Molecular Genetics and Metabolism 113 (2014) 142–147

Contents lists available at ScienceDirect

Molecular Genetics and Metabolism

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

A zebrafish model of hyperammonemia

B. Feldman [a], M. Tuchman [b], L. Caldovic [b,*]

[a] Eunice Kennedy Shriver National Institute of Child Health and Human Development, Bethesda, MD, USA
[b] Children's National Medical Center, Washington DC, USA

ARTICLE INFO

Available online 10 July 2014

Keywords:
Urea cycle
Hyperammonemia
Zebrafish
Drug screen
Neurotoxicity

ABSTRACT

Hyperammonemia is the principal consequence of urea cycle defects and liver failure, and the exposure of the brain to elevated ammonia concentrations leads to a wide range of neuro-cognitive deficits, intellectual disabilities, coma and death. Current treatments focus almost exclusively on either reducing ammonia levels through the activation of alternative pathways for ammonia disposal or on liver transplantation. Ammonia is toxic to most fish and its pathophysiology appears to be similar to that in mammals. Since hyperammonemia can be induced in fish simply by immersing them in water with elevated concentration of ammonia, we sought to develop a zebrafish (*Danio rerio*) model of hyperammonemia. When exposed to 3 mM ammonium acetate (NH_4Ac), 50% of 4-day old (dpf) fish died within 3 hours and 4 mM NH_4Ac was 100% lethal. We used 4 dpf zebrafish exposed to 4 mM NH_4Ac to test whether the glutamine synthetase inhibitor methionine sulfoximine (MSO) and/or NMDA receptor antagonists MK-801, memantine and ketamine, which are known to protect the mammalian brain from hyperammonemia, prolong survival of hyperammonemic fish. MSO, MK-801, memantine and ketamine all prolonged the lives of the ammonia-treated fish. Treatment with the combination of MSO and an NMDA receptor antagonist was more effective than either drug alone. These results suggest that zebrafish can be used to screen for ammonia-neuroprotective agents. If successful, drugs that are discovered in this screen could complement current treatment approaches to improve the outcome of patients with hyperammonemia.

1. Introduction

Ammonia is a nitrogen waste product of protein catabolism and a potent neurotoxin [1]. Six enzymes and two membrane transporters comprise the urea cycle, which converts ammonia to urea in the liver. Defects in any of the urea cycle enzymes or transporters lead to hyperammonemia, which is also a primary cause of hepatic encephalopathy due to acute and chronic liver failure [2]. Patients with hyperammonemia develop a wide range of neurocognitive abnormalities, intellectual disabilities and, in most severe cases, coma and death [3,4]. Although hyperammonemia often results in brain damage, current treatments for hyperammonemia focus almost exclusively on reducing blood ammonia levels through dietary manipulations, activation of alternative pathways of ammonia disposal or liver transplantation [5–9]. Drugs that could protect the central nervous system from damage by ammonia could transform the treatment of urea cycle disorders and also benefit patients with hepatic encephalopathy.

1.1. Ammonia toxicity to the brain

Currently, there is only a limited understanding of ammonia toxicity to the brain. Ammonia neurotoxicity has been traditionally studied in animal models of hepatic encephalopathy caused by either acute or chronic liver failure, because ammonia seems to be the most important neurotoxin in these conditions [10]. Animal models of urea cycle disorders [11–13] and animals exposed to high concentrations of ammonium salts [14–18] have also been used to study acute ammonia toxicity to the brain. These studies revealed that hyperammonemia triggers changes in metabolism, signaling pathways and gene expression. Metabolic changes associated with acute hyperammonemia include reduced levels of ATP in the brain, accumulation of lactate in the cerebrospinal fluid and increased turnover of serotonin and dopamine [10,19]. Elevated ammonia also causes biochemical and molecular changes in the endothelial cells of the blood–brain-barrier (BBB), astrocytes and neurons (Supplemental Fig. 1). Changes within the BBB include the activation of the NF-kB transcription factor, which leads to activation of matrix metalloprotease 9 and disruption of tight junctions [20,21]. Increased ammonia levels lead to increased production of glutamine in astrocytes, which may lead to astrocyte swelling and/or disruption of their mitochondrial function [22–26]. Elevated ammonia concentration also causes disruption of the Na^+K^+ATPase function, which in turn results in the increased extracellular concentration of potassium ions [11,27]. This leads to over-activation of the $Na^+K^+2Cl^-$ co-transporter

* Corresponding author at: Department of Integrative Systems Biology, Center for Genetic Medicine Research, Children's National Medical Center, 111 Michigan Avenue NW, Washington DC, 20010. Fax: +1 202 476 6014.
E-mail address: lcaldovic@cnmcresearch.org (L. Caldovic).

http://dx.doi.org/10.1016/j.ymgme.2014.07.001
1096-7192/

isoform 1 (NKCC1), which disrupts the function of the $GABA_A$-receptor complex [11]. Additionally, hyperammonemia leads to elevated extracellular glutamate concentration, which leads to over-stimulation of the NMDA receptors, increased influx of Ca^{2+} ions, activation of NO and cGMP signaling, destruction of cellular proteins and neuronal death [16,28].

Neurotoxic effects of ammonia are not restricted to the mammalian brain. Ammonia is toxic to all fish and its effects appear to be similar in fish and mammals. Although fish genomes encode genes for all six urea cycle enzymes and two transporters [29,30], hyperammonemia can be induced in fish simply by immersing them in water with an elevated concentration of ammonia, which is transferred from the water into the blood [31–35]. The enzymatic activity of glutamine synthetase (GS) and the concentration of glutamine are increased in the brains of African sharptooth catfish, rainbow trout and gulf toadfish immersed in water containing sub-lethal concentrations of ammonia [36–38]. Exposure to ammonia also affects fish neurons; MK-801, an NMDA receptor antagonist with neuroprotective effects in hyperammonemic rats [15,39], also protects goldfish from the harmful effects of acute exposure to ammonia [40]. Similar to rats, ammonia exposure leads to increased turnover of serotonin in fathead minnows [41,42].

Increased production of glutamine by GS and activation of NMDA receptors suggests that various biomolecules that inhibit the function of these two proteins could be used as drugs to potentially protect the brain from hyperammonemia. Methionine sulfoximine (MSO) is a suicide inhibitor of GS that could prevent the accumulation of glutamine in astrocytes [43]. Although MSO extended the survival of rats with hepatic encephalopathy [44,45], it may not be suitable for use as a drug because it also provoked seizures presumably due to interference with methionine metabolism in the brain [46–48]. MSO may also have undesirable side effects due to production of toxic metabolites since it could be a substrate for the cystathionine γ-lyase, L-amino acid oxidase and glutamate cysteine ligase [49,50]. Several NMDA receptor antagonists, including the FDA-approved drug memantine, prolonged the lives of rats with hepatic encephalopathy [51]. A search for additional drugs for hyperammonemia would require an animal model of hyperammonemia that is suitable for high-throughput screens of drug libraries.

1.2. Zebrafish as an animal model for drug discovery

Zebrafish (*Danio rerio*) is a vertebrate model organism that is ideally suited for high-throughput drug screens. A pair of zebrafish can produce around 200 fertilized eggs on a regular basis and thus provide the large number of fish needed for screening hundreds to thousands of compounds. Because zebrafish embryos and larvae are transparent and develop outside the mother, it is easy to observe developing organs and their defects induced by chemicals or mutations [52]. Developing zebrafish embryos and larvae are permeable to small molecules [53] and their biological response to drugs, environmental toxins and small molecules are similar to those of mammals [54–58] due to conservation of cellular physiology in vertebrates [59,60]. The brain structures, neurotransmitter systems and locomotor activity are similar in zebrafish adults and larvae [61,62]. The functions of dopaminergic, glutamatergic and GABA-ergic neurons, oligodendrocytes and astrocytes in zebrafish larvae correspond to the functions of these cell types in neonatal mice [63–69]. Zebrafish larvae have been successfully used to screen for anticonvulsants [70], antidotes for organophosphates [71], psychotropic and neuroactive drugs [72], cardiotoxic compounds [73], angiogenesis drugs [74], and compounds that protect the brain from the L-hydroxyglutaric acid toxicity [75].

2. Materials and methods

The Institutional Animal Care and Use Committees of the Eunice Kennedy Shriver National Institute of Child Health and Human Development and the Children's National Medical Center approved all procedures involving zebrafish described herein.

Adult male and female zebrafish were housed in separate tanks that were kept at 28 °C and a 14 h light and 10 h dark photoperiod. Zebrafish embryos were obtained by natural, pair-wise mating, collected between 2 and 4 h after fertilization and allowed to develop for one day in 0.006% instant ocean and 0.1% methylene blue. Between seven and ten 1 day old (dpf) embryos were arrayed in 12-well plates and allowed to develop for an additional two or three days in 3 ml of blue embryo medium (BEM; 14 mM NaCl, 0.5 mM KCl, 0.025 mM Na_2HPO_4, 0.044 mM KH_2PO_4, 1.29 mM $CaCl_2$, 0.1 mM $MgSO_4$, 4.2 mM $NaHCO_3$ and 0.1% methylene blue). Three or four day old zebrafish larvae were treated with either sodium acetate (NaAc) or ammonium acetate (NH_4Ac) at the indicated concentrations. Four day old zebrafish were treated with either 10 or 30 μM MSO, MK-801, memantine or ketamine for 20 min before the addition of either 4 mM NH_4Ac or NaAc. The combined drug treatment consisted of 10 μM MSO and either 10 μM or 30 μM MK-801 for 20 min before the addition of either 4 mM NH_4Ac or 4 mM NaAc. Zebrafish larvae were kept at 28 °C for the duration of experiment. Survival was scored every 20–30 min as judged by the presence/absence of a visible heartbeat. Leica MZ12.5 dissecting microscope was used to examine zebrafish larvae for heartbeats and brain appearance. Zebrafish larvae were photographed with the Leica MZ16.5 microscope equipped with Zeiss Axiocam/HrC camera and Zeiss Axiovision software package. Opaque appearance of the brain tissue is an indicator of cell death [76,77]. Experiments were repeated independently two or three times with 10–15 zebrafish larvae per treatment group, as indicated in figure legends. The Mantel–Cox log-rank test was used for statistical analysis of the data, which was carried out with Prism 6 software (GraphPad, Inc.).

3. Results and discussion

3.1. Zebrafish model of hyperammonemia

Our long-term goal is to discover drugs that can protect the brain from hyperammonemia. A high throughput screen for such drugs requires a simple test of a drug's ability to prolong survival of zebrafish exposed to high ammonia concentrations. Therefore, our immediate goal was to find the lowest NH_4Ac concentration that would be 100% lethal to developing zebrafish within 2 to 3 hours. Prolonged survival at a given concentration of NH_4Ac is a simple assay that can be carried out in a high throughput manner. Acute hyperammonemia was modeled in developing zebrafish by adding increasing amounts of either NH_4Ac or NaAc to their water and monitoring survival using cessation of heartbeat as an endpoint. When three-day old (3 dpf) zebrafish were exposed to 1, 5, 7.5, 10 and 20 mM NH_4Ac they succumbed to 10 mM and 20 mM NH_4Ac within 2 h while 5 and 7.5 mM NH_4Ac were 100% lethal within 3.5 h (data not shown). None of the fish exposed to 1 mM NH_4Ac died during 3.5 h of observation (data not shown). Exposure of 3 dpf zebrafish to 5 mM NH_4Ac leads to cell death in the brain (Figs. 1A and C), which is the likely cause of death. NaAc was not toxic to the 3 dpf fish and exposure to 5 mM NaAc did not result in visible effects (Figs. 1B and D).

Because high ammonia concentrations were needed for acute toxicity to 3 dpf fish, we tested whether four-day old (4 dpf) zebrafish larvae are more sensitive to ammonia toxicity. We found that lower concentrations of NH_4Ac were lethal to 4 dpf zebrafish than to 3 dpf fish. The survival curves in Fig. 2 represent one of the three biological replicas; 50% of 4 dpf zebrafish died within 3 h when exposed to 3 mM NH_4Ac, whereas, 4 mM NH_4Ac was 100% lethal. Therefore exposure of 4 dpf zebrafish to 4 mM NH_4AC was selected for the screen for drugs that can protect them from acute ammonia toxicity.

The 3 dpf fish appeared to have a degree of tolerance to acute exposure to ammonia. To confirm this, we tested survival of 3 dpf and 4 dpf zebrafish larvae at different concentrations of NH_4Ac.