cat. # IPVH00010 respectively). The blots were blocked for 1 h at room temperature (RT) in Tris-buffered saline blocking buffer (50 mM Tris-HCl, 150 mM NaCl) containing 5% bovine serum albumin followed by incubation overnight at 4 °C with primary antibodies for rabbit anti GSK3β (Cell Signaling, cat. #12456), rabbit anti phospho-GSK3β (Tyr216) (LifeSpan Biosciences, LS-C335917), rabbit anti phospho-GSK3β (Ser9) (Cell Signaling, cat. #9336, rabbit anti phospho-Tau (Ser199) (GenScript, A00894-40), mouse anti PP2A (Santa Cruz, sc-80665), rabbit anti β-Actin (Cell Signaling, cat. #4967), and rabbit anti α-Tubulin (Cell Signaling, cat. #2125). Finally, the blots were incubated in HRP-conjugated secondary antibody for 1 h at RT and visualized using enhanced chemiluminescence reagents (Bio-Rad, cat # 1705060).

2.6. Statistical analysis

All data are presented as means ± SEM. Western blot analysis was carried out using one-way ANOVA with a Newman-Keuls post-hoc test. A value of $p < 0.05$ was reported as significant. Zebrafish learning and memory was analyzed as previously described (Smith et al., 2010). The mathematical model for learning was formulated to measure the probability, P, of a correct response and the formula used is:

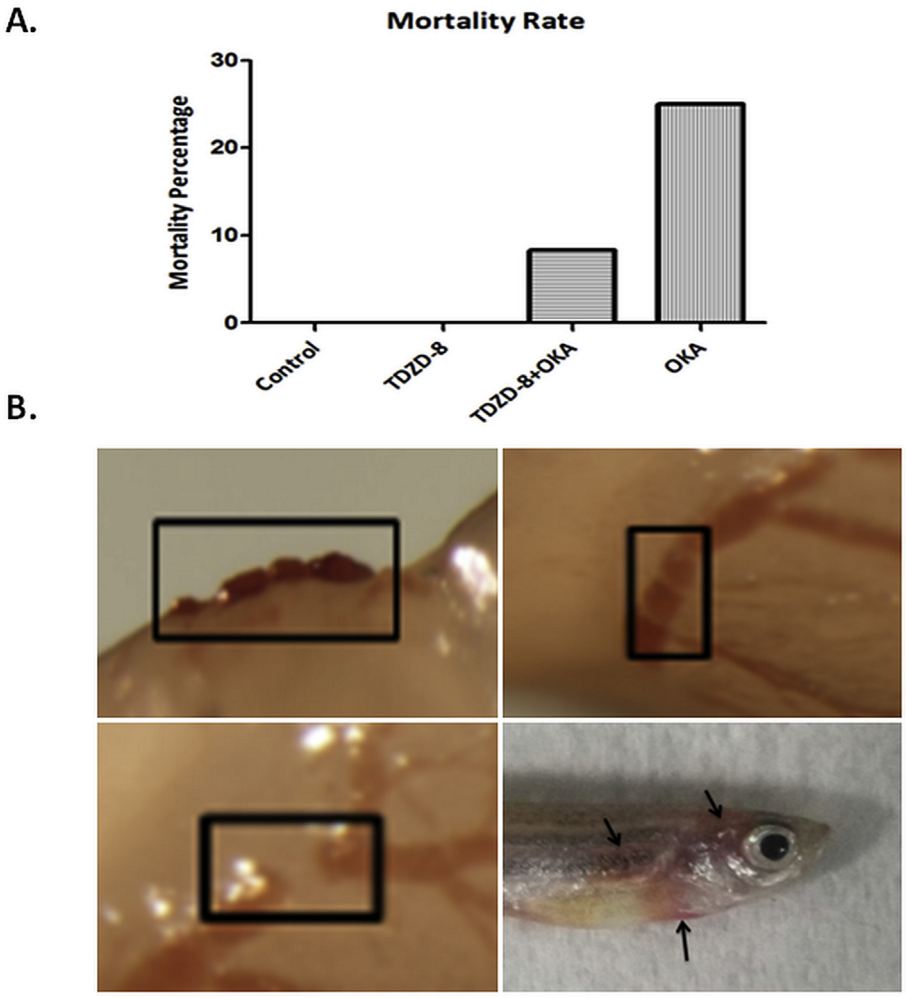

Fig. 1. Mortality rate and necropsy observations of cause of death: **A.** No fish died during the control and TDZD-8 treatments. 1 fish died during TDZD-8 + OKA treatment. 3 fish died during OKA treatment. **B.** Hemorrhage is observed to because of death for all fish that died during the experiment. Ruptured vessels are marked by black boxes and arrows.

$$P = 0.5 + \frac{b\left(\frac{t}{c}\right)^5}{1 + \left(\frac{t}{c}\right)^5}$$

Where "b" is the amount of learning that takes place, "c" is the number of trials it takes to reach half-maximum learning, and "t" is the trial number. These parameters, "b" and "c", were measured using the SAS nonlinear modeling procedure NLIN. The parameter "b" will be referenced in the paper as "maximum learning". Plots of the final prediction for P and the success frequency as functions of trial for each treated group were overlaid and used to display the fit of the estimated model.

3. Results

3.1. Treatment with TDZD-8 reduced mortality induced by OKA

Exposure to 100 nM OKA resulted in a mortality rate of 25%, while the group co-treated with 1 μM TDZD-8 + 100 nM OKA resulted in a mortality rate of 8.3% (Fig. 1A). Necropsy observations demonstrated that mortality was caused by brain and peripheral hemorrhaging (Fig. 1B).

3.2. TDZD-8 rescues the OKA induced cognition impairments

A pre-treatment test was conducted on all 4 groups, and then 10 days later, a post-treatment test was conducted. The 100 nM OKA treated zebrafish showed no ability to remember; whereas the control zebrafish, 1 μM TDZD-8 only, and the 1 μM TDZD-8 + 100 nM OKA treated zebrafish all demonstrated evidence of memory (Fig. 2). The ability for the TDZD-8 only group to demonstrate the ability to remember, exhibits that TDZD-8 at a concentration of 1 μM is not toxic to the cognitive function of the zebrafish.

3.3. Pre-treatment cognition results

The control fish demonstrated a pre-test maximum learning of about 79% which is 29% above the initial random chance of 50%. Measurement of half-maximal, which indicates a change in learning or memory of the fish, was established at around the 10th trial. The TDZD-8 only fish demonstrated a pre-test maximum learning of about 76% which is 26% above the initial random chance of 50%. Half-maximal learning was found to be at about the 8th trial. Fish treated with 1 μM TDZD-8 + 100 nM OKA had a maximum learning of about 82% which is 32% above the initial random chance of 50%. Half-maximal learning for the co-treatment group pre-test occurred around the 13th trial. Fish treated with OKA only had a maximum learning of about 85% which is 35% above the initial random chance of 50%. Half-maximal learning occurred around the 18th trial (Fig. 2A).

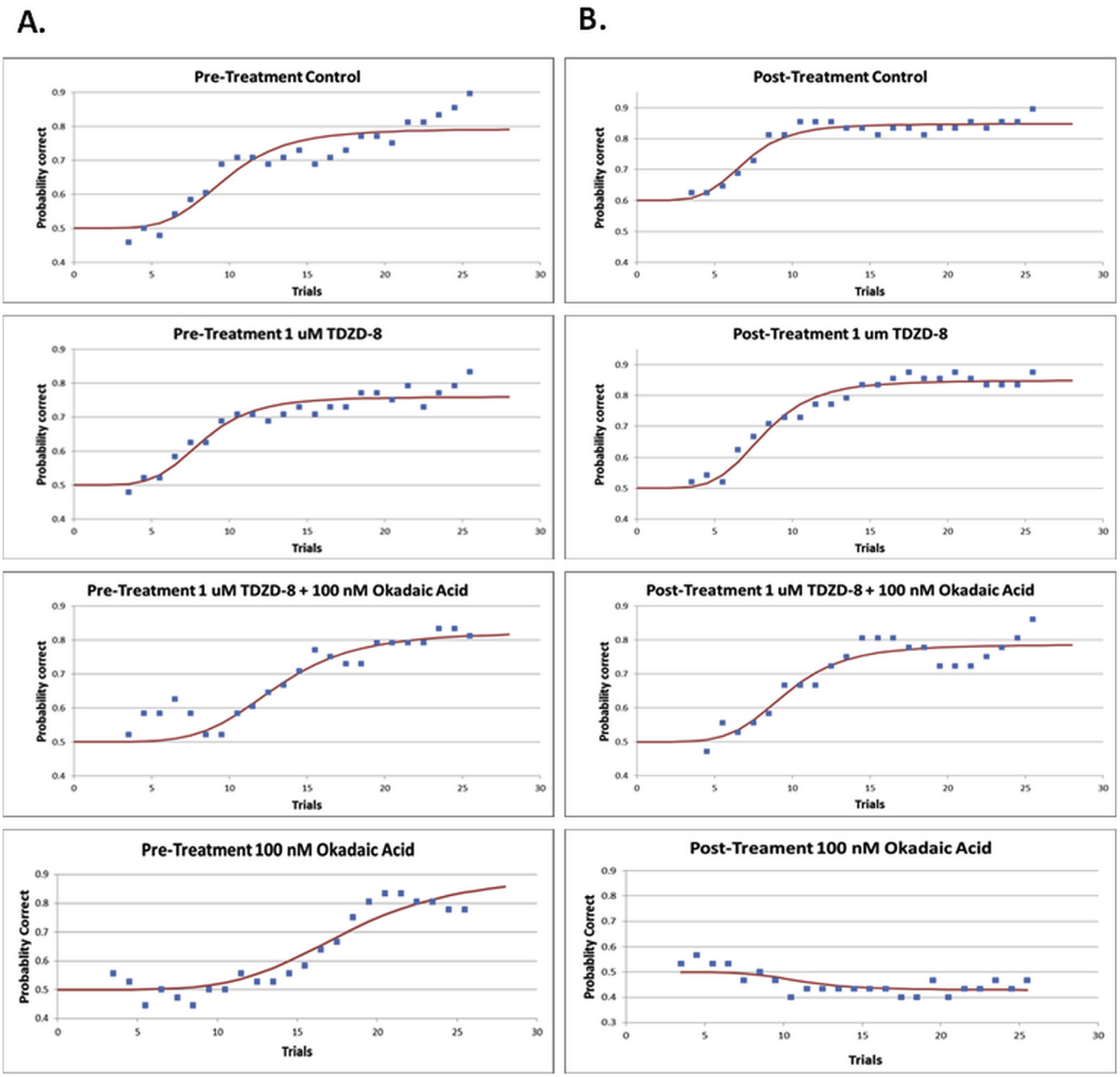

Fig. 2. Learning (pre-treatment) and memory (post-treatment) data of control, TDZD-8, TDZD-8 + OKA, and OKA treated zebrafish. The dots on each graph represent the group's running average at each trial point. The curved line represents a non-linear least-squares regression curve of the probability correct responses. **A.** Zebrafish were subject to the spatial alteration paradigm before being treated with their respective compounds. All 4 groups demonstrated the ability to learn by reaching 75% correct. **B.** After receiving their respective treatment, the zebrafish were again subject to the spatial alteration paradigm. The control group demonstrated the ability to remember by starting the behavioral task at 60% instead of the random chance probability of 50% and by reaching the 75% mark at an accelerated rate. The groups TDZD-8 and TDZD-8 + OKA demonstrated the ability to learn by reaching 75%. The OKA group did not demonstrate memory retention or the ability to learn by starting the post-treatment paradigm at random chance of 50% and never reaching a successful response of 75%. n = 12 (6 male and 6 female for pre-treatment control), n = 12 (6 male and 6 female for pre-treatment TDZD-8), n = 12 (6 male and 6 female for pre-treatment TDZD-8 + OKA), n = 12 (6 male and 6 female for pre-treatment OKA), n = 12 (6 male and 6 female for post-treatment control), n = 12 (6 male and 6 female for post-treatment TDZD-8), n = 11 (5 male and 6 female for post-treatment TDZD-8 + OKA), n = 9 (4 male and 5 female for post-treatment OKA).

3.4. Post-treatment cognition results

The control fish started the post-test at a 60% success rate which was already 10% higher than the pre-treatment test's determined random chance of 50%. The control fish performed at a maximum learning success rate of 85% and half of maximum learning (from the starting point of 60%) started at around the 7th trial. TDZD-8 only treated fish started the post-treatment test at the random success rate of 50% which was the same as the pre-treatment starting point, and performed at a maximum success rate of around 85% with half maximum performance being around the 8th trial. Fish treated with 1 μM TDZD-8 + 100 nM OKA started the post-treatment test at a 50% success rate which was the same as the pre-treatment starting point. The co-treatment group, during the post-treatment test, established a maximum success rate of about 79% and half of maximum performance was calculated to be around the 10th trial. The group of fish treated with OKA

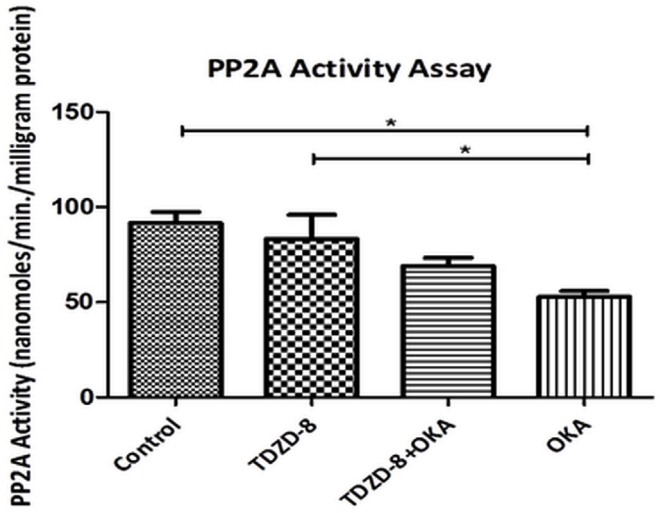

Fig. 3. Okadaic acid lowers PP2A activity in zebrafish forebrain. PP2A activity was analyzed using tissue taken from the telencephalon region of the zebrafish. PP2A activity was significantly reduced in the OKA treated zebrafish when compared to the control and TDZD-8 group. No significant difference of PP2A activity was determined between the control and TDZD-8 + OKA treated zebrafish. The bar graphs are presented as means ± SEM; *$p < 0.05$ n = 6 (3 male and 3 female for control), n = 6 (4 male and 2 female for TDZD-8), n = 5 (2 male and 3 female for TDZD-8 + OKA), n = 3 (1 male and 2 female for OKA).

only never reached a maximum performance of at least 70–75%, and therefore, it was concluded that memory was not demonstrated. Fish treated with OKA only started the post-treatment test at the random success rate of 50% and performed at a maximum success rate of around 50%. Therefore, the half maximum performance was unable to be calculated and never reached a maximum performance of at least 70–75%. Therefore it was concluded that memory was not demonstrated (Fig. 2B).

3.5. OKA treated zebrafish exhibit reduced activity of PP2A

Protein phosphatase 2A is responsible for dephosphorylating serine/threonine motifs and has been shown to be significantly decreased in patients diagnosed with AD, ultimately leading to the hyperphosphorylation of tau protein (Nicholls et al., 2016). PP2A activity in the zebrafish forebrain is significantly decreased ($p < 0.05$) by 39% when compared to the control. TDZD-8 by itself did not significantly affect the activity of PP2A. There was also no significant difference between the control and the 1 μM TDZD-8 + 100 nM OKA group, indicating that TDZD-8 treatment was able to normalize the PP2A activity (Fig. 3).

3.6. OKA treated zebrafish exhibit reduced expression of PP2A

PP2A expression is decreased by 44% ($p < 0.001$) in the OKA group when compared to the control. 1 μM TDZD-8 only and 1 μM TDZD-8 + 100 nM OKA do not alter the levels of PP2A expression when compared to the control. Compared to each respective group, PP2A expression is significantly decreased ($p < 0.001$) in the OKA group (Fig. 4A).

3.7. TDZD-8 reduces the tau kinase, GSK3β, activity: inactivity expression levels in OKA treated zebrafish

GSK3β, also known as Tau Kinase I, is a proline directed serine/threonine kinase that has been extensively studied and has strong implications in the pathogenesis of AD (Hooper et al., 2008). Its activated form, pGSKβ (Tyr216), is increased in the AD brain (Leroy et al., 2007). When compared to the control, OKA significantly increased ($p < 0.001$) the ratio of active pGSKβ (Tyr216) to inactive pGSKβ (Ser9). Compared to each respective group, the active to inactive ratio of pGSK3β is significantly increased ($p < 0.001$) in the OKA group. 1 μM of TDZD-8 does not alter the expression level of active: inactive pGSK3β when compared to the control, but 1 μM TDZD-8 when concomitantly added to 100 nM OKA seems to normalize the level of active: inactive pGSK3β back to the amount of the control (Fig. 4B).

3.8. TDZD-8 reduces the expression of phosphorylated tau in OKA treated zebrafish

Tau protein is mainly expressed in neurons and plays an essential role in stabilizing the microtubules. Phosphorylated tau disassembles from the microtubule and forms deposits correlating with cognitive decline (Chong et al., 2018; Murray et al., 2015). When compared to the control, the expression level of pTau (Ser199) in the OKA treated group was significantly increased ($p < 0.001$). The concomitant treatment of zebrafish with 1 μM TDZD-8 + 100 nM OKA normalized the pTau (Ser199) amount (Fig. 4C).

4. Discussion

In this study, it was established that TDZD-8 renders protection against OKA-induced Alzheimer's like pathology in zebrafish. These protective effects of TDZD-8 included decreased lethality, improved cognitive function, and decreased expression of several pathological hallmarks of Alzheimer's disease including p-tau and p-GSK3β. This is the first time that the effects of the GSK3β inhibitor, TDZD-8, have been demonstrated in the zebrafish OKA-induced AD model. A 1 μM dose of TDZD-8 simultaneously administered with a 100 nM dose of OKA proved to be an effectual preventive treatment against OKA-induced AD.

Currently no direct treatments for AD exist and the last FDA approved Alzheimer's drug was Namzaric® in 2014 which is not a novel drug as it is a combination pill of previously approved drugs for Alzheimer's disease. The last true new drug for Alzheimer's disease was approved in 2003 (Hung and Fu, 2017). Even though AD is an impactful disease that will continue to affect a greater number of the population in the near future, we continue to have difficulty combating this disease. A contribution to this problem is the lack of ideal animal AD models as most are only able to mimic a partial set of symptoms (Van Dam and De Deyn, 2011). Recent studies have demonstrated that when zebrafish are exposed to OKA they undergo learning and memory dysfunction and molecular changes associated with AD such as increased expression of phosphorylated tau, the deposition of Aβ-fragment, plaque formation, and cell death (Koehler et al., 2018; Nada et al., 2016).

OKA is a protein phosphatase 1 and 2A inhibitor (Bialojan and Takai, 1988). Recent therapeutic strategies for AD have shifted from reducing Aβ deposition to reducing abnormal phosphorylation of tau (Giacobini and Gold, 2013). This is influenced by the many clinical failures of Aβ directed treatments and the stronger correlation between cognitive decline and phosphorylated tau (Huber et al., 2018; Desikan et al., 2012). One of the major factors in the phosphorylation state of tau is PP2A which has been discovered to account for approximately 71% of the total tau phosphatase activity in the human brain (Goedert et al., 1995; Liu et al., 2005). Not only a key component to the phosphorylation state of tau in the healthy brain, PP2A is also implicated in the AD brain as its expression and activity levels are significantly decreased in those with AD. These changes in PP2A are thought to contribute to the hyperphosphorylation of tau and the associated cognitive decline (Liu et al., 2005; Arnaud et al., 2011; Rudrabhatla and Pant, 2011). Therefore with OKA being a PP2A inhibitor, it has been used to study various neurodegenerative diseases including AD (Valdiglesias et al., 2013; Medina et al., 2013). The administration of OKA *in vitro* and *in vivo* leads to pathologies observed in AD including Aβ deposition,

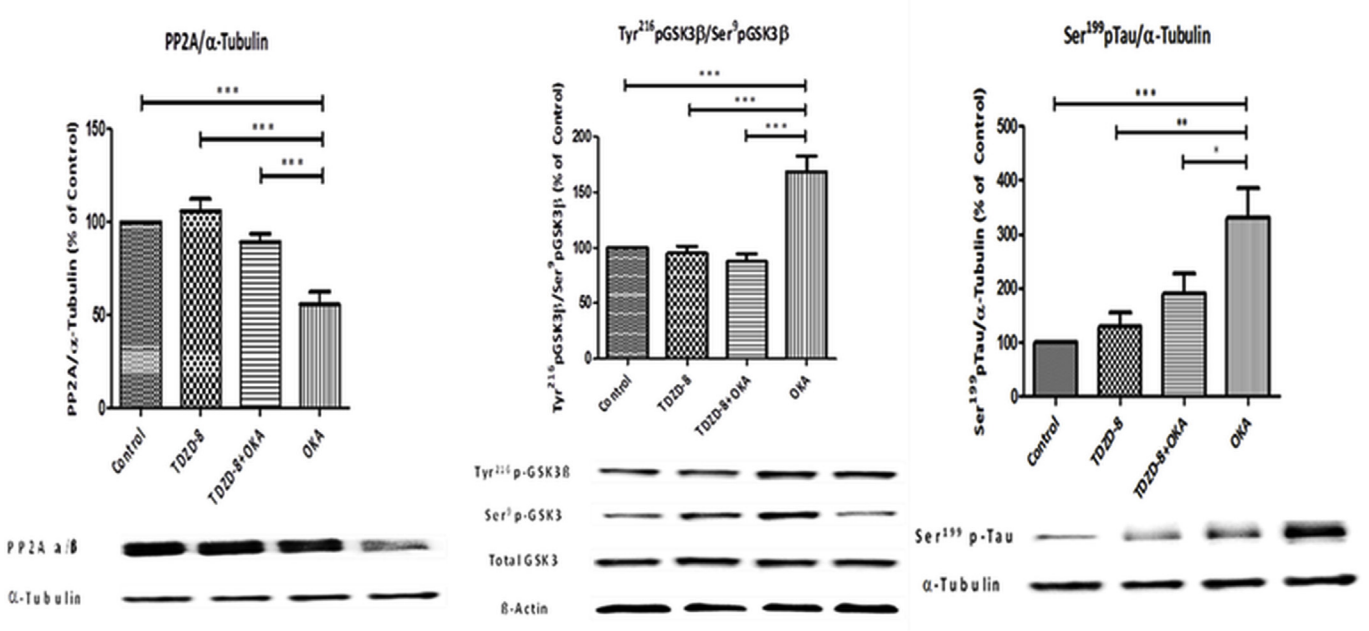

Fig. 4. TDZD-8 lowers Alzheimer's related protein expression levels increased by okadaic acid. Western blotting on tissue taken from the telencephalon region of the zebrafish forebrain are shown. **A.** Immunoblotting for PP2A shows a decrease of PP2A expression in the OKA treated group when compared to the control and the TDZD-8 group. Reduction in PP2A expression appears in the TDZD-8 + OKA group, but no significant difference was found. **B.** Immunoblotting for active pGSK3β (Tyr216) and inactive pGSK3β (Ser9) shows an increase in the ratio of active to inactive pGSK3β in OKA treated zebrafish when compared to all other groups. No difference was in the ratio of active to inactive pGSK3β between the control, TDZD-8, and TDZD-8 + OKA treated zebrafish. **C.** Immunoblotting for pTau (Ser199) shows an increase in pTau expression of OKA treated zebrafish when compared to all other groups. No difference was found in pTau expression between the control, TDZD-8, and TDZD-8 + OKA treated zebrafish. The bar graphs are presented as means ± SEM; $*p < 0.05$ $**p < 0.01$, ***p < 0.001, n = 6 (3 male and 3 female for control), n = 6 (3 male and 3 female for TDZD-8), n = 6 (3 male and 3 female for TDZD-8 + OKA), n = 6 (3 male and 3 female for OKA).

tau hyperphosphorylation, oxidative stress, inflammation, neurodegeneration, and cognitive impairments (Zhang and Simpkins, 2010a, 2010b; Tunez et al., 2003; Montilla-Lopez et al., 2002; Kamat et al., 2010). A limitation of many transgenic animal models in assessing learning and memory is the occurrence of motor dysfunction within these models. However, the use of OKA to study neurodegeneration has failed to show impairments in motor function indicating that cognitive defects are attributed to learning and memory defects and not motor dysfunction (Zhang and Simpkins, 2010b).

The effect on cognitive function of OKA, TDZD-8, and TDZD-8 + OKA was studied by a spatial alternation paradigm (Williams et al., 2002) (Fig. 2). In relation to the learning paradigm utilized, zebrafish are deemed to have learned the task if the correct choice percentage is ≥ 75%. Long-term memory is assessed in this behavioral paradigm by removing the fish from the testing apparatus for 10 days after proving their ability to learn in the initial testing phase (in this case before treatment), and then after having removed them for 10 days, they are put through the paradigm for another round of testing. Memory retention is determined by the fact that the fish begin the second testing phase above the random chance success rate of 50% and reach the ≥75% success rate in fewer trials than in the initial testing period (Williams et al., 2002).

Here the OKA only group, before treatment with OKA, demonstrated the ability to learn by reaching a maximum probability correct of 85%. Their linear regression curve resembled the pre-treatment curves of the other groups of fish. After the 9 days of OKA treatment, the OKA only group never reached a probability correct higher than 50%, indicating an inability to retain memory and proving that OKA causes a cognitive defect.

Deciphering the memory retention capacity of TDZD-8 + OKA treated fish is a bit complicated. It is apparent that their capability to learn remains, but if they retained memory of the initial testing is questionable. Their post-treatment spatial alternation task results demonstrated the ability to learn by starting the task at 50% (random chance) and performing to a maximum correct rate of 79%. This group's pre-treatment results showed a maximum learning of 82% which is higher than the maximum learning rate of the post-treatment test. However, that difference is not statistically significant. The post-treatment test indicates that half-maximal learning occurred around the 10th trial, and for the pre-treatment test half-maximal learning occurred around the 13th trial. This exhibits that the fish were able to learn at a quicker rate after the initial testing period. So it is difficult to determine if memory was retained with the treatment of TDZD-8 + OKA, but the capacity to learn unequivocally remained. Therefore it is determined that TDZD-8, when administered simultaneously with OKA, does provide protection against OKA induced cognitive impairment.

TDZD-8 is a selective non-ATP competitive glycogen synthase kinase 3β (GSK3β) inhibitor initially designed as a potential treatment of Alzheimer's disease (Martinez et al., 2002). Several reports indicate that TDZD-8 is an effective protectant against neuronal injury following ischemia and the administration of the neurotoxin 6-OHDA by increasing GSK3β (Ser9) phosphorylation and subsequently inactivating GSK3β (Collino et al., 2009; Huang et al., 2017; Xie et al., 2016). The Ser199 position of tau is phosphorylated by various kinases including GSK3β. Phosphorylation of tau at the Ser199 position might prove to be an effective biomarker for AD, and has been determined to be an early event in the development of the pathogenesis of AD (Itoh et al., 2001; Di et al., 2016; Mondragon-Rodriguez et al, 2008a, 2008b). As mentioned previously, there has been shift in the strategies in thwarting AD by targeting hyperphosphorylated tau. Consequently, inhibiting certain kinases such as GSK3β will reduce the phosphorylation of tau (Bhat et al., 2004; Churcher, 2006; Llorens-Martin et al., 2014). In the present report, TDZD-8 was able to render protection against OKA in zebrafish by decreasing the ratio of active: inactive GSK3β which decreased the amount of pTau (Ser199). Whether acted upon directly or indirectly, TDZD-8 also increased the expression and activity of PP2A which has additional potential to contribute to the decreased levels of pTau.

In summary, TDZD-8 treatment given simultaneously with OKA is able to protect against OKA induced AD pathology. TDZD-8 was able to restore cognitive dysfunction, reduce mortality, increase the expression and activity of PP2A, decrease the activity of GSK3β, and reduce the expression of pTau. It is paramount to note that the molecular analysis was done on the zebrafish forebrain, specifically the telencephalon. The telencephalon is responsible for learning and memory in teleost fish (Santana et al., 2012; Cheng et al., 2014). Showing the effectiveness of TDZD-8 in preventing OKA-induced Alzheimer's disease in zebrafish, further demonstrates the effectiveness of using the zebrafish model of OKA-induced Alzheimer's disease in drug discovery.

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