Zinc Chloride Exposure Inhibits Brain Acetylcholine Levels, Produces Neurotoxic Signatures, and Diminishes Memory and Motor Activities in Adult Zebrafish

Sreeja Sarasamma 1,2, Gilbert Audira 1,2, Stevhen Juniardi 2, Bonifasius Putera Sampurna 2, Sung-Tzu Liang 2, Erwei Hao 3,4,*, Yu-Heng Lai 5,* and Chung-Der Hsiao 1,2,6,7,*

1 Department of Chemistry, Chung Yuan Christian University, Chung-Li 32023, Taiwan; sreejakarthik@hotmail.com (S.S.); gilbertaudira@yahoo.com (G.A.)
2 Department of Bioscience Technology, Chung Yuan Christian University, No. 200, Chung-Pei Rd., Chung-Li 32023, Taiwan; stvn.jun@gmail.com (S.J.); boni_bt123@hotmail.com (B.P.S.); stliang3@gmail.com (S.-T.L.)
3 Guangxi Key Laboratory of Efficacy Study on Chinese Materia Medica, Guangxi University of Chinese Medicine, Nanning 530200, Guangxi, China
4 Guangxi Collaborative Innovation Center for Research on Functional Ingredients of Agricultural Residues, Guangxi University of Chinese Medicine, Nanning 530200, Guangxi, China
5 Department of Chemistry, Chinese Culture University, No. 55 Hwa-Kang Rd, Taipei 11114, Taiwan
6 Center for Biomedical Technology, Chung Yuan Christian University, Chung-Li 32023, Taiwan
7 Center for Nanotechnology, Chung Yuan Christian University, Chung-Li 32023, Taiwan
*
Correspondence: ewhao@163.com (E.H.); lyh21@ulive.pccu.edu.tw (Y.-H.L.); cdhsiao@cycu.edu.tw (C.-D.H.)

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Abstract: In this study, we evaluated the acute (24, 48, 72, and 96 h) and chronic (21 days) adverse effects induced by low doses (0.1, 0.5, 1, and 1.5 mg/L) of zinc chloride (ZnCl₂) exposure in adult zebrafish by using behavioral endpoints like three-dimensional (3D) locomotion, passive avoidance, aggression, circadian rhythm, and predator avoidance tests. Also, brain tissues were dissected and subjected to analysis of multiple parameters related to oxidative stress, antioxidant responses, superoxide dismutase (SOD), neurotoxicity, and neurotransmitters. The results showed that ZnCl₂-exposed fishes displayed decreased locomotor behavior and impaired short-term memory, which caused an Alzheimer’s Disease (AD)-like syndrome. In addition, low concentrations of ZnCl₂ induced amyloid beta (amyloid β) and phosphorylated Tau (p-Tau) protein levels in brains. In addition, significant induction in oxidative stress indices (reactive oxygen species (ROS) and malondialdehyde (MDA)), reduction in antioxidant defense system (glutathione (GSH), GSH peroxidase (GSH-Px) and SOD) and changes in neurotransmitters were observed at low concentrations of ZnCl₂. Neurotoxic effects of ZnCl₂ were observed with significant inhibition of acetylcholine (ACh) activity when the exposure dose was higher than 1 ppm. Furthermore, we found that zinc, metallothionein (MT), and cortisol levels in brain were elevated compared to the control group. A significantly negative correlation was observed between memory and acetylcholinesterase (AChE) activity. In summary, these findings revealed that exposure to ZnCl₂ affected the behavior profile of zebrafish, and induced neurotoxicity which may be associated with damaged brain areas related to memory. Moreover, our ZnCl₂-induced zebrafish model may have potential for AD-associated research in the future.

Keywords: zebrafish; AChE activity; zinc chloride; locomotor behavior; p-Tau; amyloid β
1. Introduction

Currently, there are more than 50 million people living with Alzheimer’s Disease (AD) in the world; this is greater than the total population of Spain and is projected to reach nearly 150 million by 2050 [1]. Loss of memory is the first symptom reported by patients suffering from AD. It was hypothesized that the pathogenesis of AD is diverse, including free-radical-mediated processes [2], dysregulated membrane metabolism [3], trace element neurotoxicity [4], or combinations of the abovementioned factors. Another study suggested that the pyruvate transporter on the inner mitochondrial membrane impacts oxidative stress and lipid metabolism, playing a significant role in the pathogenesis of AD [5]. Among these factors, one which gained significant attention is the involvement of trace elements that caused toxicity during AD progression. Previous studies showed that imbalances of trace elements in homeostasis, such as bulk copper [6], bulk iron [7], and zinc [8–10], played an important role in the formation of senile plaques in the brain.

Zinc is an essential trace element, required for the functional integrity of many organ systems, as well as for development, growth, and tissue repair [11,12]. The divalent ion forms of zinc are nutritionally essential trace elements for all living organisms, and is also a co-factor for a number of enzymes [13]. However, excessive zinc is lethal and harmful in pathological and toxicological aspects. Zinc was found as an environmental hazard in at least 985 of the 1662 waste sites proposed by the Environmental Protection Agency (EPA) National Priorities list [14]. The toxicity by zinc was reported in several fish species [15–17]. It was well documented that zinc induced embryological damage in red sea bream Pagrus major, including alterations in fecundity, low hatching rate, and high mortality [18]. Another study revealed that zinc caused toxicity in gills, which altered oxygen consumption, ammonia excretion, osmotic pressure, and the hepatopancreas in several aquatic invertebrates [19].

It was reported that zinc is neurotoxic and causes neurodegeneration in the central nervous system (CNS) [20,21]. Increases in cytosolic Zn\(^{2+}\) concentration via transport through Ca\(^{2+}\) channels triggered several downstream effects which led to neuronal cell death [22]. Despite the very low levels of free intracellular Zn\(^{2+}\), exposure of 400–600 nM Zn\(^{2+}\) results in increased toxicity [23]. Moreover, cells with over 400 nM of Zn\(^{2+}\) decreased the activity of glycolytic enzymes, such as glyceraldehyde 3-phosphate dehydrogenase (GADPH) and phosphofructokinase [24,25]. Studies also reported that intracellular free Zn\(^{2+}\) induced intracellular proton concentration that affected the function of neurons in vitro [26]. Interestingly, a number of studies revealed the significant role of heavy metals in several neurodegenerative diseases, including Alzheimer’s disease, vascular dementia, and Parkinson’s disease [21,27,28].

Reactive oxygen species (ROSs) play an important role in physiological function and are used as a biomarker of oxidative stress in toxicity studies [29,30]. Some studies already showed that the antioxidant defense system and cellular responses to oxidative stress in zebrafish were similar to mammals [31]. In the CNS, the neurotransmitter acetylcholine (ACh), involved in the cholinergic signaling system, is associated with various physiological and behavioral processes, such as motor activity, memory, and cognition, by activating metabotropic muscarinic and ionotropic nicotinic cholinergic receptors [32]. ACh is catalyzed by two cholinesterases: acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE). Zebrafish encodes only the gene for AChE, which is responsible for ACh degradation, and was already identified and functionally detected in the zebrafish brain [33]. Therefore, acetylcholinesterase (AChE) plays an active role in the nervous system [34]. Moreover, AChE is a significant biomarker for detecting environmental contaminants deposited in zebrafish [35,36]. AChE is involved in the termination of impulse exchange through rapid hydrolysis of the neurotransmitter ACh, resulting in enzyme inactivation induced by various inhibitors, and leading to disrupted neurotransmission [37]. As toxic chemicals affect non-target organisms in the aquatic biota, fish are important as bio indicators to monitor the changes in environmental toxicity compared to mammalian models.

The United States Environmental Protection Agency (USEPA) established a system as an initial screen that applied zebrafish as a model for rapid identification of chemical toxicity to evaluate
the effects from myriad chemical pollutants [38]. However, the biological effects of ZnCl₂ on neurobehavioral development in a vertebrate model are not well characterized in research (Table A1). Moreover, some reports found that genotoxic effects and gene expression changes in embryogenesis following ZnCl₂ exposure were still largely unknown. One recent study demonstrated the toxic effects of zinc in AD in a rat model, proven using histological results [20]. In addition, it was demonstrated developmental effects of ZnCl₂ during the larval stage of zebrafish [39,40]. Therefore, we sought to clarify both the short-term and long-term effects of ZnCl₂ on neurotoxicity by investigating the neurological behaviors immediately after exposure and later on by assessing the molecular parameters in adult zebrafish. In addition, there is currently no study on the effect of ZnCl₂ exposure on memory changes determined using a passive avoidance memory test, and its possible mechanisms in adult zebrafish.

The objective of this study was to determine if exposure to environmentally relevant ZnCl₂ in adult zebrafish could result in long-term impairment of brain and muscle functions, leading to neurological defects. Therefore, we used different behavioral endpoints to analyze the toxic effects of ZnCl₂, and assessed the cholinergic system by analyzing ACh and AChE activity in the zebrafish brain. Furthermore, our study was designed to assess parameters related to oxidative stress and the amino-acid neurotransmitter system after long-term exposure to ZnCl₂. Considering that long-term ZnCl₂ exposure promotes neurological dysfunction, and the cholinergic system, oxidative imbalance, and antioxidant activity are associated with neurological disorders related to human diseases, different biomarkers including oxidative and biochemical (ROS and malondialdehyde (MDA)), antioxidant parameters (glutathione (GSH), GSH peroxidase (GSH-PX), and superoxide dismutase (SOD)), neurotoxicity (ACh, AChE, amyloid β, and phosphorylated (p)-Tau), neurotransmitters (gamma-aminobutyric acid (GABA), dopamine, and melatonin), and metallothionein protein levels were studied for a better understanding of ZnCl₂-induced toxicity in the zebrafish model.

2. Results

2.1. Mortality Curve and Median Lethal Concentration for ZnCl₂ Exposure

The acute toxicity test was performed at different concentrations (2, 4, 6, 8, 10, 12, and 15 mg/L) of zinc chloride from low to high concentrations after 24, 48, 72, and 96 h of exposure (Figure 1). No deaths occurred in the untreated control group throughout the exposure experiments. Adult fish incubated at ZnCl₂ concentrations of 2 mg/L or lower showed mortality similar to the control group, while fish treated with 15 mg/L had 100% mortality after 24 h of incubation. Median lethal concentration (LC₅₀) was calculated from the percentage of fish mortality for each ZnCl₂ concentration with different exposure times (Figure A1). The 96-h LC₅₀ of ZnCl₂ was estimated around 6 ppm.

![Experimental design scheme of adult zebrafish exposed to ZnCl₂.](image-url)}
2.2. ZnCl₂ Exposure Impaired Zebrafish Three-Dimensional Locomotor Activity

The three-dimensional (3D) locomotion behavior of adult fish is a sensitive indicator for testing whether fish are suffering from external stimuli or pollution [39]. We analyzed fish 3D locomotion for five different time periods (1, 4, 24, 48, and 96 h) to demonstrate the acute changes caused by ZnCl₂ exposure. We observed that exposure to 0.5, 1.0, and 1.5 ppm ZnCl₂ resulted in a decrease in average speed (Figure 2A, p < 0.01), as well as increases in average angular velocity (Figure 2B, p < 0.01) and in meandering (Figure 2C, p < 0.001). Furthermore, treatment with 1.0 ppm ZnCl₂ for 24 h increased the time for fast latency with animals showing anxiogenic effects (Figure 2D, p < 0.01). The total distances traveled by adult zebrafish treated with ZnCl₂ were reduced (Figure 2E, p < 0.01), and the time spent in the upper zone of the tank by the animals decreased (Figure 2F, p < 0.001) at all exposure concentrations. Generally, when zebrafish are exposed to a new environment, they tend to spend more time at the tank bottom and gradually move to the upper zone after a few minutes. The time spent in the upper zone indicated an anxiolytic-like behavior [40]. Therefore, our results showed that acute ZnCl₂ exposure induced anxiety-like behavior.

2.3. ZnCl₂ Exposure Reduced Zebrafish Aggression

We tested fish aggression using the mirror-biting test. Under normal conditions, zebrafish are very aggressive, biting their mirror image [41]. In the untreated control group, we found that adult zebrafish displayed a very high percentage of time visiting/interacting with their mirror images (up to 40%, Figure 3A,B). However, ZnCl₂ exposure significantly influenced aggressive behavior as determined by the frequency of entries and time spent in the segment near the mirror images (Figure 3B–E). Quantitative data showed that ZnCl₂-treated animals displayed dose-dependent reductions in aggressive behavior compared to control fish (Figure 3A), and spent more time in the tank bottom as ZnCl₂ exposure concentration increased (Figure 3D,E; Video S1).
After being electrically shocked in the dark chamber, zebrafish could remember the pain for several days and displayed longer latency when accessing the dark chamber. We observed that short-term memory was sensitive to ZnCl$_2$ shocks to punish zebrafish to build up the passive memorial connection between the dark side and pain. The fish displayed significantly lower memory retention when exposed to very low doses of ZnCl$_2$.  

The adverse effects of zinc on memory were analyzed using the passive avoidance memory test. In the beginning, we acclimated adult zebrafish in the bright chamber after subjecting them to an experimental tank with or without ZnCl$_2$. When the separator between the light and dark sides was removed, the untreated fish immediately swam into the dark chamber. Also, we used mild electrical shocks to punish zebrafish to build up the passive memorial connection between the dark side and pain. After being electrically shocked in the dark chamber, zebrafish could remember the pain for several days and displayed longer latency when accessing the dark chamber. We observed that short-term memory was sensitive to ZnCl$_2$ exposure, and the reduction in memory retention was dose-dependent. The fish displayed significantly lower memory retention when exposed to very low doses of ZnCl$_2$ (0.1 ppm) (Figure 4).
2.5. ZnCl₂ Exposure Does Not Change Predator Avoidance Behavior

Predator avoidance is an innate behavioral response; here, we used the convict cichlid (*Amatitlania nigrofasciata*) as a predator to induce great fear/anxiety. There was no significant change in average speed (Figure 5A), predator approaching time (Figure 5B), average distance to separator (Figure 5C), or freezing/swimming time movement ratio (Figure 5D, E) between control and ZnCl₂-treated fishes. In contrast, a slightly decrease in the rapid movement time ratio was observed in the 1 ppm ZnCl₂-exposed group (Figure 5F, Video S2). This result implied that ZnCl₂ exposure at our tested concentrations did not change predator avoidance behavior in zebrafish.

2.6. ZnCl₂ Exposure Deregulated the Circadian Rhythm

The light/dark circadian test theoretically depends on the preference of staying in a dark environment, which was validated in behavioral assessment [42, 43]. When tracking zebrafish locomotion every hour, we found that untreated fish displayed active locomotion (locomotion speed between 1 and 10 cm/s) during the light cycle and sleeping-like behavior during the dark cycle (locomotion speed less than 1 cm/s) (Figure 6A, black line). However, after 1.5 ppm ZnCl₂ exposure, zebrafish displayed a reversed circadian rhythm pattern, with less locomotion activity during the light cycle, and higher activity during the dark cycle (Figure 6A, green line). We statistically summarized all locomotion patterns in the light and dark cycles with analysis of average speed during the light cycle (Figure 6C) or dark cycle (Figure 6D). Average angular velocity during the light cycle demonstrated a severe circadian deregulated effect when zebrafish were treated with 1.5 ppm ZnCl₂ (Figure 6D). Quantitation of the difference in activity revealed that, while control fish decreased their locomotor activity, the ZnCl₂-treated fish maintained almost a constant level of activity throughout the night.
By performing an ELISA assay, we found that the relative amounts of metallothionein (MT, a heavy-metal chelating protein) in the brain and gill tissues also increased significantly compared to the untreated control at ZnCl₂ exposure higher than 1 ppm. In contrast, the MT expression level in the liver tissue was low compared to muscle tissue following 1.5 ppm zinc exposure. A high level of zinc ion accumulation in the brain may induce deteriorative effects; thus, we addressed this question by measuring stress hormone levels, we found that brain catecholamine and cortisol levels were significantly elevated fivefold more than the untreated group, which indicated that a low dose of ZnCl₂ exposure was able to induce significantly high stress in fish. Next, we attempted to elucidate possible mechanisms by performing biochemical assays. No deaths were found in the treatment group and control groups after acute exposure to ZnCl₂ at low doses. However, there was a significant increase (more than 30-fold compared to the untreated control) of exogenous zinc ions in the brain after exposure to ZnCl₂ higher than 1 ppm (Figure 7A). Compared to other tissues, zinc accumulation in the brain showed the highest level (Figure A2). By performing an ELISA assay, we found that the relative amounts of metallothionein (MT, a heavy-metal chelating protein) in the brain and gill tissues also increased significantly (p < 0.001) at ZnCl₂ exposure higher than 1 ppm. In contrast, the MT expression level in the liver tissue was low compared to muscle tissue following 1.5 ppm zinc exposure (p < 0.01). A high level of zinc ion accumulation in the brain may induce deteriorative effects; thus, we addressed this question by measuring levels of oxidative species (H₂O₂ and ROS), anti-oxidative enzymes (GSH, GSH-Px, and SOD), lipid-peroxidation-related side products (MDA, thiobarbituric acid reactive substances (TBARS), and 4-hydroxynonenal (4-HNE)), DNA damage marker 8-hydroxy-2′-deoxyguanosine (8-OH-dG), and stress hormones (catecholamine and cortisol) in the brain tissue of ZnCl₂-exposed fish. Results show that oxidative species levels were elevated in the brain (Figure 7C,D). Furthermore, an excessive level of active free radicals induced lipid peroxidation, which was consistent with high levels of MDA (Figure 7E), TBARS (p < 0.001, Figure 7F), and 4-HNE (Figure 7G) in the brains of ZnCl₂-exposed fish. In addition, the persistent high oxidative stress impaired anti-oxidative capacity, which greatly reduced GSH (Figure 7I), GSH-Px (Figure 7J), and SOD (Figure 7K) enzyme activities. By measuring stress hormone levels, we found that brain catecholamine and cortisol levels were significantly elevated fivefold more than the untreated group, which indicated that a low dose of ZnCl₂ exposure was able to induce significantly high stress in fish (Figure 7L,M). The sleeping-related hormone (melatonin)
level was reduced in the brains of ZnCl\textsubscript{2}-exposed fish (Figure 7M), unlike the house-keeping protein, creatinine, which showed a constant level across tissues, providing evidence of the uniformity and consistency of protein extraction and quantification among samples (Figure 7O).

**Figure 7.** Detection of biochemical parameters in the brain. Enzymatic or ELISA-based methods were applied to detect the levels of (A) zinc ions, (B) metallothionein, (C) reactive oxygen species (ROS), (D) H\textsubscript{2}O\textsubscript{2}, (E) malondialdehyde (MDA), (F) thiobarbituric acid reactive substances (TBARS), (G) 4-hydroxynonenal (4-HNE), (H) DNA damage marker 8-hydroxy-2'-deoxyguanosine (8-OH-dG), (I) glutathione (GSH), (J) GSH peroxidase (GSH-Px), (K) superoxide dismutase (SOD), (L) catecholamine, (M) cortisol, (N) melatonin, and (O) creatinine after treatment with various concentrations of ZnCl\textsubscript{2}. Data are represented as means ± SEM of four independent experiments. Statistical analysis was compared to control using a \( t \)-test (* \( p \leq 0.05 \); ** \( p \leq 0.01 \); *** \( p \leq 0.001 \); **** \( p \leq 0.0001 \)).

2.8. ZnCl\textsubscript{2} Causes the Downregulation of Neurotransmitter and AD-Related Marker Expression

Behavioral abnormalities, such as lower memory retention, lower aggressiveness, lower locomotion activity, and circadian rhythm dysregulation in low-dose ZnCl\textsubscript{2}-exposed fish led us
to ask whether this phenotype was correlated with the downregulation of neurotransmitter and/or other AD-related markers expression. To address this question, we measured the relative content of several important neurotransmitters, such as AChE, ACh, dopamine, GABA, glutamate, glycine, and AD-related proteins like amyloid beta 42 (Aβ42) and p-Tau in the brain using ELISA assays. As shown in Figure 8A,B, AChE activity and ACh levels were detected at various concentrations (0.1, 0.5, 1, 1.5, and 5 ppm) of ZnCl₂ exposure in zebrafish brain. A significant elevation of AChE activity was detected even at a low dose of ZnCl₂ exposure at 0.1 ppm (Figure 8A). Interestingly, exposure to 1 and 1.5 ppm ZnCl₂ resulted in significantly lower ACh levels compared to control, as well as compared to 0.1 and 0.5 ppm exposure (p < 0.004, Figure 8B). Carrying out ELISA with antigen-specific antibodies, we found that dopamine (Figure 8C), glutamate (Figure 8D), and GABA (Figure 8E) levels were elevated, while glycine (Figure 8F) and histamine (Figure 8G) levels were reduced. Based on multiple behavioral endpoints measured, we hypothesized that ZnCl₂-exposed zebrafish display AD-like symptoms, including slow locomotion activity, high anxiety levels, circadian rhythm dysregulation, and lower memory retention. By measuring two AD-related biomarkers in the brain, we confirmed that ZnCl₂ exposure could significantly elevate the relative contents of Aβ42 (Figure 8H) and p-Tau (Figure 8I).

Figure 8. Detection of neurotransmitter levels in the brain. (A) Acetylcholinesterase, (B) acetylcholine, (C) dopamine, (D) glutamate, (E) gamma-aminobutyric acid (GABA), (F) glycine, (G) histamine, (H) amyloid beta 42 (Aβ42), and (I) phosphorylated (p)-Tau levels in the brain were measured using ELISA. Data are represented as means ± SEM. Statistical analysis was compared to control using a t-test (* p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001; **** p ≤ 0.0001).

3. Discussion

Zinc is widely considered as an essential trace element that is important for human health. In this study, we provided solid evidence showing that chronic ZnCl₂ exposure can induce behavioral abnormalities and neurochemical changes in adult zebrafish (Figure 9). According to previous studies, the LC₅₀ for 96 h of exposure to zinc was found to be 8.46 ppm in Fundulus heteroclitus and 1.35 ppm in white shrimp [44,45]. In addition, another study suggested that the LC₅₀ value of zinc in Fundulus heteroclitus was 9.8–75.0 ppm [46]. Our results demonstrated that the 96-h LC₅₀ in adult zebrafish was 6 ppm.
We hypothesized that minor differences in LC50 might be associated with species, developmental stage, test medium, and age. Therefore, the discrepancy observed in LC50 was still reasonable.

![Summary of the toxicological effects of ZnCl2 on cellular, locomotion, and neurobehavioral responses in adult zebrafish.](image)

**Figure 9.** Summary of the toxicological effects of ZnCl2 on cellular, locomotion, and neurobehavioral responses in adult zebrafish.

The zebrafish behavioral model is powerful, efficient, and simple for neuroscience research. Behavioral response was applied for decades as an indicator of toxicological impact within neurons [47–49]. Previous studies reported behavioral and locomotion activity alterations influenced by divalent zinc and/or other heavy metals in different species; however, only developmental toxicity of ZnCl2 in zebrafish embryos was addressed [50–52]. In contrast, our pioneer study demonstrated, for the first time, that adult zebrafish exposed to ZnCl2 had reduced locomotor behavior, average speed, entries to the upper zone, and total distance traveled. The average angular velocity and meandering of the fishes increased following 1 ppm ZnCl2 treatment compared to the control group, which was consistent with previous studies on other heavy-metal toxicity in zebrafish [53]. According to the literature research, we hypothesize that ZnCl2 exposure triggered an increase in muscle cortisol and reactive oxygen species (ROS) levels in the brains of zebrafish, which likely contributed to a stress-elicited reduction in locomotor activity [54].

Previous findings indicated that zinc was one of the potential factors causing senile plaques in the brain regions associated with AD [55]. Zinc at 300 nM showed effects of destabilizing Aβ aggregation and led to fibril formation, which caused damage in the cholinergic system. Our findings demonstrated that low concentrations (0.1 and 1 ppm) of ZnCl2 significantly impaired short-term memory in the inhibitory avoidance task, suggesting it caused significant neurotoxic effects. To investigate the mechanism related to the memory impairment induced by ZnCl2, we evaluated the activity of biomarkers, acetylcholine esterase (AChE), and acetylcholine (ACh) levels. We found that a low dose of ZnCl2 significantly elevated AChE activity and reduced ACh content in the brain tissue of zebrafish. A possible explanation is that ACh reduction may be caused by the excess hydrolysis activity of AChE, thereby altering neuromuscular activity and behavioral response. In addition, our biochemical results showed that levels of the metal-binding protein metallothionein, as well as those of ROS, glutamate, and glycine, were increased, which were associated with neuronal death and caused neurological defects [56]. Therefore, our zinc-induced zebrafish model showed potential as an AD-like model, and may provide a solid platform in neurological research.

Heavy metals are known to affect behavior, including impulsive aggression and anxiety [57–59]. In this study, to assess the effects of chronic ZnCl2 exposure on social behavior, we performed two behavioral paradigms, the mirror-biting and predator avoidance tests, and observed that the
mirror-biting time was significantly reduced by sub-chronic exposure to ZnCl₂ in adult animals (Figure 6B). Adult zebrafish responded promptly by escape and avoidance, which included freezing, erratic movements, jumping, or shoaling. Nonetheless, predator avoidance was not observed in ZnCl₂-treated animals sub-chronically in our study; instead, anxiety and fear response toward the predator was expressed in zebrafish with an elevation of cortisol and catecholamine levels in the brain. On the other hand, circadian rhythms can be regulated by circadian genes in the brain or pineal gland in several vertebrates, including zebrafish [60,61]. In our study, the 1.5 ppm ZnCl₂-exposed fish showed significantly different circadian patterns, with decreased locomotion during the light phase and increased locomotion during the dark phase. Our results showed that GABA expression levels increased and melatonin levels decreased in the brains of fish after exposure to ZnCl₂. According to a previous study, GABA signals blocked by an antagonist increased circadian rates and rhythm in suprachiasmatic nucleus (SCN) neuron cells [62]. Moreover, disturbance of the pineal melatonin secretion played a significant role in the pathogenesis of Alzheimer’s disease, which was postulated as a circadian disorder [63]. Therefore, we hypothesized that the difference during light/dark phases might be correlated with the inhibition of melatonin in the brains of treated fish, which warrants further investigation in the future.

Previous studies demonstrated that metallothionein (MT) plays an important role in metal detoxification [64] and the homeostasis of zinc and copper [65,66]. Therefore, MTs are considered as a biomarker of environmental pollution of metals, which is consistent with the induction of MTs after chronic exposure to ZnCl₂ in our study [67,68]. Furthermore, we provided a comprehensive collection of data to show that chronic ZnCl₂ exposure elevated oxidative stress, as well as lipid oxidation and reactive oxygen species (ROS) levels, which resulted in MT induction.

AD is highly associated with oxidative stress and the overproduction of amyloid Aβ, while ROS levels are significantly higher than seen in healthy normal brains [69]. Over-accumulation of metal ions, inflammation, and aggregation of amyloid Aβs contribute to the high level of ROS in the brain. H₂O₂, a major component of ROS, is produced through the Fenton reaction of amyloid Aβ with metal ions and causes the accumulation of inflammatory cytokines such as tumor necrosis factor α (TNFα) and interleukin-1β (IL-1β), which attract active plaques [70]. To investigate the possible mechanism related to memory and other behavioral deficits induced by ZnCl₂ exposure in zebrafish, we evaluated the impact of ZnCl₂ on the cholinergic system in the brain. Our results showed that adult animals exposed to ZnCl₂ had increased activity of neurotransmitters, such as GABA, dopamine, and serotonin, in the brain. Moreover, the inhibition of ACh content in the brain tissue caused behavioral abnormalities. ZnO and Al₂O₃ nanoparticles induced DNA damage in human peripheral blood lymphocytes [71]. In this study, single-stranded DNA damage was identified in the brains of ZnCl₂-treated fish. Our results showed that the brain might be very sensitive to damage at the DNA level following ZnCl₂ exposure, which impaired memory, decreased locomotion performance, and increased anxiogenic behavior, as well as inducing oxidative stress in brain. Therefore, we suggest that the oxidative stress in the CNS due to zinc exposure may induce changes in social behavior through irreversible DNA damage.

In summary, behavioral and neurotoxicological evaluations of ZnCl₂ were assessed using multiple biomarker endpoints in our model. The underlying mechanism of ZnCl₂ toxicity in adult zebrafish resulted from an increase in oxidative stress biomarker responses (ROS and H₂O₂) and a decrease in antioxidant biomarker responses (SOD, GSH, and GSH-Px). We report novel behavioral and neurotoxic effects upon exposure to divalent zinc in adult zebrafish. Our results demonstrated significant differences in neuroinflammatory effects (TNF α and IL-1β) and single-stranded DNA damage, as well as reductions in locomotion and memory, and alterations in circadian rhythm, in adult zebrafish. Moreover, our study showed that an increase in zinc could serve as a catalyst for increased ROS production, which might be important in the toxicity of amyloid Aβ and p-Tau proteins in brain pathogenesis. These deficits may result in the elevated concentration of MTs and antioxidants within brain tissue, as well as AChE expression in the cholinergic transmission system. Due to the advantage
of it being relatively easy to induce an AD-like disease model in adult zebrafish using low-dose 
ZnCl₂ exposure, our AD-like neurotoxicological model may benefit future neurodegenerative studies. 
In addition, it would be worthwhile to unveil the mechanism of Aβ aggregation and Tau pathology 
using the zinc-induced fish model and applying it to clinical research.

4. Materials and Methods

4.1. Animal Ethics

All experimental protocols and procedures involving zebrafish were approved by the Committee 
for Animal Experimentation of the Chung Yuan Christian University (Number: CYCU104024, issue 
date 21 December 2015). All experiments were performed in accordance with the guidelines for 
laboratory animals.

4.2. Zebrafish Rearing

Wild-type AB strain adult zebrafish (*Danio rerio*) were maintained in a recirculating aquatic system 
at 28.5 °C with a 10/14-h dark/light cycle according to standards. Circulating water in the aquarium 
was filtered by reverse osmosis (pH 7.0–7.5). The zebrafish were fed twice a day with lab-grown brine 
shrimp. For behavioral tests, we used adult zebrafish aged around 6–7 months.

4.3. Acute Toxicity Test

Zinc chloride (ZnCl₂) was purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Healthy 
adult zebrafish were separated into groups of 14 animals aged between six and seven months, and 
were housed in 3-L tanks prior to treatment. To determine the LC₅₀, the following ZnCl₂ concentrations 
were used: 2, 4, 6, 8, 10, 12, and 15 mg/L at 24, 48, 72 and 96 h of exposure.

4.4. Chronic Toxicity Test

The concentrations of ZnCl₂ (below 5 mg/L) were chosen according to the USEPA guidelines 
for the disposal of ZnCl₂ as an industrial effluent in the environment. Adult fish were exposed to a 
nominal concentration of 0 (control group), as well as concentrations of 0.1, 0.5, 1, and 1.5 ppm ZnCl₂ 
for 24 h, 96 h, and 21 days for acute, sub-chronic, and chronic exposures, respectively. The exposure 
water was changed daily. The zebrafish were fasted during the entire experiment’s duration. A series 
of behavioral tests (3D locomotion, predator avoidance, memory, aggression, and circadian rhythm) 
were conducted in all groups, and performance was compared between ZnCl₂-treated and control 
groups (procedures are summarized in Figure 1).

4.5. Adult Neurobehavioral Analysis

Five behavioral assays were used to assess neuron function in adult zebrafish upon systematical 
exposure to low concentrations of ZnCl₂. The test battery consisted of 3D locomotor activity (for acute 
toxicity test), passive avoidance (on day 9 of ZnCl₂ exposure), aggression (on day 9 of exposure), 
predator escape/avoidance (on day 13 of exposure), and circadian rhythm (on day 21 of exposure) 
assays which are described below.

4.6. Zebrafish 3D Locomotor Activity

Three-dimensional locomotor activity was tracked at five separate time points after exposure 
to ZnCl₂ for 1, 4, 24, 48, and 96 h. The tracking strategy for zebrafish 3D locomotion followed the 
protocol described in our previous study [39]. In each test, we routinely tested the 3D locomotion 
activity of six fish at each time point while holding them in an acrylic tank (20 × 20 × 20 cm with a 
15-cm-high water level). We tested in triplicate and had a total number of 18 fish for both the untreated 
and ZnCl₂-treated groups.
4.7. Passive Avoidance Test (Short-Term Memory Test)

To evaluate the effects of ZnCl$_2$ on short-term memory impairment in zebrafish, the passive avoidance test was carried out after 96 h of exposure, following a previous protocol with minor modification [72]. The test included two sessions with an interval of 24 h between sessions. In each session, fish were placed individually in an acrylic tank (30 × 20 × 20 cm) divided by a door into black and white compartments with equal size. In the training session, the fish were placed in the white compartment with the door closed for one minute for environmental acclimatization and recognition. Next, the door between the compartments was lifted and the fish were allowed to cross over to the dark side of the tank. When fish passed into the dark compartment the door was again closed, and a mild electric shock (25 V, 1 mA) was subjected to the fish with a 5-s interval. When fish passed into the white compartment, fish were pushed back into the dark compartment and shocked again until the fish returned into the white compartment. The training was repeated for a maximum of three times or until the fish passed to the white side. The fish that completed the training were stored in a small plastic container (15 × 10 × 5 cm) and kept at 28 °C. After 24 h, the fish were put into the training apparatus and tested for latency to pass into the dark area. The latency observed in the test session was used as an indicator of short-term memory retention.

4.8. Aggressiveness Test

Aggressive behavior was determined in adult zebrafish based on the mirror test as previously described with minor modification [73,74]. The test tank (28 × 5 × 15 cm) was filled with 1 L of water where a mirror was located at the side of the tank. One minute after the fish were introduced into the tank, aggressive behaviors (average speed, maximum and minimum speed, distance traveled, freezing, swimming, and rapid time movement percentage, mirror-biting time percentage, and longest duration in mirror side percentage) were recorded for a period of 5 min.

4.9. Predator Avoidance Test

To analyze fear-like and escaping behavior in zebrafish, the predator avoidance test was performed based on the mirror test described previously with minor modification [75]. The test tank (28 × 5 × 15 cm) was filled with 1 L of water where a transparent separator was in the middle of the tank. The predator convict cichlid (Amatitlania nigrofasciata) was put into the left area and zebrafish were put into the right area. After the fish were introduced into the tank, the predator avoidance behaviors (average speed, maximum and minimum speed, distance traveled, freezing, swimming, and rapid time movement percentage, top/bottom ratio of time spent and traveling distance, predator approaching time percentage, and distance to predator separator on average) were recorded for a period of 5 min.

4.10. Circadian Rhythm Test

To analyze zebrafish locomotion activity during the day and night, we performed a circadian rhythm test based on a previously published method with some modifications [76]. To prevent any external disturbance, the tested fish were moved into a temperature-controlled incubator and kept at 28 °C. One specially designed light-emitting diode (LED) light box with conventional and infrared LED arrays was used as the bottom light source. One infrared-sensitive charge-coupled device (CCD; detection window: 700–1000 nm) with a maximum resolution of 1920 × 1080 pixels and a 30-fps frame rate was used for video recording (3206_1080P module, Shenzhen, China). In this experiment, we recorded zebrafish locomotion activity (average speed, average angular velocity, and meandering) for 1 min every hour and used the idTracker software to track fish movement trajectories according to our previously published method [39].
4.11. Biochemical Parameters

4.11.1. Tissue Preparation and Total Protein Determination

After performance of the behavioral experiments, fish were euthanized with a high concentration of 200 mg/L tricaine solution (MS-222). For each independent assay, the whole brain and tissue from the caudal peduncle were extracted. A pool of three zebrafish tissues was used to prepare an independent homogenate preparation for each sample, which was homogenized on ice in 50 volumes (v/w) of phosphate-buffered saline (PBS) at pH 7.2. Samples were then centrifuged at 15,000 rpm for 15 min at 4 °C, and the supernatant was kept in microtubes at −80 °C for further assays. Total protein concentration was determined using a Pierce BCA Protein Assay Kit (23225, Thermo Fisher Scientific, Waltham, MA, USA). The color formation was analyzed at 562 nm using a microplate reader (Multiskan GO, Thermo Fisher Scientific, Waltham, MA, USA).

4.11.2. Analysis of Zinc and Metallothionein (MT) Content in Tissues

Concentrations of zinc in the brain and other tissues were determined using a zinc calorimetric quantification kit (E011, Nanjing Jiancheng Bio-Engineering Institute Co., Ltd, Nanjing, China). Tissue samples were extracted and prepared using PBS according to the method described in the above section. Calibration curves were prepared using serial dilutions of stock solutions of 100 mg/L zinc standard. The color formation was analyzed at 630 nm using a microplate reader (Multiskan GO, Thermo Fisher Scientific, Waltham, MA, USA). A fish metallothionein (MT) ELISA kit was used to measure total MT in tissues according to the manufacturer’s instructions (ZG-E1562, Zgenebio Company, Taipei, Taiwan). The color change was measured spectrophotometrically at a wavelength of 450 nm. MT content was expressed as pg/mL.

4.11.3. Determination of Acetylcholine and Acetylcholinesterase in Brain Samples

ZnCl₂-treated zebrafish were anesthetized and euthanized using 200 mg/L tricaine. The whole-brain tissue was homogenized on ice in 50 volumes (v/w) of PBS at pH 7.2 using a bullet blender tissue homogenizer (Next Advance, Inc., Troy, NY, USA). For the AChE activity analysis, a pool of brain tissue from three individual fish was considered as one sample. The tests were performed in triplicate using a total of nine fish per group to ensure consistency. The activity of the homogenate was measured using an AChE calorimetric kit (A024, Nanjing Jiancheng Bio-Engineering Institute Co., Ltd., Nanjing, China) in accordance with the instructions. Briefly, 10 µL of brain homogenate was placed onto the plate. Then, 100 µL of working solution containing AChE enzyme, assay buffer, dye reagent, and enzyme mix was added into each well. After incubation for 15 min, the color formation was analyzed at 412 nm using a microplate reader (Multiskan GO, Thermo Fisher Scientific, Waltham, MA, USA) and the AChE activity was expressed in µmol. ACh levels were determined using an ACh ELISA kit (ZG-E1585, Zgenebio Company, Taipei, Taiwan) according to the specifications of the manufacturer. The absorbance was analyzed at 450 nm using a microplate reader (Multiskan GO, Thermo Fisher Scientific, Waltham, MA, USA). Data were expressed as U/mg of total protein.

4.11.4. Quantification of Hydrogen Peroxide and Reactive Oxygen Species Levels in Brain Samples

Hydrogen peroxide (H₂O₂) (ZG-E1602, Zgenebio Company, Taipei, Taiwan) and the reactive oxygen species (ROS) ELISA kit (ZG-E1561, Zgenebio Company, Taipei, Taiwan) were applied to detect their respective components according to the manufacturer’s instructions, and absorbance was determined at 450 nm. These assay kits are based on the sandwich ELISA method that involves a specific antibody for the detection of the chemicals of interest.
4.11.5. Determination of MDA, TBARS, 4-HNE, 8-OH-dG, Catecholamine, and Cortisol Levels in Brain Samples

Under oxidative stress, levels of biomarkers for lipid peroxidation (malondialdehyde, TBARS, and 4-HNE), DNA damage marker 8-OH-dG, and stress hormones catecholamine and cortisol were measured in the brain homogenate samples. The production of MDA, TBARS, 4-HNE, and 8-OH-dG in the samples was measured using a commercial ELISA kit (ZG-E1592, ZG-E1605, ZG-E1603 and ZG-E1564, Zgenebio Company, Taipei, Taiwan) according to the manufacturer’s protocol. The levels of catecholamine (CA) and cortisol in the brain homogenate were determined using an ELISA kit (ZG-E1590 and ZG-E1575, Zgenebio Company, Taipei, Taiwan) according to the manufacturer’s protocol. The color change was measured spectrophotometrically at a wavelength of 450 nm.

4.11.6. Determination of Antioxidant Capacity in Brain Samples

The exposure of fish to ZnCl$_2$ promotes the production of glutathione (GSH), glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD) in the fish brain, and the relative amounts of each were measured using specific ELISA kits (ZG-E1622, ZG-E1601, ZG-E1604, Zgenebio Company, Taipei, Taiwan). The color change was measured spectrophotometrically at a wavelength of 450 nm.

4.11.7. Evaluation of Neurotransmitter Levels in Brain Samples

Levels of neurotransmitters (GABA, glutamate, glycine, dopamine, 5-hydroxytryptamine (5-HT), norepinephrine, histamine and melatonin) in the fish brain were determined using commercially available ELISA kits (ZG-E1574, ZG-E1588, ZG-E1587, ZG-E1753, ZG-E1572, ZG-E1571, ZG-E1586, ZG-E1597, Zgenebio Company, Taipei, Taiwan) according to the manufacturer’s instructions. The color change was measured spectrophotometrically at a wavelength of 450 nm.

4.11.8. Estimation of Amyloid Beta (Aβ-42) and p-Tau Levels in the Brain

Aβ42 and p-Tau are two markers for Alzheimer’s Disease, and their concentrations in the brain tissues were detected using ELISA kits (ZG-E1607 and ZG-E1618, Zgenebio Company, Taipei, Taiwan) according to the manufacturer’s instructions. The absorbance was analyzed at 450 nm using a microplate reader (Multiskan GO, Thermo Fisher Scientific).

4.11.9. Statistical Analysis

For statistical analysis, the experimental values were compared between groups and data were expressed as means ± standard error of the mean (SEM). The Prism software (Graph pad Software version 7, La Jolla, CA, USA) was used to determine significant differences between treated and control groups. Data were compared using the t-test, ANOVA, or non-parametric test, depending on data normality for significance determination. A significant difference was considered to be $p < 0.001$.

**Supplementary Materials:** The following are available online at [http://www.mdpi.com/1422-0067/19/10/3195/s1](http://www.mdpi.com/1422-0067/19/10/3195/s1), Video S1: Mirror-biting assay of adult zebrafish after exposure to different concentrations of ZnCl$_2$. Video is sped up $10 \times$; Video S2: Predator avoidance test of adult zebrafish after exposure to different concentrations of ZnCl$_2$. Video is sped up $10 \times$; Video S3: Circadian rhythm test of adult zebrafish after exposure to 1.5 ppm ZnCl$_2$. Video is sped up $5 \times$.

**Author Contributions:** E.H., Y.H.L. and C.D.H. conceived and designed the study. S.S., G.A., and S.J. performed the experiments. G.A., S.J. and B.P.S. analyzed the data. S.S. and S.T.L. wrote the paper. E.H., Y.H.L. and C.D.H. reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Appendix A

Figure A1. LC50 test of ZnCl2 in adult zebrafish. Adult zebrafish were incubated with different concentrations of ZnCl2 (2, 4, 6, 8, 10, 12, and 15 ppm) and the fish number was recorded every day to build up the mortality curve. LC50 curves at 24, 48, 72 and 96 h are labeled in blue, red, green, and purple, respectively.

Figure A2. Comparison of zinc levels in different tissues after chronic exposure to ZnCl2 at different concentrations for 21 days. Adult zebrafish were incubated with different concentrations of ZnCl2, and the relative amounts of zinc accumulation in (A) brain, (B) muscle, (C) gill, and (D) liver tissues were measured using enzymatic assays. Data are represented as means ± SEM. Statistical analysis was compared to control using a t-test (**p ≤ 0.01; ****p ≤ 0.0001).

Table A1. Comparison of present study with other animal models exposed to ZnCl2.

| Toxicant | Model Organism | Concentration | Study Behavior | LC50 | References |
|----------|----------------|---------------|----------------|------|------------|
| ZnCl2    | Mice           | 7.5, 10, 15 mg/kg | In vivo | No Cytotoxicity, head abnormalities | NA [77] |
| ZnCl2    | Neuronal cells | 0.15 mmol/L    | In vitro | No Loss of viability | NA [78] |
| ZnCl2 + ZnO | MDKC cells | 100 μg/mL | In vitro | No Cytotoxicity, genotoxicity | NA [79] |
| ZnCl2    | Mice           | 10 ppm         | In vivo | Yes Depressive Behavior | NA [80] |
| Zinc     | Mice equimolar | In vitro | No | Stimulate hippocampal microglia activation | NA [20] |
| ZnCl2    | D. magna      | 0.16 ppm       | In vivo | No | Toxic effects on growth and reproduction | NA [81] |
| ZnCl2 + ZnO | C. elegans larvae | 614 μM | In vivo | No Genotoxicity, Apoptosis | NA [82] |
Table A1. Comparison of present study with other animal models exposed to ZnCl₂.

| Toxicant | Model Organism | Concentration | Study | Behavior Endpoints | Toxic Effects | LC₅₀ | References |
|----------|----------------|---------------|-------|--------------------|--------------|------|------------|
| ZnCl₂    | Mice           | 7.5, 10, 15 mg/kg | In vivo | No        | Cytotoxicity, head abnormalities | NA     | [77]       |
| ZnCl₂    | Neuronal cells | 0.15 mmol/L | In vitro | No | Loss of viability | NA | [78] |
| ZnCl₂ + ZnO | MDKC cells      | 100 µg/ml. | In vitro | No | Cytotoxicity, genotoxicity | NA | [79] |
| ZnCl₂    | Mice           | 10 ppm       | In vivo | Yes        | Depressive Behavior | NA     | [80] |
| Zinc     | Mice           | equimolar     | In vitro | No | Stimulate hippocampal microglia activation | NA | [20] |
| ZnCl₂    | D. magna       | 0.16 ppm     | In vivo | No | Toxic effects on growth and reproduction | NA | [81] |
| ZnCl₂ + ZnO | C. elegans larvae | 614 µM | In vivo | No | Genotoxicity, Apoptosis | NA | [82] |
| Zinc     | Red sea bream  | 2.5 ppm      | In vivo | No | Toxic effects on development and growth | 4.3 ppm | [18] |
| ZnCl₂    | Zebrafish embryo | 0.5 ppm | In vivo | No | Skeletal anomalies | NA     | [83] |
| ZnCl₂    | Zebrafish embryo | 0.1–0.5 ppm | In vivo | No | Developmental deformities | NA | [84] |
| This study Low concentration of ZnCl₂ | Adult zebrafish | 0.1–1.5 ppm | In vivo | Yes, we used 5 behavioral parameters | Memory impairment, locomotor deficit, and high AChE content in brain | 6 ppm | This study |

References

1. Martins, R.N.; Villemagne, V.; Sohrabi, H.R.; Chatterjee, P.; Shah, T.M.; Verdile, G.; Fraser, P.; Taddei, K.; Gupta, V.B.; Rainey-Smith, S.R. Alzheimer’s Disease: A Journey from Amyloid Peptides and Oxidative Stress, to Biomarker Technologies and Disease Prevention Strategies—Gains from AIBL and DIAN Cohort Studies. *J. Alzheimers Dis.* 2018, 62, 965–992. [CrossRef] [PubMed]
2. Markesbery, W.R. Oxidative stress hypothesis in Alzheimer’s disease. *Free Radic. Biol. Med.* 1997, 23, 134–147. [CrossRef]
3. Nitsch, R.M.; Blusztajn, J.K.; Pittas, A.G.; Slack, B.E.; Growdon, J.H.; Wurtman, R.J. Evidence for a membrane defect in Alzheimer disease brain. *Proc. Natl. Acad. Sci. USA* 1992, 89, 1671–1675. [CrossRef] [PubMed]
4. Lovell, M.A. Zinc and Zinc Transport and Sequestration Proteins in the Brain in the Progression of Alzheimer’s Disease. In Neurochemical Mechanisms in Disease; Springer: Berlin, Germany, 2011; pp. 669–693.
5. Divakaruni, A.S.; Wiley, S.E.; Rogers, G.W.; Andreyev, A.Y.; Petroseyan, S.; Loviscach, M.; Wall, E.A.; Yadava, N.; Heuck, A.P.; Ferrick, D.A. Thiazolidinediones are acute, specific inhibitors of the mitochondrial pyruvate carrier. *Proc. Natl. Acad. Sci. USA* 2013, 110, 5422–5427. [CrossRef] [PubMed]
6. Tandon, L.; Ni, B.-F.; Ding, X.; Ehmnn, W.; Kasarskis, E.; Markesbery, W. RNAA for arsenic, cadmium, copper, and molybdenum in CNS tissues from subjects with age-related neurodegenerative diseases. *J. Radioanal. Nucl. Chem.* 1994, 179, 331–339. [CrossRef]
7. Thompson, C.; Markesbery, W.; Ehmnn, W.; Mao, Y.; Vance, D. Regional brain trace-element studies in Alzheimer’s disease. *Neurotoxicology* 1988, 9, 1–7. [PubMed]
8. Lovell, M.; Robertson, J.; Teesdale, W.; Campbell, J.; Markesbery, W. Copper, iron and zinc in Alzheimer’s disease senile plaques. *J. Neurol. Sci.* 1998, 158, 47–52. [CrossRef]
9. DeGrado, T.R.; Kemp, B.J.; Pandey, M.K.; Jiang, H.; Gunderson, T.M.; Lindscheid, L.R.; Woodwick, A.R.; McConnell, D.M.; Fletcher, J.G.; Johnson, G.B. First PET imaging studies with 63Zn-zinc citrate in healthy human participants and patients with Alzheimer disease. *Mol. Imaging* 2016, 15. [CrossRef] [PubMed]
10. Butterfield, D.A.; Swomley, A.M.; Sultana, R. Amyloid β-peptide (1–42)-induced oxidative stress in Alzheimer disease: Importance in disease pathogenesis and progression. *Antioxid. Redox Signal.* 2013, 19, 823–835. [CrossRef] [PubMed]
11. Murgia, C.; Lang, C.; Truong-Tran, A.; Grosser, D.; Jayaram, L.; Ruffin, R.; Perozzi, G.; Zalewski, P. Zinc and its specific transporters as potential targets in airway disease. *Curr. Drug Targets* 2006, 7, 607–627. [CrossRef] [PubMed]

12. Zalewski, P.D. Zinc metabolism in the airway: Basic mechanisms and drug targets. *Curr. Opin. Pharmacol.* 2006, 6, 237–243. [CrossRef] [PubMed]

13. Zastrow, M.L.; Pecoraro, V.L. Designing hydrolytic zinc metalloenzymes. *Biochemistry* 2014, 53, 957–978. [CrossRef] [PubMed]

14. Fay, M. Exposure to contaminant mixtures at US hazardous waste sites. *WIT Trans. Ecol. Environ.* 2005, 85. [CrossRef]

15. Stankevičiūtė, M.; Sauliutė, G.; Svecevičius, G.; Kazlauskienė, N.; Baršienė, J. Genotoxicity and cytotoxicity response to environmentally relevant complex metal mixture (Zn, Cu, Ni, Cr, Pb, Cd) accumulated in Atlantic salmon (*Salmo salar*). Part I: Importance of exposure time and tissue dependence. *Ecotoxicology* 2017, 26, 1051–1064. [CrossRef] [PubMed]

16. Sharaf, M.S.; Stevens, D.; Kamunde, C. Zinc and calcium alter the relationship between mitochondrial respiration, ROS and membrane potential in rainbow trout (*Oncorhynchus mykiss*) liver mitochondria. *Aquat. Toxicol.* 2017, 189, 170–183. [CrossRef] [PubMed]

17. Liu, L.; Xiao, Y.-Y.; Ji, Y.-H.; Liu, M.-Z.; Chen, Y.; Zeng, Y.-L.; Zhang, Y.-G.; Jin, L. CuInS$_2$/ZnS QD exposure induces developmental toxicity, oxidative stress and DNA damage in rare minnow (*Gobiocypris rarus*) embryos and larvae. *Comp. Biochem. Physiol. Part C Toxicol. Pharmacol.* 2017, 198, 19–27. [CrossRef] [PubMed]

18. Huang, W.; Cao, L.; Shan, X.; Xiao, Z.; Wang, Q.; Dou, S. Toxic effects of zinc on the development, growth, and survival of red sea bream *Pagrus major* embryos and larvae. *Arch. Environ. Contam. Toxicol.* 2010, 58, 140–150. [CrossRef] [PubMed]

19. Elumalai, M.; Antunes, C.; Guilhermino, L. Enzymatic biomarkers in the crab *Carcinus maenas* from the Minho River estuary (NW Portugal) exposed to zinc and mercury. *Chemosphere* 2007, 66, 1249–1255. [CrossRef] [PubMed]

20. Lee, M.-C.; Yu, W.-C.; Shih, Y.-H.; Chen, C.-Y.; Guo, Z.-H.; Huang, S.-J.; Chan, J.C.; Chen, Y.-R. Zinc ion rapidly induces toxic, off-pathway amyloid-β oligomers distinct from amyloid-β derived diffusible ligands in Alzheimer’s disease. *Sci. Rep.* 2018, 8, 4772. [CrossRef] [PubMed]

21. Yang, T.C.; Wu, P.c.; Chung, I.f.; Jiang, J.H.; Fann, M.J.; Kao, L.S. Cell death caused by the synergistic effects of zinc and dopamine is mediated by a stress sensor gene Gadd45b–implication in the pathogenesis of Parkinson’s disease. *J. Neurochem.* 2016, 139, 120–133. [CrossRef] [PubMed]

22. Cai, A.L.; Zipfel, G.J.; Sheline, C.T. Zinc neurotoxicity is dependent on intracellular NAD$^+$ levels and the sirtuin pathway. *Eur. J. Neurosci.* 2006, 24, 2169–2176. [CrossRef] [PubMed]

23. Choi, D.W.; Koh, J.Y. Zinc and brain injury. *Annu. Rev. Neurosci.* 1998, 21, 347–375. [CrossRef] [PubMed]

24. Ikeda, T.; Kimura, K.; Morioka, S.; Tamaki, N. Inhibitory effects of Zn$^{2+}$ on muscle glycolysis and their reversal by histidine. *J. Nutr. Sci. Vitaminol.* 1980, 26, 357–366. [CrossRef] [PubMed]

25. Krotkiewska, B.; Banas, T. Interaction of Zn$^{2+}$, and Cu$^{2+}$, Ions with glyceraldehyde-3-phosphate dehydrogenase from bovine heart and rabbit muscle. *Int. J. Biochem.* 1992, 24, 1501–1505. [CrossRef]

26. Dineley, K.; Brocard, J.; Reynolds, I. Elevated intracellular zinc and altered proton homeostasis in forebrain neurons. *Neuroscience* 2002, 114, 439–449. [CrossRef]

27. Conte-Daban, A.; Day, A.; Fallar, P.; Hureau, C. How Zn can impede Cu detoxification by chelating agents in Alzheimer’s disease: A proof-of-concept study. *Dalton Trans.* 2016, 45, 15671–15678. [CrossRef] [PubMed]

28. Tanaka, K.-I.; Kawahara, M. Copper enhances zinc-induced neurotoxicity and the endoplasmic reticulum stress response in a neuronal model of vascular dementia. *Front. Neurosci.* 2017, 11, 58. [CrossRef] [PubMed]

29. Du, Z.; Zhu, L.; Dong, M.; Wang, J.; Wang, J.; Xie, H.; Liu, T.; Guo, Y. Oxidative stress and genotoxicity of the ionic liquid 1-octyl-3-methylimidazolium bromide in zebrafish (*Danio rerio*). *Arch. Environ. Contam. Toxicol.* 2014, 67, 261–269. [CrossRef] [PubMed]

30. Costa-Silva, D.; Nunes, M.M.; Wallau, G.; Martins, I.; Zemolin, A.P.; Cruz, L.; Rodrigues, N.; Lopes, A.; Posser, T.; Franco, J. Oxidative stress markers in fish (*Astyanax* sp. and *Danio rerio*) exposed to urban and agricultural effluents in the Brazilian Pampa biome. *Environ. Sci. Pollut. Res.* 2015, 22, 15526–15535. [CrossRef] [PubMed]
31. Mohanty, R.; Das, S.K.; Patri, M. Modulation of benzo[a]pyrene induced anxiolytic-like behavior by retinoic acid in zebrafish: Involvement of oxidative stress and antioxidant defense system. *Neurotox. Res.* 2017, 31, 493–504. [CrossRef] [PubMed]

32. Lawrence, J.J. Cholinergic control of GABA release: Emerging parallels between neocortex and hippocampus. *Trends Neurosci.* 2008, 31, 317–327. [CrossRef] [PubMed]

33. Bertrand, C.; Chatonnet, A.; Takke, C.; Yan, Y.; Postlethwait, J.; Toutant, J.-P.; Cousin, X. Zebrafish acetylcholinesterase is encoded by a single gene localized on linkage group 7 gene structure and polymorphism; molecular forms and expression pattern during development. *J. Biol. Chem.* 2001, 276, 464–474. [CrossRef] [PubMed]

34. Audira, G.; Sampurna, B.P.; Juniardi, S.; Liang, S.-T.; Lai, Y.-H.; Hsiao, C.-D. A simple setup to perform 3D locomotion tracking in zebrafish by using a single camera. *Inventions* 2018, 3, 11. [CrossRef] [PubMed]

35. Azizullah, A.; Richter, P.; Häder, D.-P. Comparative toxicity of the pesticides carbofuran and malathion to the freshwater flagellate *Euglena gracilis*. *Ecotoxicology* 2011, 20, 1442–1454. [CrossRef] [PubMed]

36. Garaventa, F.; Gambardella, C.; Di Fino, A.; Pittore, M.; Faimali, M. Swimming speed alteration of *Artemia* sp. and *Brachionus plicatilis* as a sub-lethal behavioural end-point for ecotoxicological surveys. *Ecotoxicology* 2010, 19, 512–519. [CrossRef] [PubMed]
51. Makaras, T.; Svecevičius, G.; Kazlauskienė, N.; Montvydienė, D. Rapid Detection of Sublethal Toxicity Using Locomotor Activity of Rainbow Trout Juveniles. *Bull. Environ. Contam. Toxicol.* **2018**, *100*, 221–227. [CrossRef] [PubMed]

52. Tu, H.; Fan, C.; Chen, X.; Liu, J.; Wang, B.; Huang, Z.; Zhang, Y.; Meng, X.; Zou, F. Effects of cadmium, manganese, and lead on locomotor activity and neurexin 2a expression in zebrafish. *Environ. Toxicol. Chem.* **2017**, *36*, 2147–2154. [CrossRef] [PubMed]

53. Abreu, M.S.; Giacomini, A.C.; Rodriguez, R.; Kulueff, A.V.; Barcellos, L.J. Effects of ZnSO₄-induced peripheral anemia on zebrafish behavior and physiology. *Behav. Brain Res.* **2017**, *320*, 275–281. [CrossRef] [PubMed]

54. Mocelin, R.; Hermann, A.P.; Marcon, M.; Ramon, C.L.; Rohden, A.; Bevilaqua, F.; de Abreu, M.S.; Zanatta, L.; Elisabetsky, E.; Barcellos, L.J. N-acetylcysteine prevents stress-induced anxiety behavior in zebrafish. *Pharmacol. Biochem. Behav.* **2015**, *139*, 121–126. [CrossRef] [PubMed]

55. Deibel, M.; Ehmann, W.; Markesbery, W. Copper, iron, and zinc imbalances in severely degenerated brain regions in Alzheimer’s disease: Possible relation to oxidative stress. *J. Neurol. Sci.* **1996**, *143*, 137–142. [CrossRef]

56. Tekkök, S.B.; Ye, Z.; Ransom, B.R. Excitotoxic mechanisms of ischemic injury in myelinated white matter. *J. Cereb. Blood Flow Metab.* **2007**, *27*, 1540–1552. [CrossRef] [PubMed]

57. De Matos Mansur, B.; Cavalcante, C.N.S.; dos Santos, B.R.; Gouveia, A. Effects of mercury chloride (HgCl₂) on Betta splendens aggressive display. *Span. J. Psychol.* **2012**, *15*, 442–450. [CrossRef]

58. Weber, D.N.; Ghorai, J.K. Experimental design affects social behavior outcomes in adult zebrafish developmentally exposed to lead. *Zebrafish* **2013**, *10*, 294–302. [CrossRef] [PubMed]

59. Scott, G.R.; Sloman, K.A. The effects of environmental pollutants on complex fish behaviour: Integrating behavioural and physiological indicators of toxicity. *Aquat. Toxicol.* **2004**, *68*, 369–392. [CrossRef] [PubMed]

60. Knapen, D.; Reynders, H.; Bervoets, L.; Verheyen, E.; Blust, R. Metallothionein gene and protein expression as a biomarker for metal pollution in natural gudgeon populations. *Aquat. Toxicol.* **2007**, *82*, 163–172. [CrossRef] [PubMed]

61. Noche, R.R.; Lu, P.-N.; Goldstein-Kral, L.; Glasgow, E.; Liang, J.O. Circadian rhythms in the pineal organ persist in zebrafish larvae that lack ventral brain. *BMC Neurosci.* **2011**, *12*, 7. [CrossRef] [PubMed]

62. Aton, S.J.; Huettner, J.E.; Straume, M.; Herzog, E.D. GABA and Gi/o differentially control circadian rhythms. *Int. J. Mol. Sci.* **2018**, *19*, 3195. [CrossRef] [PubMed]

63. Cardinali, D.P.; Furio, A.M.; Brusco, L.I. Clinical aspects of melatonin intervention in Alzheimer’s disease progression. *Curr. Neuropharmacol.* **2010**, *8*, 218–227. [CrossRef] [PubMed]

64. Huang, P.; Morris, S.; Dinman, J.; Pine, R.; Smith, B. Role of metallothionein in detoxification and tolerance to transition metals. In *Metallothionein II*; Springer: Berlin, Germany, 1987; pp. 439–446.

65. De Lisle, R.C.; Sarras, M.P., Jr.; Hidalgo, J.; Andrews, G.K. Metallothionein is a component of exocrine pancreas secretion: Implications for zinc homeostasis. *Am. J. Physiol. Cell Physiol.* **1996**, *271*, C1103–C1110. [CrossRef] [PubMed]

66. Pearce, L.L.; Wasserloos, K.; Croix, C.M.S.; Gandy, R.; Levitan, E.S.; Pitt, B.R. Metallothionein, nitric oxide and zinc homeostasis in vascular endothelial cells. *J. Nutr.* **2000**, *130*, 1467S–1470S. [CrossRef] [PubMed]

67. Deibel, M.; Ehmann, W.; Markesbery, W. Copper, iron, and zinc imbalances in severely degenerated brain regions in Alzheimer’s disease: Possible relation to oxidative stress. *J. Neurol. Sci.* **1996**, *143*, 137–142. [CrossRef]

68. Tu, H.; Fan, C.; Chen, X.; Liu, J.; Wang, B.; Huang, Z.; Zhang, Y.; Meng, X.; Zou, F. Effects of cadmium, manganese, and lead on locomotor activity and neurexin 2a expression in zebrafish. *Environ. Toxicol. Chem.* **2017**, *36*, 2147–2154. [CrossRef] [PubMed]

69. Scott, G.R.; Sloman, K.A. The effects of environmental pollutants on complex fish behaviour: Integrating behavioural and physiological indicators of toxicity. *Aquat. Toxicol.* **2004**, *68*, 369–392. [CrossRef] [PubMed]

70. Knapen, D.; Reynders, H.; Bervoets, L.; Verheyen, E.; Blust, R. Metallothionein gene and protein expression as a biomarker for metal pollution in natural gudgeon populations. *Aquat. Toxicol.* **2007**, *82*, 163–172. [CrossRef] [PubMed]

71. Deibel, M.; Ehmann, W.; Markesbery, W. Copper, iron, and zinc imbalances in severely degenerated brain regions in Alzheimer’s disease: Possible relation to oxidative stress. *J. Neurol. Sci.* **1996**, *143*, 137–142. [CrossRef] [PubMed]
72. Blank, M.; Guerim, L.D.; Cordeiro, R.F.; Vianna, M.R. A one-trial inhibitory avoidance task to zebrafish: Rapid acquisition of an NMDA-dependent long-term memory. *Neurobiol. Learn. Mem.* 2009, 92, 529–534. [CrossRef] [PubMed]

73. Gerlai, R.; Lahav, M.; Guo, S.; Rosenthal, A. Drinks like a fish: Zebra fish (*Danio rerio*) as a behavior genetic model to study alcohol effects. *Pharmacol. Biochem. Behav.* 2000, 67, 773–782. [CrossRef] [PubMed]

74. Moretz, J.A.; Martins, E.P.; Robison, B.D. The effects of early and adult social environment on zebrafish (*Danio rerio*) behavior. *Environ. Biol. Fishes* 2007, 80, 91–101. [CrossRef]

75. Bass, S.L.; Gerlai, R. Zebrafish (*Danio rerio*) responds differentially to stimulus fish: The effects of sympatric and allopatric predators and harmless fish. *Behav. Brain Res.* 2008, 186, 107–117. [CrossRef] [PubMed]

76. Steenbergen, P.J.; Richardson, M.K.; Champagne, D.L. Patterns of avoidance behaviour in the light/dark preference test in young juvenile zebrafish: A pharmacological study. *Behav. Brain Res.* 2011, 222, 15–25. [CrossRef] [PubMed]

77. Gupta, T.; Talukder, G.; Sharma, A. Cytotoxicity of zinc chloride in mice in vivo. *Biol. Trace Elem. Res.* 1991, 30, 95–101. [CrossRef] [PubMed]

78. Zysk, M.; Bielarczyk, H.; Gul-Hinc, S.; Dyš, A.; Gapys, B.; Ronowska, A.; Sakowicz-Burkiewicz, M.; Szutowicz, A. Phenotype-dependent interactions between N-acetyl-L-aspartate and acetyl-coa in septal sn56 cholinergic cells exposed to an excess of zinc. *J. Alzheimer’s Dis.* 2017, 56, 1145–1158. [CrossRef] [PubMed]

79. Kononenko, V.; Repar, N.; Marušič, N.; Drašler, B.; Romih, T.; Hočevar, S.; Drobne, D. Comparative in vitro genotoxicity study of zno nanoparticles, zno macroparticles and ZnCl$_2$ to mdck kidney cells: Size matters. *Toxicol. In Vitro* 2017, 40, 256–263. [CrossRef] [PubMed]

80. Manosso, L.M.; Moretti, M.; Colla, A.R.; Ribeiro, C.M.; Dal-Cim, T.; Tasca, C.I.; Rodrigues, A.L.S. Involvement of glutamatergic neurotransmission in the antidepressant-like effect of zinc in the chronic unpredictable stress model of depression. *J. Neural Transm.* 2016, 123, 339–352. [CrossRef] [PubMed]

81. Adam, N.; Schmitt, C.; Galceran, J.; Company, E.; Vakurov, A.; Wallace, R.; Knapen, D.; Blust, R. The chronic toxicity of zno nanoparticles and ZnCl$_2$ to daphnia magna and the use of different methods to assess nanoparticle aggregation and dissolution. *Nanotoxicology* 2014, 8, 709–717. [PubMed]

82. O’donnell, B.; Huo, L.; Polli, J.R.; Qiu, L.; Collier, D.N.; Zhang, B.; Pan, X. From the cover: Zno nanoparticles enhanced germ cell apoptosis in *Caenorhabditis elegans*, in comparison with ZnCl$_2$. *Toxicol. Sci.* 2016, 156, 336–343.

83. Salvaggio, A.; Marino, F.; Albano, M.; Pecoraro, R.; Camiolo, G.; Tibullo, D.; Bramanti, V.; Lombardo, B.M.; Saccone, S.; Mazzei, V. Toxic effects of zinc chloride on the bone development in *Danio rerio* (hamilton, 1822). *Front. Physiol.* 2016, 7, 153. [CrossRef] [PubMed]

84. Küçükoğlu, M.; Binokay, U.S.; Pekmezekm, A.B. The effects of zinc chloride during early embryonic development in zebrafish (*Brachydanio rerio*). *Turkish J. Biol.* 2013, 37, 158–164.

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