Published in final edited form as:

Mol Genet Metab. 2014 ; 113(0): 142–147. doi:10.1016/j.ymgme.2014.07.001.

A zebrafish model of hyperammonemia

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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 neurocognitive 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 (NH₄Ac), 50% of 4-day old (dpf) fish died within 3 hours and 4 mM NH₄Ac was 100% lethal. We used 4 dpf zebrafish exposed to 4 mM NH₄Ac 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.

Keywords

Urea cycle; Hyperammonemia; Zebrafish; Drug screen; Neurotoxicity

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

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Conflict of Interest

The authors declare that they have no conflict of interest.

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- κ B 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 $\text{Na}^+\text{K}^+\text{ATPase}$ function, which in turn results in the increased extracellular concentration of potassium ions [11,27]. This leads to over-activation of the $\text{Na}^+\text{K}^+2\text{Cl}^-$ co-transporter 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-hydroxyglutamic 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 Na₂HPO₄, 0.044 mM KH₂PO₄, 1.29 mM CaCl₂, 0.1 mM MgSO₄, 4.2 mM NaHCO₃ and 0.1% methylene blue). Three or four day old zebrafish larvae were treated with either sodium acetate (NaAc) or ammonium acetate (NH₄Ac) at the indicated concentrations. Four day old zebrafish were treated with either 10 or 30 μ M MSO, MK-801, memantine or ketamine for 20 min before the addition of either 4 mM NH₄Ac or NaAc. The combined drug treatment consisted of 10 μ M MSO and either 10 μ M or 30 μ M MK-801 for 20 min before the addition of either 4 mM NH₄Ac 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 NH₄Ac concentration that would be 100% lethal to developing zebrafish within 2 to 3 hours. Prolonged survival at a given concentration of NH₄Ac 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 NH₄Ac 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 NH₄Ac they succumbed to 10 mM and 20 mM NH₄Ac within 2 h while 5 and 7.5 mM NH₄Ac were 100% lethal within 3.5 h (data not shown). None of the fish exposed to 1 mM NH₄Ac died during 3.5 h of observation (data not shown). Exposure of 3 dpf zebrafish to 5 mM NH₄Ac 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 NH₄Ac 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 NH₄Ac, whereas, 4 mM NH₄Ac was 100% lethal. Therefore exposure of 4 dpf zebrafish to 4 mM NH₄Ac 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 . Fig. 3 shows that 3 dpf fish survive longer than 4 dpf fish when exposed to 3, 4 and 5 mM NH_4Ac respectively. All fish immersed in 3, 4 and 5 mM NaAc survived (data not shown). Greater tolerance of ammonia in 3 dpf zebrafish could be due to greater ability to convert ammonia into urea at three days than at four days post fertilization [78, 79]. Alternatively, biomolecules and pathways in the brain that are affected by high ammonia may not yet be present or susceptible in 3 dpf zebrafish.

3.2. Protection of zebrafish larvae from ammonia toxicity

We sought to determine whether mechanisms of ammonia toxicity in developing zebrafish could be similar to those documented in gold-fish and mammals. Therefore, we tested whether NMDA-receptor antagonists and MSO prolong survival of 4 dpf zebrafish exposed to high ammonia. The fish were first exposed to two doses (10 μM and 30 μM) of the GS inhibitor, MSO, and three NMDA receptor antagonists, MK-801, memantine and ketamine, followed by the addition of 4 mM NH_4Ac . All four compounds were effective in prolonging the survival of 4 dpf zebrafish in 4 mM NH_4Ac (Figs. 4A–D). Higher doses of all three NMDA receptor antagonists appeared to be even more effective in protecting 4 dpf zebrafish from ammonia toxicity (Figs. 4B–D) whereas 30 μM MSO appeared to be less protective than 10 μM MSO (Fig. 4A). None of the tested compounds were in and of themselves toxic, since fish survival was not affected by exposure to MSO, MK-801, memantine and ketamine in 4 mM NaAc (data not shown). These observations suggest that certain concentrations of MSO combined with exposure to ammonia may be toxic to developing zebrafish. Increased survival of zebrafish larvae in the presence of MSO and NMDA receptor antagonists is not surprising because their targets, GS and NMDA receptors, are expressed in 4 dpf fish [80,81]. The zebrafish genome encodes three GS paralogs: *glula*, *glulb* and *glulc* [82]. While *glulc* is expressed in adult zebrafish [82], expression of both *glula* and *glulb* in 4 dpf zebrafish is restricted to the brain, retina and peripheral nervous system [81], and the two genes encode functional enzymes, GSa and GSb, with 94% amino acid similarity. The GSb protein abundance and enzymatic activity increase when adult zebrafish are placed in hyperammonemic conditions [82]. Our finding that MSO mitigates ammonia-induced toxicity in zebrafish larvae would be consistent with the block of glutamine accumulation in astrocytes due to the inhibition of glutamine synthetase activity in the brains of 4 dpf zebrafish. Zebrafish have ten genes that encode subunits of the NMDA receptor [80] due to genome duplication that occurred in the teleost lineage [83–85]. The amino acid sequences of the NMDA receptor 1 subunits from zebrafish are more than 90% identical to the corresponding human proteins while zebrafish NMDA receptor 2 subunits have between 35 and 50% sequence identity to the human homologs [80]. The role of NMDA receptors in brain function and the effects of MK-801, ketamine and memantine on learning, memory and behavior appear to be similar in zebrafish and mammals [86–94]. This suggests that mechanism(s) of neuroprotection against hyperammonemia by NMDA receptor antagonists may also be similar in zebrafish and mammals.

Because MSO and NMDA-receptor antagonists protect the brain from ammonia toxicity by different mechanisms [15,39,44,45,51], we tested whether the combination of MSO and

MK-801 is more effective in prolonging the survival of 4 dpf zebrafish in 4 mM NH₄Ac than either drug alone. Only the lower dose of MSO was tested because 30 μM MSO appeared to be less effective than 10 μM MSO in protecting zebrafish from ammonia toxicity. The combination of 10 μM MK-801 together with 10 μM MSO indeed protected 4 dpf zebrafish from ammonia toxicity better than either drug alone (Fig. 5A). Similarly, treatment with 30 μM MK-801 and 10 μM MSO was more effective than either drug alone, although the added benefit of combining 10 μM MSO with a 30 μM MK-801 treatment is less striking than with a 10 μM MK-801 treatment (compare Fig. 5B to A). Synergistic protection of developing zebrafish by MSO and MK-801 is consistent with the effects of ammonia on multiple pathways and cell types in the brain.

4. Concluding remarks

Despite our still rudimentary understanding of the mechanisms of ammonia toxicity to the brain, available data suggest that biomolecules such as GS, NMDA receptors, the NLCC1 co-transporter and possibly the GABA_A receptor are affected by elevated ammonia and might therefore be effectively targeted with drugs to achieve neuroprotection. Preclinical studies in mice with hyperammonemia have shown effectiveness of the FDA-approved drugs memantine, ketamine and bumetanide in mitigating the toxic effects of hyperammonemia [11,15,39,51]. There may be other FDA-approved drugs that could be used to protect the brain from hyperammonemia. However, identifying such drugs would require a high-throughput screening approach. Our studies suggest that zebrafish larvae are well suited for high throughput chemical screen for drugs that protect against hyperammonemia because zebrafish brain cells appear to be as sensitive to ammonia as the human brain. Moreover, since zebrafish larvae have a fully functioning central nervous system [95] and BBB [96,97], they should be well suited for the identification of drugs with distinct mechanisms of neuroprotection against ammonia toxicity and for the subsequent testing of whether combinations of distinctly acting drugs have synergistic effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the Eunice Kennedy National Institute for Child Health and Human Development and by the Public Health Service grant R21 DK099476.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.ymgme.2014.07.001>.

Fig. 1. Brain cell death in zebrafish larvae exposed to ammonia. 3 dpf zebrafish larvae exposed to either 5 mM NH_4Ac (A and C) or 5 mM NaAc (B and D). Fish were monitored for heartbeats and photographed immediately after cessation of heartbeat in fish immersed in NH_4Ac . Arrows indicate necrotic brain tissue.

Fig. 2.

Survival of 4 dpf zebrafish immersed in NH_4Ac . Fish were monitored for heartbeats every 30 min. Each survival curve represents one of three biological replicas with similar results.

Fig. 3. Survival of 3 dpf and 4 dpf zebrafish exposed to ammonia. Zebrafish larvae were exposed to 3, 4 and 5 mM NH₄Ac. Each survival curve represents one of three biological replicates with similar results.

Fig. 4.

Survival of 4 dpf fish treated with either MSO or NMDA receptor antagonists and then exposed to NH₄Ac. Zebrafish larvae were treated with either 10 μ M or 30 μ M MSO (A and E), MK-801 (B and F), memantine (C and G) and ketamine (D and H) for 20 min followed by addition of either 4 mM NH₄Ac. Fish were monitored for heartbeats every 20–30 min. Solid black line — survival of fish in 4 mM NH₄Ac; dashed gray line — survival in 10 μ M drug and 4 mM NH₄Ac; solid gray line — survival in 30 μ M drug and 4 mM NH₄Ac. Survival curves represent one of two biological replicates with similar results.

Fig. 5.

Survival of 4 dpf zebrafish treated with either MSO or MK-801 or both drugs and exposed to NH_4Ac . A. 4 dpf zebrafish were treated with 10 μM MK-801 (dashed dark gray line), 10 μM MSO (dashed light gray line) or 10 μM MK-801 and 10 μM MSO (solid gray line) for 20 min followed by addition of 4 mM NH_4Ac . Control fish were treated with NH_4Ac alone (solid black line). Fish were monitored for the presence of heartbeat every 20–30 min. B. 4 dpf zebrafish were treated with 30 μM MK-801 (dashed dark gray line), 10 μM MSO (dashed light gray line) or 30 μM MK-801 and 10 μM MSO (solid gray line) for 20 min followed by addition of 4 mM NH_4Ac . Control fish were treated with NH_4Ac alone (solid black line). Fish were monitored for heartbeats every 20–30 min. Survival curves represent one of two biological replicates with similar results.