Aquatic Toxicology 194 (2018) 167–175

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aqtox

Role of neurexin2a in lead-induced locomotor defect in developing zebrafish

Hongwei Tu[a,1], Tao Peng[a,1], Jiaxian Liu[a,1], Xiaohui Chen[b], Chengji Fan[a], Zhibin Huang[b], Yiyue Zhang[b], Fei Zou[a,*], Xiaojing Meng[a,*]

[a] Department of Occupational Health and Occupational Medicine, Guangdong Provincial Key Laboratory of Tropical Disease Research, School of Public Health, Southern Medical University, Guangzhou, Guangdong, China

[b] Key Laboratory of Zebrafish Modeling and Drug Screening for Human Diseases of Guangdong Higher Education Institutes, Department of Developmental Biology, School of Basic Medical Sciences, Southern Medical University, Guangzhou, China

ARTICLE INFO

Keywords:
Lead (Pb)
Neurexin2a
Locomotor behavior
Zebrafish

ABSTRACT

Low-dose chronic lead (Pb) exposure interferes with the development of the nervous system, which may lead to learning disabilities, behavioral abnormalities, and mental retardation. Neurexins (Nrxns) are synaptic cell-adhesion molecules associated with neurological disorders. We hypothesized that Pb can affect the expression of *nrxns* during synapse formation and alter the phenotype behavior. Here, apoptosis, *nrxns* mRNA expression, and alterations of locomotion were examined after exposure to Pb in zebrafish embryos/larvae. To confirm the function of *nrxn2a*, rescue experiments were performed using *β-nrxn2a* mRNA microinjection. Pb exposure increased apoptosis and altered locomotor behavior in zebrafish larvae. Quantitative PCR showed that among several synaptic adhesion molecules, only *nrxn2a* were affected by Pb exposure. Moreover, exposure to Pb at 10 μmol/L upregulated mRNA expression of *nrxn1a* and *nrxn3a* at 24 h post fertilization (hpf) and downregulated expression at 48 hpf, whereas the expression remained unchanged at 72 hpf. Only the two isoforms of *nrxn2a* were downregulated by Pb at 10 μmol/L at all three time points. Rescue experiments showed that *β-nrxn2a* mRNA injection recovered the decreased locomotor activity and the increased apoptosis induced by Pb. In addition, overexpression of *β-nrxn2a* mRNA upregulated *α-nrxn2a*. These data indicated that Pb inhibited the expression of *nrxn2a* genes, which play a critical role in neural development, and further altered the behavior of zebrafish embryos/larvae.

1. Introduction

Lead (Pb) is an old environmental metal with many applications worldwide, which has a high potential for biological toxicity and bioaccumulation (Andrade et al., 2015). Because of its extensive applications, Pb pollution in the environment is increasing, resulting in a high burden for environmental protection in recent decades. Pb poisoning causes a variety of symptoms and injury to more than one body system, which depend on the exposure pathway and dose. Acute Pb poisoning occurs mainly in the human digestive system and nervous system, whereas chronic exposure can cause damage to the hematopoietic and nervous systems (Andrade et al., 2015; Assi et al., 2016). Unlike the case in adults, the developing central nervous system (CNS) is particularly vulnerable to Pb because of an increase in hand to mouth activity, intestinal absorption, and the rapid development of the CNS with an incompletely developed metabolic system during childhood (Wirbisky et al., 2014). Epidemiological studies reported the hazards and prevalence of Pb exposure in childhood. In these reports, the associations between high Pb exposure levels and a decreased intelligence quotient revealed the strong neurotoxicity of Pb to the CNS of children, and an increased odds ratio for developing attention deficit hyperactivity disorder was observed (Eubig et al., 2010; Koller et al., 2004; Surkan et al., 2007). Despite strategies targeting Pb poisoning, long-term and low-dose chronic exposure is ignored in daily life, which may further cause long-lasting effects, especially on cognitive function and behavior (Neal and Guilarte, 2013).

Neural signaling depends on the structure of synapses between neurons and neurons/muscles, whereas synaptic adhesion molecules (SAMs) play key roles in synapse formation and function in target recognition, synaptic specialization, and the regulation of synaptic structure and function (Yamagata et al., 2003). In mature neurons, SAMs are involved in the stabilization of the synaptic ultrastructure, neurotransmitter release regulation, synaptic remodeling and plasticity (Leshchyns'ka and Sytnyk, 2016). Neurexins (Nrxns), which include

* Corresponding authors.
E-mail addresses: zoufei616@163.com (F. Zou), xiaojingmeng@smu.edu.cn (X. Meng).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.aquatox.2017.11.011
Received 29 August 2017; Received in revised form 18 November 2017; Accepted 21 November 2017
Available online 22 November 2017
0166-445X/

three different genes (*Nrxn1*, *Nrxn2*, and *Nrxn3*) and two protein isoforms for each gene in mammals, are a family of evolutionarily conserved SAMs. They participate in synapse formation, including synapse specialization, establishment, maturation, and plasticity. All *nrxns* mRNAs are extensively alternatively spliced and are involved in a variety of neural activities (Treutlein et al., 2014). A study by Missler et al. showed that most newborn mice did not survive for 1 week after triple/double knock-out of *nrxns* in the embryo stage (Missler et al., 2003). Nrxns were shown to be associated with neurological disorders in recent studies, including autism spectrum disorders, schizophrenia, and mental retardation (Born et al., 2015; Dachtler et al., 2015; Reichelt et al., 2012). The Nrxns trans-synaptic connexus acts as a gatekeeper of behavioral control and provides a basis for the molecular and clinical characterization and stratification of behavioral disorders (Clarke and Eapen, 2014).

The zebrafish (*Danio rerio*) is an important vertebrate model organism for developmental research, which has considerable advantages such as small size, a short-term reproduction cycle, transparent embryos, rapid growth, and low culturing cost, etc. Moreover, Zebrafish Nrxn2a has two isoforms, a long α-isoform and a short β-isoform, which are orthologs of Nrxn2α and Nrxn2β in mammals, respectively (Rissone et al., 2007). In our previous study, we examined the characteristics of acute toxicity and alterations in *nrxn2a* expression in zebrafish after Pb exposure (Tu et al., 2017). The malformation and mortality rates were increased in a concentration-dependent manner, and the expression of the *α-nrxn2a* and *β-nrxn2a* genes, was significantly inhibited after exposure to 10 μmol/L Pb. However, whether changes in *nrxn2a* gene expression induced by Pb played a role in the induced phenotypes and behavior changes remained unclear. Here, we performed apoptosis assays, quantitative real-time polymerase chain reaction (qPCR), and rescue experiments to confirm the association between behavioral alterations and *nrxns* defects after Pb exposure in zebrafish embryos/larvae.

2. Materials and methods

2.1. Fish husbandry and embryo collection

The wild-type AB strain and the neural-specific beta tubulin (NBT)-DsRed transgenic strain of zebrafish were raised according to the protocol described by Westerfield (Westerfield, 2000). All procedures were approved by the Key Laboratory of Zebrafish Modeling and Drug Screening for Human Diseases Institute, Southern Medical University (Guangzhou, China). Briefly, zebrafish embryos and larvae were housed in a recirculating tank system on a 14: 10 h light/dark cycle. Water quality was maintained at 28.5 °C (pH 7.2–7.6; salinity 0.03%–0.04%).

2.2. Embryo and larvae exposure

Fifty embryos [< 1 h post fertilization (hpf)] were exposed to treatment solutions of Pb (0, 2.5, 5, or 10 μmol/L $(CH_3COO)_2Pb$; Sigma–Aldrich, MO, USA), in zebrafish medium (3.50 g/L NaCl, 0.05 g/L KCL, 0.05 g/L $NaHCO_3$, and 0.10 g/L $CaCl_2$), with three replicates. At 48 and 72 hpf, the hatched larvae were counted and the hatching rate was analyzed by the ratio of hatched numbers/total exposed numbers × 100. The abnormal and dead zebrafish embryos or larvae were monitored. Embryos were collected at 24, 48 and 72 hpf, immediately frozen in liquid nitrogen, and stored at −80 °C until RNA extraction.

2.3. Locomotion analysis

A ViewPoint Zebrabox behavior testing system (ViewPoint Life Sciences, Lyon, France) and the quantization setting were used to monitor and record the locomotion activity as described by Chen et al. (2012). In brief, 24 larvae which do not exhibit any phenotypic defects from the indicated groups were transferred to a 96-well plate (1 fish per well) at 6 dpf before the behavior assessment. Spontaneous movements in the larvae were observed over a time period of 15 min. Three independent experiments were performed.

2.4. Apoptotic staining

Larvae at 72 hpf from the indicated groups were treated with acridine orange (AO) (Sigma–Aldrich). A stock solution was prepared by dissolving 1 mg of AO powder into 1 mL of distilled water. Larvae were incubated with 5 μg/mL AO at room temperature for 30 min in the dark. Larvae were washed three times with zebrafish medium for 10 min each. The transgenic fish were treated the same way as the wild-type fish. Apoptotic cells appeared as bright green fluorescent spots, and neural cells appeared as bright red fluorescent spots (in the transgenic zebrafish line), and were identified with a fluorescence stereomicroscope (Olympus Corporation, Tokyo, Japan).

2.5. Inductively coupled plasma-mass spectrometry (ICP-MS)

Embryo/larvae samples (~100, n = 3) were collected, washed 3 times in ultrapure water and weighed. After transformed into the digestion tube, 3 mL of concentrated HNO_3 and 2 mL H_2O_2 were then added to the embryos/larvae for acid digestion and subsequent release of metal ions. Then the samples were placed at 100 °C for 8 h. Samples were diluted with ultrapure water to reach a final concentration of 1% HNO_3 with a total volume of 25 mL. The concentrations of Pb were measured by using an ICP-MS (X Series 2, Thermo Fisher Scientific, Waltham, MA, USA). The intake of Pb was quantified as micrograms per gram with internal standard correction, as previously described (Zhang et al., 2011).

2.6. RNA extraction and qPCR

Whole embryos or larvae at each indicated time point were homogenized and RNA was isolated using the RNAiso Plus kit (TaKaRa, Dalian, China) and transcribed to cDNA with the PrimeScript RT reagent kit with gDNA Eraser (TaKaRa). Samples were stored at −80 °C until they were processed. RNA was extracted by chloroform phase separation followed by isopropanol precipitation, and RNA quality and quantity were checked with a Nanodrop-2000 (ThermoFisher Scientific, MA, USA). Relative levels of *nrxn* and other SAMs gene expression were measured by qPCR, and expression was normalized to *gapdh*. qPCR was performed with the SYBR green polymerase chain reaction core reagent kit (TaKaRa) according to the manufacturer's instructions on the Stratagene MX3000P (Agilent, La Jolla, CA, USA). Primers for *nrxn* and other SAMs gene were generated using the Primer3 program (Rozen and Skaletsky, 2000). The primers used for the gene amplification are shown in Table 1. To ensure the consistency of the results, the tests were performed at least in triplicate, using a total of 50 embryos or larvae per group.

2.7. In situ hybridization

Zebrafish larvae were used for *in situ* hybridization (ISH) as described by Samuel et al. (Peterson et al., 2013). Antisense *α-nrxn2a and β-nrxn2a* riboprobes labeled with digoxigenin (Roche, Basel, Switzerland) were synthesized from linearized template DNA by T7 RNA Polymerase (Roche) using *in vitro* transcription systems. Representative images were recorded with a fluorescence stereomicroscope (Olympus Corporation).

2.8. RNA synthesis and microinjections

For rescue experiments, full-length cDNA for *β-nrxn2a* of zebrafish was amplified and cloned into pCS2+ (Invitrogen). Capped mRNAs were *in vitro* transcribed using the Message Machine kit (ThermoFisher