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Changes in Brain Water Content in the Rainbow Trout (Oncorhynchus mykiss) and in the Goldfish (Carassius auratus) due to High External Ammonia Exposure

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By

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ABSTRACT

In fishes, hyperammonemia may occur following feeding or exposure to abnormally high concentrations of environmental ammonia due to sewage effluents, agricultural run-off and in crowded aquaculture pens. Increased internal ammonia can result in hyperactivity, convulsions, coma and death. In mammals, it is also associated with potentially fatal brain edema, in which the accumulation of intracellular water results in swelling, increased intracranical pressure and herniation leading to death. Recently it was shown that rainbow trout (*Oncorhynchus mykiss*) and goldfish (*Carassius auratus*) experience brain swelling following exposure to high external ammonia (HEA). However, the mechanism of ammonia-induced brain swelling in fishes remains unsolved. The goal of this thesis was to determine the underlying mechanisms of ammonia-induced swelling in the fish brain. The specific research objectives were to (i) determine if intracellular glutamine accumulation contributed to brain swelling by increasing intracellular osmolarity; (ii) ascertain what role NMDA receptor over-activation might play in mediating ammonia-induced brain swelling, and (iii) determine if changes in brain water content were accompanied by disturbances to ion balance and transport processes in the brain. Exposure of rainbow trout (1mM NH_4Cl) and goldfish (5mM NH_4Cl) to 48 h HEA lead to a 12-15% increase in brain water content, 10-fold increase in plasma ammonia [T_{Amm}], and 5-6-fold increase in brain glutamine. Dry brain Na^+ content displayed similar trends to brain water content, increasing approximately 45-70 %, while K^+ was reduced by approximately 10-24 %. These changes were accompanied by simultaneously, 50 % reductions in rainbow trout brain Na^+/K^+-ATPase activity after 48 h high external ammonia (HEA). Goldfish were able to withstand higher concentrations of external

ammonia compared to the rainbow trout, which was consistent with greater tolerance observed in previous studies. Remarkably, brain water content was restored to control levels following 24 h recovery in ammonia-free well water. Administration of MK801 (dizocilpine) an NMDA receptors antagonist prior to HEA prevented significant brain swelling in the rainbow trout but not in goldfish. Additionally, the administration of MK801 caused a reduction from control levels in brain Na^+ content in both species. Lastly, the administration of glutamine synthetase inhibitor, methionine sulfoximine (MSO) to each species followed by 48 h HEA did not prevent significant swelling. In conclusion, HEA resulted in water accumulation in the brain, but this was not due to increased glutamine production. Rather, brain swelling appears to be mediated by NMDA receptors followed by ionic disturbances, resulting in brain swelling.

ACKNOWLEDGEMENTS

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Additionally, I would like to express my deepest appreciation to Dr. Jonathan M. Wilson who has given a great deal of time to teach different analytical methods and for his encouragement and guidance. I also would like to thank Dr. Andrea Lister for her valuable advice, guidance, and continued support. Lastly, I also thank Lisa Sorensen, David Lisser, Benjamin Hlina, Laura Tessier, Malcolm Glennie, Scott Hepditch, Christopher White and Dr. Stephanie DeWitte-Orr at Wilfrid Laurier University and the Laurier Institute for Water Science for their technical assistance and animal care. This work was supported by a by NSERC Discovery Grant to Dr. M.P. Wilkie.

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List of Abbreviations

AA	atomic absorption
ALF	acute liver failure
ANOVA	analysis of variance
ATP	adenosine-triphosphate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BBB	blood brain barrier
$C_6H_{12}O_6$	glucose
$CaCl_2$	calcium chloride
Cl^-	chloride
CNS	central nervous system
cNOS	constitutive nitric oxide
CSCl	cesium chloride
CSF	cerebral spinal fluid
DNA	deoxyribonucleic acid
EAAT	excitatory amino acid transporter
ECF	extracellular fluid
EDTA	ethylenediaminetetraacetic acid
Fe^{3+}	iron (iii)
FHF	fulminant hepatic failure
GDH	glutamate dehydrogenase
GLN	glutamine

GLNase	glutaminase
GLNT	glutamine transporter
GLU	glutamate
GS	glutamine synthetase
H_2O	water
H_2O_2	hydrogen peroxide
HCl	hydrochloric acid
HE	hepatic encephalopathy
HEA	high external ammonia
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
HNO_3	nitric acid
ICF	intracellular fluid
ICP	intracranial pressure
IP	intra-peritoneal
K^+	potassium
$[K^+]_i$	intracellular potassium
KCA	α-ketoglutaric acid
Km	concentration of substrate
KCl	potassium chloride
KOH	potassium hydroxide
LTP	long-term potentiation
MAPK	mitogen-activated protein kinase
MCA	middle cerebral artery

Mg^{2+}	magnesium chloride
$MgSO_4$	magnesium sulfate
MK801	dizocilpine
mRNA	messenger RNA
MRI	magnetic resonance imaging
MS222	tricaine methanesulfonate
MSO	methionine sulfoximine
N_2	nitrogen
Na^+	sodium
$[Na^+]_i$	intracellular sodium
NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate
NaH_2PO_4	monosodium phosphate
NADPH	nicotinamide adenine dinucleotide phosphate reduced
$NADH^+$	nicotinamide adenine dinucleotide phosphate
NH_3	ammonia
NH_4^+	ammonium
NKA	Na^+/K^+-ATPase
NKCC	$Na^+/K^+/Cl^-$ cotransporter
NF-κB	nuclear factor kappa-B protein
NMDA	N-Methyl-D-Aspartate
NO	nitric oxide
NOS	nitric oxide synthase

NOX	NADPH oxidase
NSCC	non-selective cation channels
$O_2^{\cdot -}$	superoxide anion radical
$OH^{\cdot}$	hydroxyl radical
OUC	ornithine-urea cycle
PAG	phosphate activated glutaminase
PCA	perchloric acid
PVC	polyvinyl chloride
Rh	rhesus glycoprotein
ROS	reactive oxygen species
RONS	reactive nitrogen species
RNA	ribonucleic acid
SEM	standard error of mean
SN1	glutamine transporter
SN2	glutamine transporter
SOD	superoxide dismutase
$[T_{Amm}]$	total ammonia concentration
V_{max}	rate of enzyme reaction

Chapter 1

General Introduction

INTRODUCTION

Ammonia production & excretion in mammals and fish

Ammonia is the major nitrogenous waste product generated by most vertebrates due to the catabolism of excess proteins, which leads to the generation of smaller peptides and amino acids followed by their subsequent deamination to produce ammonia. Many excess amino acids that are produced from the proteolysis of proteins, are converted to amino acid glutamate by transaminase enzymes, and then oxidatively deaminated by glutamate dehydrogenase (GDH), to generate of ammonium (NH_4^+) and α-ketoglutarate (**Fig 1.1.**; Mommsen and Walsh 1991; Wright 1995). Ammonia can exist as either un-ionized NH_3 or as ionized NH_4^+, but with a pKa of approximately 9.5, most total ammonia exists as NH_4^+ at physiological pH in fishes (pH 7.8; Cameron and Heisler 1983). In freshwater fish, nitrogenous waste products are mainly excreted across the gill epithelium by Rhesus glycoprotein (Rh; Wright and Wood 2009).

Recently Wright and Wood (2009) summarized how ammonia excretion likely takes place via Rhesus (Rh) glycoproteins in freshwater fish. They suggested that the process was driven by acid excretion, which trapped NH_3 as NH_4^+ thus, maintaining the NH_3 diffusion gradient across the gills. There are three types of Rh glycoprotein in ammonia excretion by fishes. The Rhag facilitates NH_3 movement out of erythrocytes, while Rhbg moves NH_3 across the basolateral membrane of the branchial ionocyte (mitochondria rich cell), specialized cells involved in maintaining ion and acid-base balance in the body. Apical excretion of NH_3 involves an apical "Na^+/NH_4^+ exchanger complex" consisting of the Rhcg and the V-type H^+- ATPase or Na^+/H^+-exchanger (NHE-2), which work together as a metabolon to promote acid trapping of NH_3 as NH_4^+

(**Fig 1.2**; Wright and Wood 2009). Consequently, when pH or external ammonia is elevated, internal NH_3 concentrations markedly increases due to the reduction of the NH_3 diffusion gradient across the gills (**Fig 1.2**; Schenone et al. 1982; Wright and Wood 1985; Wilkie and Wood 1991; Wilkie, 2002; Wilkie and Wood 1991; Ip et al. 2001 Ip et al. 2004a, 2004b).

Un-ionized NH_3 is most toxic to fish when they are exposed to high environmental ammonia (HEA) because it can lower or reverse the NH_3 diffusion gradient between the blood and water that drives ammonia excretion across the gill (**Fig 1.2**; Ip et al. 2001; Wilkie 2002; Weihrauch et al. 2009). This subsequently leads to simultaneous uptake of exogenous ammonia across the gill down a favorable NH_3 partial pressure gradient and accumulation of endogenous ammonia (Felipo and Butterworth 2002). As a result, there may be several-fold increases in blood and tissue ammonia when fish are exposed to HEA. Other factors that contribute to higher endogenous ammonia include postprandial increases after meals, when rates of proteolysis and amino acid catabolism are elevated (Wicks and Randal 2002). Additionally, vigorous exercise can also lead to large increases in ammonia due to the deamination of purines (Driedzic and Hochachka 1978; Wang et al. 1995).

Ammonia toxicity and disorders

Internal ammonia concentrations can be markedly elevated due to a variety of pathological conditions, resulting in hyperammonemia. In mammals, hyperammonemia can be caused by liver defects triggered by alcoholism and defects in the ornithine urea cycle (Felipo and Butterworth 2002). Increased internal ammonia may elicit irreversible damage to the central nervous system (CNS) due to ventricular enlargement and cortical

atrophy, which can lead to seizures, cognitive impairment, coma and death (Braissant 2012; Felipo and Butterworth 2002). Brain swelling is more often observed in patients with acute liver failure (ALF), and is referred to clinically as hepatic encephalopathy (HE). In this condition, the liver is unable to convert ammonia to urea, resulting in excessive ammonia accumulation in the bloodstream, which often necessitates a liver transplant (Cordoba and Blei 1995).

In most vertebrates the liver is the most important site of ammonia detoxification **(Fig 1.1;** Felipo and Butterworth 2002; Mommsen and Walsh 1992**)**. Defects in the liver's ability to detoxify ammonia may arise from prehepatic "portal" hypertension, inborn genetic errors of the OUC and liver damage arising from alcoholism, hepatitis, or other ingestible toxins (Randall and Tsui 2002). As a result, the levels of ammonia in the blood become dangerously elevated. Freshwater fishes may experience hyperammonemia due to exposure to sewage effluents, agricultural run-off, and the degradation of organic matter resulting from overcrowding and overfeeding in aquaculture facilities (Ip et al. 2001; Eddy 2005), each of which cause water ammonia concentrations to increase. Compared to humans, fish are more tolerant to increased internal ammonia. In humans, plasma ammonia concentrations range from 10-65$\mu molL^{-1}$, whereas in fish plasma ammonia concentrations range from 50-300$\mu molL^{-1}$ (Wilkie and Wood, 1991; Wicks and Randall, 2002a,b; Ip and Chew 2010). At high blood ammonia concentrations, ammonia probably enters the brain via diffusion. Diffusion of ammonia into the brain is promoted by the lower intracellular pH of brain tissue, in which NH_3 is trapped as ionized NH_4^+ and unable to move back across the BBB due to its positive charge (Goldbecker 2010; Walsh 2007).

Competing theories of ammonia action on the central nervous system in mammals

The effects of ammonia in glutamate-glutamine cycle & astrocyte swelling

Recent evidence suggests that increased ammonia may alter signal transduction pathways, nitric oxide synthesis, oxidative stress and neurotransmission in the central nervous system (CNS). In vertebrates the CNS is comprised of the brain and spinal cord and two main classes of cells, neurons and glial cells. Glial cells are greater in density and number compared to neurons, but the exact distribution and number of these cells are undetermined in fishes (Allen and Barres 2009; Sidhu 2012). Glial cells play a number of roles in the CNS including the nourishment and support of neurons, insulation for neurons through the formation of myelin, and protection from pathogens (Kandel et al. 2000). One type of glial cells, the astrocytes, play an essential role in controlling neurotransmitter action and duration by mediating the removal of some neurotransmitters such as glutamate, from the synaptic cleft between pre- and post-synaptic neurons. In mammals, astrocytes are believed to be the most prone to neurophysiological effects of increased ammonia because majority of the cell composition in the brain is mainly comprised of astrocytes (Pope 1978; Blei et al. 1994; Norenberg 1995,1998; Brusilow 2002; Veauvy et al. 2005; Albrecht and Norenberg 2006). Direct evidence of ammonia-induced brain swelling was observed in cultured mammalian astrocytes, which displayed significant swelling when treated with ammonia (Norenberg 1998). In addition, Butterworth (2001) used primary cultures of mammalian astrocytes to demonstrate that these cells underwent significant swelling when treated with ammonia. It remains unclear why astrocyte cells are prone to swelling during acute hyperammonemia.

However, glutamine synthetase (GS) enzyme located in astrocytes cells may be the link to astrocytic swelling.

Traditionally, GS has been thought to play a role in the detoxification of ammonia in the brain. By lowering ammonia levels in the blood and tissues, glutamine synthesis is believed to protect vertebrates against ammonia-toxicity (**Fig 1.3.** Felipo and Butterworth 2001; Randall and Tsui 2002). However, some have suggested that increased glutamine within the intracellular fluid of the astrocyte could be an important factor that increases the cell's osmolarity, driving water uptake (**Fig 1.3.** Brusilow 2002; Walsh et al. 2007). Patients suffering from fulminant hepatic failure (FHF) demonstrated that arterial ammonia concentrations were significantly correlated between brain glutamine content and intracranial pressure (ICP; Glassford et al. 2011). Indeed, some fishes are known to increase glutamine synthetase (GS) activity in responses to ammonia exposure (Wicks and Randall 2002; Ip et al. 2005). However, these hypotheses have been challenged by Wilkie's group, who demonstrated that the inhibition of GS by methionine sulfoximine (MSO) did not mitigate ammonia-induced brain swelling in the goldfish (*Carassius auratus*; Wilkie et al. 2015). These findings suggest that accumulation of glutamine does not directly lead to increases in intracellular osmolarity in the goldfish brain, and that other factors cause brain swelling. Albrecht and Norenberg (2006) suggested that glutamine acts as a silent carrier ("Trojan horse") of ammonia, which is taken up along with glutamate and converted, to glutamine by glutamine-synthetase in astrocytes. They argue that the glutamine is then converted to glutamate and ammonia within the mitochondria by phosphate activated glutaminase (PAG), with the ammonia triggering production of reactive oxygen species (ROS) through the induction of the mitochondria

permeability transition (MPT), which subsequently causes cell swelling and protein damage. Regardless of glutamine`s mechanism of action, this evidence suggests that it may in fact contribute to toxicity and brain swelling in the CNS. A goal of the present study was to better understand if glutamine contributes to ammonia-induced brain swelling in the goldfish and rainbow trout.

The effects of ammonia on central nervous system and NMDA receptors

A second theory of ammonia toxicity is that ammonia induces glutamate excitotoxicity (Fan and Szerb 1993; Hermenegildo et al. 1996). Electrophysiological studies by Fan and Szerb (1993) using hippocampal slices, and more recently by Wilkie et al. (2011) using slices of goldfish telencephalon, have demonstrated that ammonia potentiates NMDA receptor currents, which can initiate glutamate excitotoxicity through over-activation of the receptors on neurons. As a consequence, there can be excess calcium entry into post-synaptic neurons, leading to the activation of proteases and lipases, and the generation of ROS, such as nitric oxide (NO), hydrogen peroxide (H_2O_2) and superoxide radicals (O_2^-) (Kosenko et al. 1997; Norenberg et al. 2006; Norenberg et al. 2009; Walsh et al. 2007; Wilkie at al. 2011). These reactive molecules are the byproducts of oxidative metabolism, which can severely damage proteins and lipids in the brain.

Under normal conditions, NMDA receptors are ionotropic receptors, which act as cation channels that are most permeable to calcium when triggered. NMDA receptors play a critical role in the process of CNS development, neuroplasticity, learning and memory (Wenthold et al. 2003). The development of learning and memory is promoted by a process known as long-term potentiation (LTP), where long lasting signal transfers

occur between the pre-synaptic and post-synaptic neuron (**Fig 1.4**). LTP can be elicited by the activation of NMDA glutamate receptors (DiScenna and Teyler 1987; System 1992). Activation of the NMDA receptor occurs following the release of glutamate into the synaptic cleft from the pre-synaptic neuron. The glutamate then binds the NMDA receptor and also to the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor, which acts as a Na^{+}/Ca^{2+} channel. The binding of glutamate to AMPA receptors triggers the AMPA channels to open, depolarizing the cell due to the influx of sodium into the neuron. The NMDA receptor is typically not activated until the resulting depolarization arising from AMPA receptor activation resulting in the displacement of Mg^{2+} from the channel of the NMDA receptors, which allows an influx of Ca^{2+} into the post-synaptic cell (**Fig 1.4**; Wenthold et al. 2003).

Despite extensive work by many research groups, the mechanism(s) of ammonia toxicity remains uncertain, however, ammonia and associated oxidative and nitrosative stress (RONS) seem to play a role in astrocyte swelling. Previous *in vivo* work revealed that during experimental hyperammonemia, ammonia toxicity triggered increase RONS productions in cultured astrocyte (Norenberg 2003; Jayakumar et al. 2009). Kosenko et al. 2007, reported that hyperammonemia reduced levels of antioxidant enzymes and lipid peroxidation in the brain. Additionally, oxidative stress and the effects of free radicals cause swelling in cultured astrocyte and brain slices (Chan et al. 1989; Chan et al. 2000; Jayakumar et al. 2009). Similarly, in the presence of ammonia, oxidative damage to proteins and lipids has been demonstrated in astrocyte cultures (Murphy et al. 1992; Norenberg et al. 2009, Jayakumar et al. 2009). An initial key event during ammonia

toxicity in cultured astrocytes is the increase in intracellular Ca^{2+} (Schliess et al. 2002; Rose et al. 2005).

It remains unclear how or if ammonia induced-activation of NMDA receptors triggers the cascade of events that lead to brain swelling in mammals, let alone fishes. Researchers suspect that ammonia depolarizes neurons which results in the release of Mg^{2+} from the ion channel of the receptor (Fan and Szerb 1993), which normally prevents the opening of NMDA channels, thereby preventing Ca^{2+} from entering the cell under resting conditions (Wenthold et al. 2003). Hemenegildo et al. (1996) injected mice with various NMDA receptor antagonists, such as dizocilpine (MK801), phencyclidine, and ketamine, which act on different sites of the NMDA receptors, following the intraperitoneal (IP) injection of the mice with lethal doses of ammonia. The MK801 treated mice survival was extended, suggesting that acute ammonia toxicity is linked to the over-activation of NMDA receptors. However, it is not known if the NMDA receptor-mediated excitotoxicity explains the brain swelling that accompanies ammonia toxicity in vertebrates. Thus, another goal of this work was to determine if over-activation of NMDA receptors contributes to brain swelling in the ammonia tolerant goldfish, compared to the more ammonia-sensitive trout during exposure to high environmental ammonia.

Osmotic disturbances during high external ammonia

Maintenance of the brain's osmotic, ionic and volume balance of these fluids is essential for proper function of the CNS. The composition of the CSF and ICF are tightly controlled due to the presence of the blood brain barrier (BBB), which, maintain a narrow and stable homeostatic neuronal environment required for normal brain function (Serlin

et al. 2015). The BBB is comprised of brain capillary endothelial cells and their junctional complexes (Nguyen 2012). The ICF of neurons and astrocytes are controlled by a variety of ion transporters. In addition to the Na^+/K^+-ATPase, which is ubiquitous, another important transporter is the Na^+-K^+-Cl^- cotransporters (NKCC), which are a class of membrane proteins that transport Na^+, K^+ and Cl^- ions into and out of cells. Brain swelling during HE and ALF may be due to the similar radii between K^+ and NH_4^+ and their effects on NKCC1 and Na^+/K^+-ATPase. With a pKa of ~9.0 at 37^{o}C only a small percentage will exist as NH_3 at a physiological pH (7.4) therefore, majority will exist as NH_4^+ and able to compete with K^+ on NKCC1 and Na^+/K^+-ATPase (Aickin et al. 1982; Moser 1987; Hertz et al. 2015). Additionally, rise in glutamate due to the over-activation of NMDA receptors results in the increased inward transfer of glutamate into astrocytes, by a Na^+/glutamate co-transporter EAAT-1 with a K^+ counter-transport (Zerangue and Kavanaugh 1996; Levy et al. 1998; Hertz et al. 2015). Elevated EAAT activity may increase extracellular K^+, which act on NKCC1, Na^+/K^+-ATPase and endogenous ouabain pathway. The combination of these disturbances activates the production of ROS and nitrosative agents, which trigger various ion channels to activate, leading to cell swelling (Hertz et al. 2015). Lastly, studies by Chen et al. (2013) in swamp eel, *Monopteris albus* found that exposure to ammonia lead to a significant decrease in mRNA expression of Na^+/K^+-ATPase and overall protein abundance. Furthermore, brain Na^+/NH_4^+-ATPase activities were significantly lower than Na^+/K^+-ATPase. Suggesting, that *M. albus* impressive ammonia-tolerant capability may be due to their ability to down-regulate mRNA expression and overall protein abundance. Thus one goal of this work is to investigate Na^+/K^+-ATPase activity during HEA.

Changes in the BBB permeability to Na^+ may also play a predominant role in ischemic brain edema. Gotoh et al. (1985) occluded the middle cerebral artery (MCA) in rats, which resulted in significant swelling of the brain as demonstrated by increased brain water content, and parallel increases in dry brain Na^+ and decreases in dry brain K^+ content. However, ionic and osmotic disturbances during ammonia-induced brain swelling in fish remains poorly understood. Thus, the last goal of this work is to determine the role of ionic changes during HEA.

Hypothesis and Objectives:

With this background, the overall goal of my M.Sc. thesis was to determine the mechanisms of ammonia-induced brain swelling in the rainbow trout and goldfish. My specific objectives were to:

Objective I: To determine if ammonia-induced brain swelling is due to the increase concentration of intracellular glutamine.

Objective II: To determine if ammonia-induced brain swelling is due to the overactivation of NMDA receptors.

Objective III: To analyze the role of brain ionic disturbances during the development of ammonia-induced brain swelling.

Objective IV: To characterize the effects of ammonia on Na^+/K^+-ATPase protein activity during ammonia-induced brain swelling.

To test these hypotheses, rainbow trout and goldfish were exposed to HEA and brain tissue water content was measured. To further explore the underlying mechanism of brain swelling, rainbow trout and goldfish were injected with an NMDA receptor antagonist (MK801) and exposed to HEA. Following treatment and exposure, brain tissue water, plasma ammonia, ammonia and glutamine content were measured. Based on previous studies by Wilkie et al. (2011), I predicted that MK801 would mitigate brain swelling in ammonia-sensitive rainbow trout but not in ammonia-tolerant goldfish because ammonia-sensitive fish may be more susceptible to neuronal depolarization. Whereas, ammonia-tolerant fish may have adaptations that allow them to be less tolerant to neuronal depolarization in the presence of ammonia (Wilkie et al. 2011). I also predicted that the inhibition of glutamine synthetase by MSO would not mitigate brain

swelling due to intracellular accumulation of glutamine in both rainbow trout and goldfish. Lastly, I predicted that there would be significant ionic/osmotic disturbances during ammonia-induced brain swelling in the rainbow trout and goldfish.

Figure 1.1 Ammonia production and excretion pathways in mammals, reptile and fish. The production of ammonia arises from the breakdown of proteins followed by the subsequent deamination of amino acids. (1) In mammals, the liver is responsible for detoxifying ammonia. In which ammonia is converted to urea by the ornithine-urea cycle and excreted by the kidney. (2) Reptiles excrete nitrogenous waste in the form of uric acid via the 15-reaction pathway. (3) Most fishes excrete ammonia directly across the gill due to its high water solubility.

FIGURE 1.1.

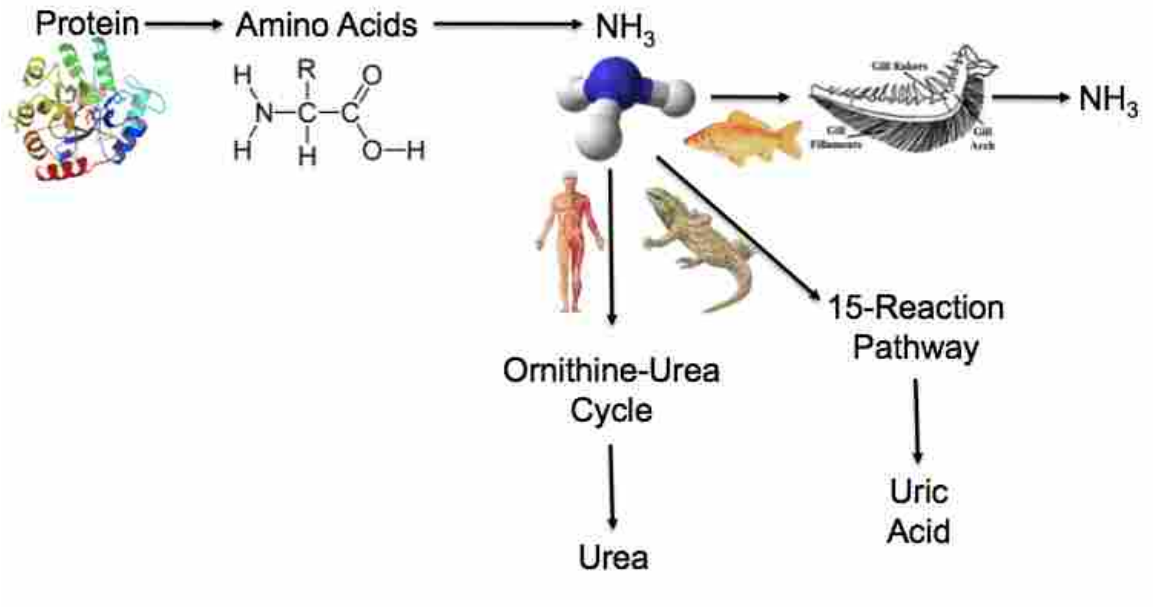

Protein
Amino Acids
NH3
NH3
15-Reaction Pathway
Ornithine-Urea Cycle
Uric Acid
Urea

Figure 1.2. Schematic diagram of Rh glycoprotein mediated ammonia excretion in freshwater fish. Three types of Rh protein are involved in the movement of ammonia from the blood to the water via the gill. First, ammonia that is present in red blood cells is transferred from the blood to the extracellular fluid via the Rhag, which is then taken up by gill epithelial cells across the basolateral membrane via Rhbg, before it is subsequently excreted to the water via apically-located Rhcg proteins. Under normal conditions, the H^+ generated at the gill surface by CO_2 hydration and the H^+-ATPase traps NH_3 as $NH4^+$, which maintains a steady NH_3 partial pressure diffusion gradient across the apical membrane of the gill cell (Adapted from Wright and Wood 2009).

FIGURE 1.2.

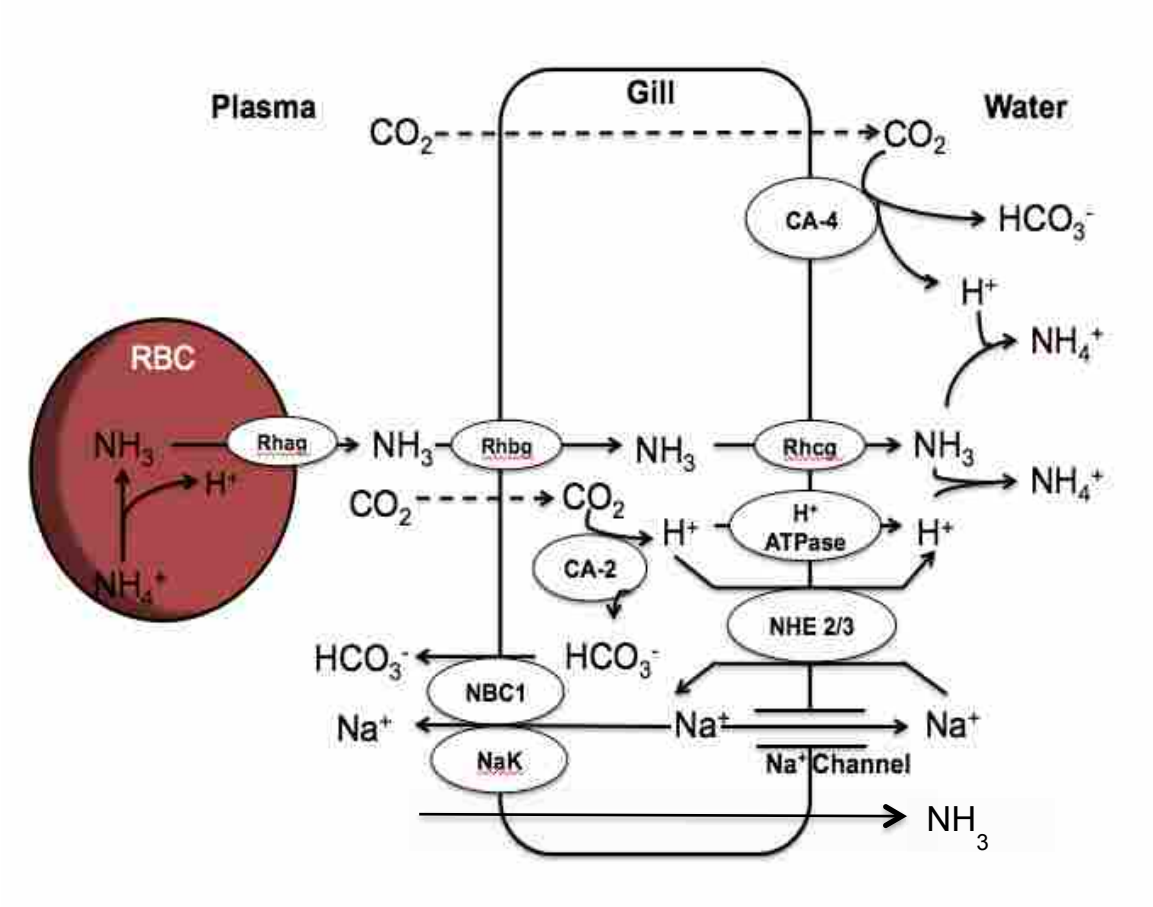

Plasma
Gill
Water
CO_2
CO_2
CA-4
HCO_3^-
H^+
NH_4^+
RBC
NH_3
Rhag
NH_3
Rhbg
NH_3
Rhcg
NH_3
NH_4^+
H^+
CO_2
CO_2
H^+
H^+ ATPase
H^+
CA-2
NHE 2/3
NH_4^+
HCO_3^-
HCO_3^-
NBC1
Na^+
Na^+
Na^+
NaK
Na^+ Channel
NH_3

Figure 1.3. Glutamate-Glutamine Cycling. 1. Glutamate (GLU) is released into the synaptic cleft from the excitatory presynaptic neuron. **2.** Excess glutamate is taken up by nearby astrocytes via Na^+/glutamate co-transporter EAAT-1 and EAAT-2, terminating excitatory signals. **3.** Within astrocytes, glutamate is converted to glutamine by catalyzing NH_4^+, ATP and glutamate via glutamine synthetase (GS). **4.** The glutamine product is exported into the extracellular space via Na^+/glutamine co-transporter (SN1) and taken up in the **5.** presynaptic neuron (GlnT) and packaged into vesicles (Figure adapted from Walsh et al. 2007).

FIGURE 1.3.

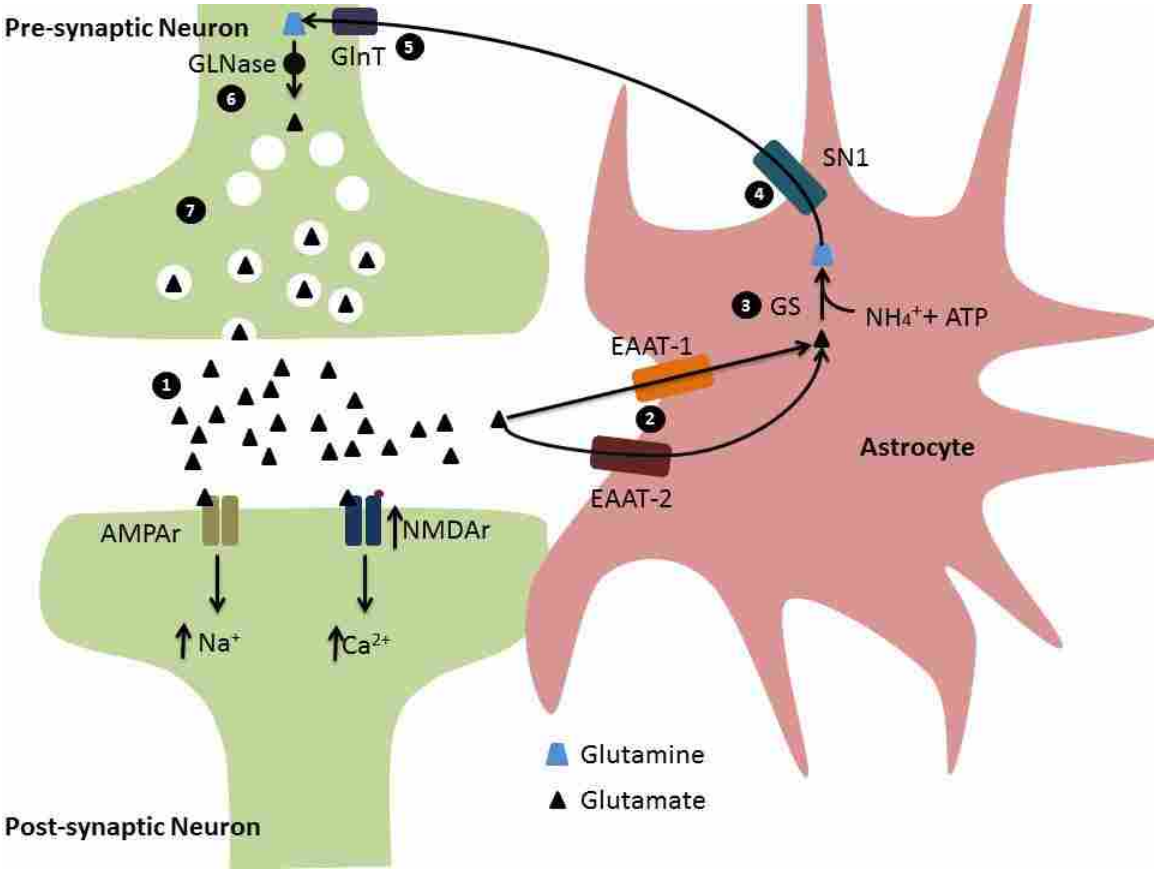

Pre-synaptic Neuron
GLNase
GlnT
5
6
7
SN1
4
3 GS
NH4+ + ATP
EAAT-1
1
2
Astrocyte
EAAT-2
AMPAr
NMDAr
Na+
Ca2+
Glutamine
Glutamate
Post-synaptic Neuron

Figure 1.4. Glutamate Cycling and NMDA receptors Function. 1. Glutamine is converted to glutamate via glutaminase (GLNase) catalyzed reaction and packaged into synaptic vesicles **2.** On the post synaptic cell there are two receptors that are usually coupled together, the AMPA receptor and NMDA receptor. These receptors are activated by glutamate, which is released by the pre-synaptic neuron into the synaptic cleft. **3.** Glutamate binds to the AMPA receptor causing the channels to open, leading to an influx of Na^+ ions leading to the depolarization of the cell. **4.** The AMPA-induced depolarization causes the repulsion of Mg^{2+} from the central channel of the NMDA receptors, which normally prevents the NMDA receptors from opening. As a result, Ca^{2+} enters the post-synaptic neuron down its electrochemical gradient, resulting in the activation of numerous second messenger pathways involved in learning and memory, and the regulation of physiological processes including generation of rhythms for respiration and locomotion.

FIGURE 1.4.

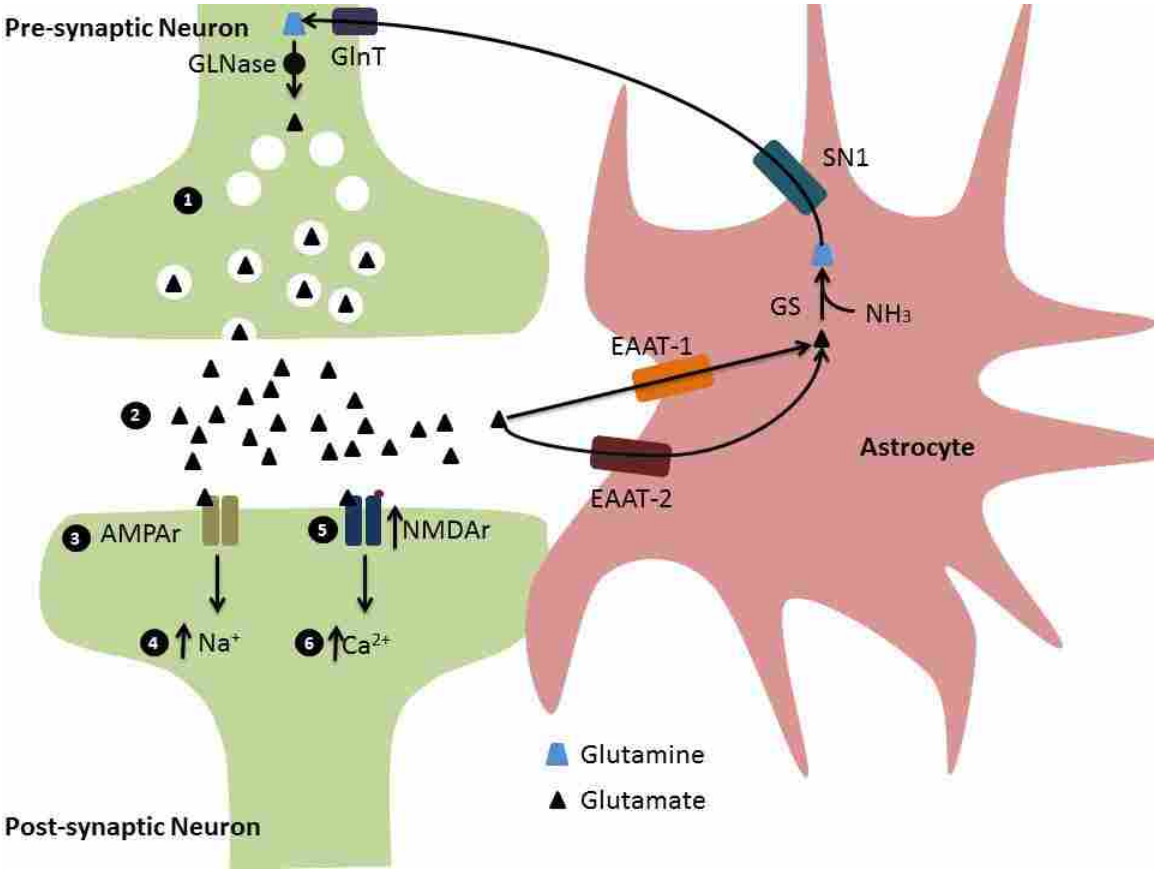
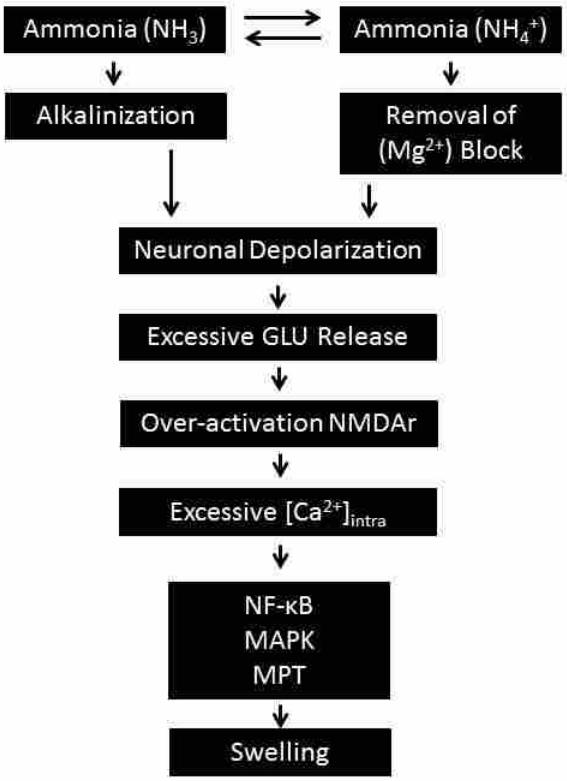

Pre-synaptic Neuron
GLNase
GlnT
SN1
1
GS
NH3
EAAT-1
2
Astrocyte
EAAT-2
3 AMPAr
5 NMDAr
4 Na+
6 Ca2+
Glutamine
Glutamate
Post-synaptic Neuron

Figure 1.5. Proposed mechanism of ammonia toxicity overview. Ammonia can either exist as NH_3 or NH_4^+. Intracellular alkalization can occur due to increase entry rate and concentration of NH_3. NMDA receptors are more susceptible to activation during HEA resulting in neuronal depolarization. The over-activations of NMDA receptors results in increased intracellular calcium, which can lead to ROS generation. Additionally, studies proposed that the generation of ROS might result from the induction of the mitochondrial permeability transition pore leading to ROS production.

FIGURE 1.5.


Ammonia (NH_3)
Ammonia (NH_4^+)
Alkalinization
Removal of (Mg^{2+}) Block
Neuronal Depolarization
Excessive GLU Release
Over-activation NMDAr
Excessive $[Ca^{2+}]_{intra}$
NF-κB
MAPK
MPT
Swelling

Chapter 2

Role of N-Methyl-D-Aspartate Receptor and Na^+/K^+-ATPase in Ammonia-Induced Brain Swelling in the Rainbow Trout *(Oncorhynchus mykiss)*

INTRODUCTION

Ammonia arises from the deamination of excess amino acids, but due to its high toxicity it must either be excreted or converted to less toxic end-products such as urea to prevent it from increasing to toxic levels (Mommsen and Walsh 1991; Wright 1995; Weihrauch et al. 2009). However, elevated levels of internal ammonia in mammals, hyperammonemia, can arise from liver damage, defects in the ornithine urea cycle or other causes such as, Reye's syndrome leading to brain swelling (encephalopathy), and subsequent damage to the central nervous system (CNS) triggering hyperexcitability, seizures, coma and death (Felipo and Butterworth 2002: Braissant 2012). In contrast to mammals, in which internal ammonia concentrations are tightly regulated, fishes may experience transient elevations in internal ammonia following feeding or vigorous exercise (Driezic and Hochachka 1978; Wang et al. 1996; Wicks and Randall, 2002a; Randal and Tsui 2002). Such natural spikes in plasma ammonia concentrations [T_{Amm}] may even approach those associated with death due to exposure to high external ammonia (HEA) in fasted animals (Wicks and Randall 2002a, b). Wilkie et al. (2015) recently reported that goldfish (*Carassius auratus*) and the closely related crucian carp (*Carassius carassius*), were not only highly tolerant to ammonia, but that these fish experienced 20-30 % increases brain water volume in response to HEA exposure. Moreover, they noted that brain water volume was restored following recovery in clean (ammonia-free) water. However, the underlying causes of ammonia-induced brain swelling in fishes, not to mention mammals, still remain unclear.

One possible cause of ammonia-induced brain swelling is increased glutamine production within the astrocytes of the brain (Brusilow 2002). Glutamine synthesis is

mediated by the ATP-dependent conversion of ammonia plus glutamate to form glutamine via glutamine synthetase in the brain (Wick and Randall 2002b; Randal and Tsui 2002; Kosenko et al. 2003; Albrecht and Norenberg 2006; Jayakumar et al. 2006; Norenberg et al. 2007; Sanderson et al. 2010). Although glutamine production is normally thought to protect against ammonia toxicity, it has been hypothesized to cause osmotic shifts of water into the intracellular space of astrocytes by increasing the osmolarity of the cell cytosol (Brusilow 2002). However, administration of the glutamine synthetase inhibitor (intraperitoneal; IP), methionine sulfoximine (MSO), failed to prevent ammonia-induced brain swelling in goldfish, despite preventing rises in brain glutamine concentration suggesting that glutamine only plays a minor, if any, role in this process. One goal of the present study was to further investigate what role, if any, glutamine accumulation played in mediating brain swelling in a more ammonia-sensitive rainbow trout (*Oncorhynchus mykiss*), by measuring changes in brain water volumes in trout that had been exposed to sub-lethal concentrations of high external ammonia (HEA) following the administration of MSO.

Ammonia is also known to mediate its toxic effects through the overactivation the of N-methyl-D-aspartate (NMDA) receptors (Fan and Szerb 1993; Hermengildo et al. 1996, 2000). NMDA receptors are glutamate-gated Ca^{2+}channels found primarily on neurons and they play a critical role in many biological processes including the development of the central nervous system (CNS), and the initiation of rhythms for breathing and locomotion. They also play a critical role in the process of learning, neuroplasticity and memory development (Wenthold et al. 2003; Kemp and McKernan 2002). The over-activation of NMDA receptor is thought to cause excitotoxic cell death

in which uncontrolled increases in intracellular Ca^{2+} lead to the generation of reactive oxygen species (ROS) by mitochondria and/or through various Ca^{2+} dependent enzymes such as NADPH oxidase (NOX) (Suzuki et al. 1985; Kosenko et al. 2001, 2003; Norenberg et al. 2009; Jayakumar et al. 2009). Indeed, HEA exposure leads to oxidative damage in the brain of the goldfish characterized by lipid peroxidation and the generation of protein carbonyls (Lisser 2016). Yet, the potential role of the NMDA receptor in ammonia-induced brain swelling remains unresolved. Accordingly, another goal of the present study was to determine if administration of the NMDA receptor antagonist dizocilpine (MK801) prevented ammonia-induced brain swelling from taking place in the rainbow trout.

Ultimately, in order for brain swelling to occur, an osmotic gradient must be generated to trigger fluid shifts from the extracellular to intracellular space of astrocytes and/or neurons. For instance, increased brain water content in rats following experimentally induced ischemia is accompanied by simultaneous increases in dry Na^+ and decreases in dry K^+ content (Gotoh et al. 1985). There are also numerous reports implicating electrolyte imbalances in the development of cerebral edema (e.g. Brunson et al. 1973; Shibata et al. 1974; Hossmann 1980; Gotoh et al. 1985; Katzman et al. 1997). However, the mechanistic basis of ionic and osmotic disturbances during ammonia-induced brain swelling in fish and mammals remain poorly understood. Thus, the final goal of the present study was to better characterize how potential shifts in Na^+ and K^+ balance contributed to changes in brain water content in rainbow trout during HEA. A particular aim was to test the hypothesis that ammonia inhibited the Na^+/K^+-ATPase,

which is integral to controlling intracellular Na^+ and K^+ balance in cells, but could be potentially inhibited by the production of ROS.

METHODS AND MATERIALS

Experimental Animals and Holding

Juvenile rainbow trout (*Oncorhynchus mykiss*; 50-100 g) were purchased from a commercial supplier (Rainbow Springs Trout Hatchery, Thamesford, Ontario) and transported to the Wilfrid Laurier University Animal Care Facility, where they were held in 220 L cylindrical holding tanks continuously receiving aerated well water (dissolved oxygen > 80% saturation) at 12 ± 1^{o}C and pH 7.8 ± 0.4. The animals were acclimated to the facility for a minimum of 2 weeks, during which they were fed 3 times a week, at a ration of 2% body weight, with commercially available dried pellets (3.0mm Martin Profishent^{TM} Fish Food for Aquaculture, Elmira, Ontario). Prior to experiments, the fish to be used were fasted for a period of 3-5 to eliminate the effects that feeding history might have on ammonia excretion and tolerance to build-ups of internal ammonia (Fromm, 1963; Hillaby and Randall 1979; Wright and Wood, 1985; Wicks and Randall 2002a), before being transferred to individual darkened, fish-holding containers (3-3.5 L in volume), where they were left to acclimate for 12-24 h. The fish were carefully examined for evidence of disease or stress that could have adversely affected fish health or experiments. All animal care and experiments conducted were approved by the Wilfrid Laurier University Animal Care Committee and followed the guidelines of the Canadian Council of Animal Care.

Experimental Set-Up

The experimental system was constructed with an angle iron frame, upon which was placed on a large PVC tray, which held the individual fish holding containers. An overhanging head tank drained into a flow-splitter that fed into each of the well aerated,

individual holding chambers, which received a continuous flow of water at a rate of 0.5 to 1.0 L per minute. Water drained out of the boxes into the PVC and then to a lower reservoir, from which the water was returned to the head tank using a submersible water pump. Water pH was carefully regulated between 7.9-8.1 during the experiment to ensure that the relative proportions of NH_3 and NH_4^+ to which the fish were exposed was constant. This was done using a pH-stat system comprised of a GK2401C pH electrode (Radiometer, Copenhagen, Denmark) running from a PHM82 pH meter Radiometer connected to a TTT80 auto-titrator (Radiometer). When pH deviated from the set point, 0.5M HCl was added into the head tank by opening a solenoid valve (Cole Parmer Instruments Co., IL. USA), activated by the auto-titrator.

Experimental Protocols

Experiment 1: Characterization of the Effects of HEA Exposure on Brain Water Content, Ion Concentration and Na^+/K^+-ATPase Activity in Rainbow Trout

The night before experiments, rainbow trout were transferred into individual 3.0-3.5 L dark rectangular holding chambers and left to acclimate overnight. The next morning ammonia exposure was initiated by cutting-off water flow to each holding chamber and to the entire recirculating system. An appropriate amount of 5 M NH_4Cl stock solution was then added to into each container, and to the entire system to establish a nominal target total ammonia concentration ($T[_{Amm}]$ = sum of NH_3 plus NH_4^+) of 1 mmol L^{-1} total ammonia in the water. Water pH in the system was monitored using the pH-stat set-up and at regular intervals (0, 4, 6, 12, 24, 36, and 48 h) in the holding chambers using a handheld pH meter (Oakton Instruments, pH 11 Model, Vernon Hills, IL). At each sampling period, sub-sets of animals were euthanized with 1g L^{-1} tricaine

methanesulfonate buffered with 2.0g L^{-1} $NaHCO_3$, and blood samples were collected by caudal puncture using a 1mL-heparinized (7 mg heparin in 25 mL Cortland's saline to counteract coagulation) syringe and 21 gauge needle, transferred to 1.5 mL microcentrifuge tubes, and then centrifuged at 10,000 X *g* for 3 minutes. The plasma was then transferred to clean microcentrifuge tubes, snap-frozen in liquid nitrogen (N_2) and stored at -80°C until it was quantified for plasma $T[_{Amm}]$ concentration. The cranial cavity was then exposed and the whole intact brain (telencephalon, cerebellum, vagal lobes and optic tectum) carefully extracted, transferred to pre-weighed 1.5 mL microcentrifuge tubes, and then dried to constant weight at 60^{o}C (~ 72 h) for the determination of brain water content. The brains of additional subsets of fish exposed to ammonia were also removed, snap frozen in liquid N_2 and stored at -80°C for later analysis of glutamine, ammonia, and Na^+-K^+-ATPase activity.

Experiment 2: The Effects of MSO on Ammonia-Induced Brain Swelling

To characterize what role glutamine played in brain swelling in rainbow trout, the fish were administered the glutamine synthetase inhibitor, MSO. As described for Experiment 1, the fish were transferred to their individual holding chambers the night before experiments. The next morning, however, subsets of fish were anesthetized (0.1g L^{-1} tricaine methanesulfonate buffered with 0.2g L^{-1} $NaHCO_3$) and injected intraperitoneally (IP) with MSO (10 mg kg^{-1}, dissolved in Cortland's saline; dose chosen based on previous experiments performed by Sidhu (2009)), Cortland's saline (7.25 g - NaCl, 0.38 g - KCl, 0.23 g - $CaCl_2 \cdot 2H_2O$, 0.23 g - $MgSO_4 \cdot 7H_2O$, 0.41 g - $NaH_2PO_4 \cdot H_2O$, and 1.0 g - $C_6H_{12}O_6$) or subjected to a sham treatment, in which anaesthetized fish were handled in an identical manner but not injected with solution. The next day the fish

were exposed to HEA (1 mmol L^{-1} total ammonia) as described above, followed by brain and blood collection at 48 h of exposure. Brain tissue water was determined as described under Experiment 1, but the brains of sub-sets of animals were also preserved in liquid N_2, and saved at -80^{o}C for later quantification of ammonia and glutamine.

Experiment 3: The Effects of MK801 on Ammonia-Induced Brain Swelling and Osmotic and Ion Regulation

To determine if NMDA receptor over-activation contributed to ammonia-induced brain swelling fish were administered the NMDA receptor antagonist MK801. As described above, fish were transferred to individual holding chambers and acclimated for 24h. Different groups of fish were then anesthetized with an anaesthetic dose of 0.1 g L^{-1} tricaine methanesulfonate and 0.2 g L^{-1} $NaHCO_3$ and administered (IP) MK801 (5 mg kg^{-1}, dissolved in Cortland's saline; dose chosen based on previous experiments performed by Wilkie et al. (2011)) or Cortland's saline or sham treatment. After 24 h recovery, the goldfish were exposed to ammonia (1 mmol L^{-1} HEA), and then sampled exactly as described for experiment 1. The brain was then dried to constant weight as described above, and blood was processed and saved for determination of plasma ammonia concentration.

Analytical Techniques

Water Ammonia Concentration

Water ammonia concentrations were determined spectrophotometrically using the salicylate-hypochlorite assay at 650nm (Verdouw et al. 1978) using a 96 micro-well plate spectrophotometer (Spectramax 190, Molecular Devices, Sunnyvale, CA).

Brain Tissue Water Measurements

After brains were dried to constant mass, the measured decrease in wet tissue mass (converted to mL of H_2O) was divided by the original wet tissue mass (g) to yield tissue water content per gram wet mass (mL H_2O g^{-1} wet mass). Brain water content was then expressed as ml H_2O/g dry mass by using the following equation:

$$\text{Water Content} = \frac{\text{ml } H_2O/\text{g wet mass}}{\text{g dry mass/ g wet mass}} \quad (1)$$

where brain water content is expressed in mL H_2O g^{-1} dry mass (Wilkie et al. 2015).

Brain Na^+ and K^+ Quantification

Following determination of brain water content, the dried brains were digested in 6 volumes of 1 N HNO_3, and a sub-sample of the digest diluted 1000 times using deionized water, and then acidified with 1% with 16 N, HNO_3, and quantified using flame atomic absorption (AA) spectrophotometry (PinAAcle 900, Perkin Elmer, Woodbridge, Ontario) and expressed as mmol kg^{-1} dry mass. Samples processed for K^+ quantification were further treated with 0.1% cesium chloride to improve analytical resolution on the Flame AA.

Determination of Plasma Concentration

Plasma ammonia concentrations were determined enzymatically (L-glutamate dehydrogenase; *Proteus sp.*; Sigma-Aldrich, G4387) with commercial reagents (AA0100, Sigma-Aldrich) and absorbance's determined using a plate spectrophotometer at 340 nm. Quality control checks were performed using commercial ammonia standard (A0978; 588µmol, Sigma-Aldrich).

Brain Glutamine and Ammonia Concentration Determination

Tissue Homogenization

Whole brain tissue was prepared for glutamine and ammonia analysis using a bead mill homogenizer (Precelly ® 24 Bead Mill Homogenizer). Briefly, frozen brain samples were weighed, and then transferred to chilled 2 mL polypropylene centrifuge tubes, followed by the addition of 5 volumes of ice-cold 7% perchloric acid (PCA) containing 1 mmol L^{-1} ethylenediaminetetraacetic acid (EDTA). Three ceramic homogenizing beads were then added to each tube, which was then transferred to the bead mill homogenizer, and homogenized for two-15 seconds intervals, separated by a 30 second cooling interval. The homogenized samples were immediately transferred to a 4^{o}C refrigerated centrifuged, left for 10 minutes, centrifuged for 8 min at 12,000 X *g*. The supernatant drawn-off and and then neutralized with 0.5 volumes of KOH (2N) to ensure pH was between 7-8. The neutralized supernatant was then used for the enzymatic quantification of ammonia as described above, or for glutamine concentration. Glutamine quantification was determined using glutamine synthetase and hydroxylamine, which leads to the generation of γ-glutamylhydroxymate which forms a brown-yellow color complex in the presence Fe^{3+}, and is measured at 540nm on the plate spectrophotometer.

Brain Na^{+}/K^{+}/ATPase Activity Determination

Frozen brain samples were weighed, and transferred into 2.0 mL polypropylene centrifuge tubes, to which three ceramic beads and 25-times volume of ice-cold SEID (1X concentrate; 0.025 g sodium deoxycholate in 100 mL SEI; 250 mM sucrose, 10 mM Na_2EDTA, 50 mM imidazole, pH 7.5) were added. The tubes containing the brain tissue were then immediately transferred to the bead mill homogenizer (described above), and homogenized for two-15 seconds intervals. The resulting homogenates were then

centrifuged for 8 minutes at 16,000 X *g* in a refrigerated centrifuge at 4°C (Thermoscientific, Micro21R), placed on ice, and the supernatant used to measure Na^+/K^+-ATPase activity within 30 minutes.

Measurements of brain Na^+/K^+-ATPase activity were based on McCormick (1993), in which Na^+/K^+-ATPase activity was calculated as the difference between uninhibited total ATPase activities minus ouabain-inhibited ATPase activity. To measure total ATPase activity, brain tissue homogenate was added to an assay solution containing pyruvate kinase (375 units), lactate dehydrogenase (200 units), NADH (0.22 mM), phosphenolpyruvate (2.8 mM), and ATP (0.7 mM), in imidazole buffer (50 mM). An identical solution, but also containing ouabain (0.7 mM) was used to measure ouabain-inhibited ATPase activity. All assay mixtures were brought to room temperature (25°C) immediately prior to enzyme activity determinations.

Total ATPase and ouabain-inhibited ATPase activity were determined using 96-well microplates that were placed on a dry ice pack during assay preparation containing 2.5µL of brain tissue homogenate in each well (in quadruplicate). Assay mixture, with or without ouabain, was then added to each well, at which time the microplate was placed into the temperature-controlled microplate spectrophotometer (25°C), and ATPase activity was determined based on the rate of NADH disappearance (oxidation) measured at 340 nm over 10 minutes. An NADH standard curve (0-40 nmoles/well) was used to determine the extinction coefficient of NADH at 340 nm, and the protein concentration of each sample was determined using the Bradford assay (Bradford 1976). Wet weight Na^+/K^+-ATPase activity was calculated as the difference of activity in the presence and absence of ouabain, which was divided by the protein content of each sample to yield the

specific Na^+/K^+-ATPase activity in μmol ADP mg $protein^{-1}h^{-1}$.

Statistical Analysis

All data are expressed as the mean ± 1 standard error of mean (SEM). With all the assumptions met, differences between treatments were analyzed using one-way analysis of variance (ANOVA) to determine significance and variation between means, followed by a Holm-Sidak post-hoc test. All significant differences were determined at the $p < 0.05$ level using GraphPad InStat, Version 3 (GraphPad Software, Inc., San Diego, CA, USA).

RESULTS

Effects of HEA Exposure on Brain Water Content, Ion Concentration and Na^+/K^+-ATPase Activity in Rainbow Trout

Ammonia-sensitive rainbow trout were able to survive a nominal total ammonia (T_{Amm}) concentration of 1 mmol L^{-1} for 48 h with approximately a 7% mortality rate. In surviving fish, HEA exposure brain water content increased by approximately 12% from 4.40 ± 0.12 to 4.90 ± 0.03 mL H_2O g^{-1} dry mass, after 48 h HEA exposure. But, it recovered to pre-exposure concentrations of 4.51 ± 0.08 mL H_2O g^{-1} dry mass within 24 h of depuration in clean, (nominally) ammonia-free water (**Figure 2.1A**).

The increases in brain water content, were accompanied by marked increases of plasma and brain tissue ammonia concentration ($[T_{Amm}]$), which increased approximately 13-fold from 133 ± 19 to 1670 ± 79 μmol L^{-1}. After 24 h recovery in ammonia-free water, however, plasma $[T_{Amm}]$ had returned to control levels of approximately 214 ± 26 μmol L^{-1} (**Figure 2.1B**).

Similarly, brain $[T_{Amm}]$ increased by approximately 2-fold from 1213 ± 89 to 2185 ± 188 μmol kg^{-1} after 48 h of HEA exposure, but returned to control concentrations of 1263 ± 103 μmol kg^{-1} (**Figure 2.2A**). Glutamine concentrations increased markedly, from control concentrations of 3486 ± 556 μmol kg^{-1} wet mass and increased to 16095 ± 851 μmol kg^{-1} wet mass following 48 h HEA (**Figure 2.2B**), but concentrations were partially reversed following 24 h recovery in ammonia-free water, decreasing to approximately 5156 ± 581 μmol kg^{-1} wet mass (**Figure 2.2B**).

Brain dry mass Na^+ and K^+ content of rainbow trout were significantly influenced by HEA exposure. Brain Na^+ content increased from approximately 320 ± 2 to 537 ± 20

μmol g^{-1} dry mass, but returned to control levels of 337 ± 30 μmol g^{-1} dry mass after 24 h of depuration in ammonia-free water (**Figure 2.3A**). In contrast to Na^+, brain K^+ content in HEA exposed rainbow trout demonstrated an inverse relationship. The K^+ content of controls was 429 ± 21 μmol g^{-1} dry mass compared to HEA exposed groups of 334 ± 16 μmol g^{-1} dry mass. After 24 h recovery in ammonia-free water brain K^+ was unchanged compared to the HEA exposure group (**Figure 2.3B**). It was notable, that these changes in brain ion content were accompanied by changes in Na^+/K^+-ATPase activity, which was 50% lower after 48 h HEA, and remained so after 24 h of recovery in ammonia-free water (**Figure 2.3C**).

The Inhibition of GS Effects on Brain Tissue Water Content

The inhibition of GS with MSO injection during 48 h HEA exposure did not significantly dampen or inhibit the accumulation of water in the rainbow trout brain. As expected, administration of MSO significantly dampened glutamine accumulation by approximately 88% in rainbow trout brain following exposure to 48 h HEA (**Table 2.2).** Despite the lack of glutamine accumulation, brain water content still increased from 4.48 ± 0.07 to 4.75 ± 0.06 mL H_2O g^{-1} dry mass after 48 h HEA exposure, but the increase in brain water content, 6 %, was less than measured in untreated fish (Compare **Figure 2.1A** to **Figure 2.4**). In sham and saline-injected fish, brain water content also increased with HEA exposure; approaching 4.92 ± 0.02 and 4.83 ± 0.07 mL H_2O g^{-1} dry mass, respectively (**Figure 2.4)**. As with brain water content, rainbow trout plasma [T_{Amm}] increased approximately 14-fold when exposed to 48 h HEA rising to an average of 1506 μmol L^{-1} in all exposed groups (**Table 2.3**).

In normal control sham and saline treated animals, brain Na^+ contents was 377 ± 30 and 386 ± 29 μmol g^{-1} dry mass, respectively (**Table 2.3**). Following 48 h HEA exposure brain Na^+ content in sham-treated and saline-treated controls were significantly increased by roughly 38% to 519 ± 18 μmol g^{-1} dry mass (**Table 2.3**). However, saline-treated exposed groups displayed minor increases of brain Na^+ content to 463 ± 20 μmol g^{-1} dry mass but, these increases were not significantly different (**Table 2.3**). The administration of MSO did not prevent the increase of brain Na^+ content in fish exposed to 48 h HEA. MSO-treated fish brain Na^+ content increased approximately 40% to 543 ± 26 μmol g^{-1} dry mass (**Table 2.3**). Unlike Na^+, brain K^+ content in rainbow trout exposed to 48 h HEA did not significantly change in this series of experiments, and was highly variable amongst the different treatment groups (**Table 2.3**).

Effects of MK801 on Brain Tissue Water Content and Brain Ion Content

Rainbow trout injected with MK801 (5mg kg^{-1}) did not demonstrate significant accumulation of water in the brain, remaining near control values of approximately 4.51 ± 0.04 mL H_2O g^{-1} dry mass (**Figure 2.5A**). In saline-injected trout and sham-injected trout, on the other hand, brain tissue water content was approximately 7% higher compared to control, saline and sham-injected fish (**Figure 2.5A**).

The treatment with MK801 did, however, alter other physiological responses to HEA including brain and plasma [T_{Amm}], which increased 8-fold from pre-exposure values, before returning to pre-exposure values during the recovery period (**Figure 2.5B**).

Notably, MK801-treated fish did not exhibit significant increases in brain Na^+ concentration. Similarly Na^+ content did not significantly increase following depuration

in ammonia-free water (**Figure 2.6A**). Similarly, dry brain Na^+ content in control sham and saline-treated fish significantly increased following 48 h HEA exposure.

Brain K^+ content in rainbow trout exposed to 48 h HEA did not demonstrate any significant changes between all groups (**Figure 2.6B**).

DISCUSSION

Ammonia-induced brain swelling in ammonia-sensitive rainbow trout

The present study demonstrates that the sub-lethal accumulation of ammonia by rainbow trout lead to significant brain swelling, which was completely reversed following reintroduction into ammonia-free water. Similar observations were recently reported for the goldfish and crucian carp (*Carassius carassius*), in which brain tissue water increased by approximately 20 % following exposure to 5 mmol L^{-1} and 20 mmol L^{-1} ammonia, respectively (Wilkie et al. 2015). The ability for fish to restore brain water volume, with no obvious evidence of damage or adverse behavioural effects may be because the relative volume of the cranial cavity of these fish is much larger compared to the size of their brain (Van der Linden et al. 2001). This would reduce the risk of brain damage arising from increases in intracranial pressure and/or compression of the brain due to contact with the walls of the cranium, which commonly results neuropathology and sometimes death in mammals (Eddy, 2005; Walsh et al. 2007).

Due to the absence of an ornithine urea cycle in most teleost fishes they lack the capacity to efficiently convert ammonia to less toxic urea in response to increases in internal ammonia (Mommsen and Walsh 1989; Wright 1995; Wood 2001). Rather ammonia must either be excreted or temporarily tolerated. The relatively large cranial cavities of fishes such as the salmonids and cyprinids may therefore be important for allowing fish to cope with upward spikes in internal ammonia elevations following feeding and perhaps even exhaustive exercise, both of which can result in large increases in internal ammonia (Driedzic and Hochochka 1978; Wang et al. 1996; Wicks and Randall 2002a,b). However, fluctuations in brain water volume may also be caused by

other stressors, and may not be unusual in fish. Indeed, magnetic resonance imaging (MRI) did demonstrate that, brain water volume increased by approximately 6.5 % in common carp (*Cyprinus carpio*) following 2 h anoxia exposure (Van der Linden et al. 2001). More recently, 10 % increases in brain water content were reported for crucian carp following 48 h of anoxia (Wilkie et al. 2015). Despite the fact that brain encephalopathy, or brain swelling, does occur in at least some fishes, little is known about the underlying causes.

Brain glutamine concentration and osmotic disturbances

Although glutamine is normally considered to be involved in the detoxification of ammonia (Cooper and Plum 1989; Mommsen and Walsh 1991; Ip et al. 2001), a number of groups have suggested that glutamine accumulation in the presence of elevated internal ammonia may be in fact toxic (Brusilow 2002; Albrecht and Norenberg 2006). As expected, brain glutamine concentration increased during HEA exposure, supporting the suggestion that excess NH_4^+ was converted to glutamine by glutamine synthestase (GS) via an ATP-dependent reaction of $NH_4^+$and glutamate (E.g. Mommsen and Walsh 1992; Felipo and Butterworth 2002; Wicks and Randall 2002; Ip et al. 2005; Walsh et al. 2007). This enzymatic processes has been proposed to be an essential factor for the detoxification of ammonia in mammals (Cooper and Plum 1989; Felipo and Butterworth 2002), and numerous fishes, including rainbow trout (Wicks and Randall 2002a; Sanderson et al. 2010), as well as goldfish (Wilkie et al. 2011, 2015), common carp (*Cyprinus carpio;* Dabrowska and Wlasow, 1986), Lake Magadi tilapia (*Oreochromis alcalicus grahami*; Randall et al. 1989), toadfish (*Opsanus beta*; Mommsen and Walsh 1989; Veauvy et al. 2005), Amazonian freshwater stingray (*Potamotrygon motoro*; Ip et

al. 2009), and the mudskipper (*Boleophthalmus boddarti*; Peng et al. 1998) all demonstrate substantial brain glutamine accumulation following HEA exposure.

In addition to being a metabolic waste product, ammonia is also involved in the glutamate-glutamine pathway between neurons and astrocytes (Felipo and Butterworth 2002; Kelly and Christine 2010). However, a number of studies in the last decade or so have suggested that the accumulation of glutamine leads to osmotic disturbances leading to subsequent water influx and cell swelling (Brusilow 2002). Indeed, preventing glutamine accumulation by inhibiting glutamine synthetase with MSO attenuated brain swelling in rats undergoing experimentally induced hyperammonemia (Takahashi et al. 1991; Willard-Mack et al. 1996; Tofteng et al. 2006). Certainly, glutamine and brain tissue water content appeared to be correlated with one another in the rainbow trout exposed to HEA (**Figure 2.1A and 2.1D**). At first glance, these observations would support the hypothesis that glutamine acts as an intracellular osmolyte, promoting the influx of water and swelling in the brain (Brusilow 2002). However, the administration of MSO did not prevent increases in brain water content, despite marked reductions in glutamine accumulation during HEA exposure (**Figure 2.4 & Table 2.2**). Similarly, Wilkie et al. (2015) reported that the suppression of glutamine synthesis in goldfish exposed to HEA (5 mmol L^{-1}) following MSO administration did not attenuate ammonia-induced increases in brain tissue water. These observations tend to suggest that glutamine accumulation does not directly cause the increases in intracellular osmolarity required to drive the osmotic influx of water that results in ammonia-induced encephalopathy in these fishes.

Another possibility is that brain swelling due to hyperammonemia may be an indirect consequence of glutamine production in the brain. Albrecht and Norenberg (2006) proposed that glutamine may acts as a silent carrier ("Trojan horse") of NH_3, which is taken up along with glutamate and converted, to glutamine via GS in astrocytes. They suggest that much of this glutamine is then hydrolyzed back to glutamate and NH_3 by phosphate activated glutaminase (PAG) by the mitochondria, where NH_3 triggers the production of ROS through the induction of the mitochondria permeability transition (MPT), ultimately resulting in astrocytic swelling. In goldfish, exposure to HEA results in oxidative damage characterized by protein carbonyl formation and lipid peroxidation in warmer (14 ºC) water but oxidative damage and brain swelling are attenuated during HEA in cold (4 ºC) water, supporting the hypothesis that hyperammonemia results in ROS production (Lisser 2016). While these findings strongly implicates that ammonia-induced brain swelling is related to ROS production, the underlying mechanisms of ROS production with hyperammonemia have not yet been resolved. While the findings of the present study suggest that the accumulation of intracellular glutamine does not directly lead to swelling in the rainbow trout brain, an indirect role for glutamine through the production of ROS cannot yet be ruled out.

Protection in ammonia-sensitive rainbow trout from ammonia toxicity

It remains widely accepted that ammonia toxicity involves NMDA receptor over-activation (Fan and Szerb 1993; Hermenegildo et al. 1996, 2000; Wilkie et al. 2011). In mammals, the overactivation of NMDA receptor results in marked increases of intracellular Ca^{2+} and Na^{+}, which can stimulate the production of ROS through various Ca^{2+} dependent enzymes such as, NADPH oxidase (NOX) (Suzuki et al. 1985;

Jayakumar et al. 2009) forming cNOS which, generates NO and free radicals (Nakamura et al. 1989; Mayer et al. 1990; Kramer and Sharp 1997; Cocco et al. 1999; Kosenko et al. 2001; Jayakumar et al. 2009). As described above, the overproduction of ROS may in turn trigger the initiation of mitochondrial permeability transition (MPT; Rama Rao et al. 2005), as well as the activation of MAP Kinase (MAPK), and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) leading to lipid peroxidation, protein damage, RNA/DNA oxidation, and necrosis/apoptosis, astrocytic swelling (Norenberg et al. 2009).

The findings of the present study also show that NMDA receptor likely plays a key role in the response of the trout to hyperammonemia. The observation that administration of the NMDA receptor antagonist MK801, prevented ammonia-induced brain swelling in rainbow trout strongly suggests that the NMDA receptor plays a key role in the ammonia toxicity response of fishes. Further evidence supporting this conclusion includes previous observations showing that MK801 enhanced survival rate in ammonia-sensitive rainbow trout exposed to HEA (Wilkie et al. 2011), as well in ammonia tolerant weather loach (*Misgurnus anguillicaudatus*) injected with ammonium acetate (Tsui 2004). Likewise, the administration of MK801 to a plainfin midshipman (*Porichtys notatus*), increased delayed the unconsciousness during exposure to 10 mM ammonium chloride (Veauvy et al. 2005; Walsh et al. 2007). Notably, however, the survival of some ammonia-tolerant fishes is not affected by prior MK801 administration when internal ammonia concentrations are elevated. This includes the ammonia-tolerant African sharptoothcatfish (*Clarias gariepinus*), in which MK801 treatment (IP injection) failed to increase survival time following a lethal dose of ammonium acetate (20 μmol g^{-}

[1]; Wee et al. 2007), and MK801 treated goldfish exposed to 8 mM total ammonia (Wilkie et al. 2011). Similar observations were made in two species of mudskippers (*Periophthalmodon schlosseri* and *Boleophthalmus boddaerti*), where MK801 treatment prior to lethal injection of ammonium acetate had no protective effect (Ip et al. 2005). *In vitro* electrophysiology experiments have shown that MK801 does irreversibly inhibit NMDA receptor activity in the goldfish (Wilkie et al. 2008), suggesting that goldfish uses other mechanisms to prevent excitotoxicity in the presence of ammonia. Indeed, NMDA receptor downregulation, characterized by a 50 % reduction in the NR1 subunit of abundance, may be one factor that protects the goldfish from ammonia toxicity during longer-term exposure to HEA (Wilkie et al. 2011). Nevertheless, the weight of evidence suggest that in more ammonia-sensitive fishes such as the trout, ammonia-induced brain swelling is related to over-excitation NMDA receptor that lead to homeostatic disturbances, which compromises the osmoregulatory capacity of the brain.

Ionoregulation and disturbances during HEA

The significant increase in brain water content that was observed in the rainbow trout during HEA exposure was accompanied by marked increases in dry Na^+ concentration along with lesser decreases in dry K^+. Such findings are consistent with an impairment of ionoregulatory processes in neurons and/or astrocytes, which could have led to a net increase in intracellular Na^+ content, and an increase in intracellular osmolarity sufficient to cause cell swelling. Further support for this hypothesis was that ammonia exposure resulted in 50 % reduction in brain Na^+/K^+-ATPase activity, after 48 h. This finding is consistent with previous reports displaying the similar relationship between brain swelling and elevated Na^+ content in mammalian models following

ischemia (Gotoh et al. 1985; Rose and Ransome 1996a, 1997; Pisani et al. 1998; Kelly and Christine 2010).

The brain edema observed in response to hyperammonemia in trout and in mammals is similar to that demonstrated in models of mammalian ischemia/hypoxia, in which the rapid accumulation of intracellular Na^+ leads to brain edema (Hansen 1985; Karpiak et al. 1989). Under anoxic conditions, cessation of aerobic ATP production leads to ionic disturbances characterized by increased intracellular Na^+ and Ca^{2+}, which result in osmotic shifts of water into the intracellular space of neurons and neuronal swelling (Lutz et al. 2003; Walsh et al. 2007; Bickler and Buck 2009). However, swelling is thought to be restricted to the astrocytes rather than the neurons with ammonia toxicity (Felipo and Butterworth 2002; Albrecht and Norenberg 2006; Walsh et al. 2008). Studies on cultured rat astrocyte cells, demonstrated that 5 mM NH_4Cl treatment lead to increased Na^+-K^+-Cl^- co-transporter (NKCC1) abundance and swelling, which was prevented in following inhibition with the NKCC1 antagonist bumetanide (Jayakumar et al. 2008). These findings suggests that disturbances to intracellular ion balance arising from changes in the kinetics of different ion transporters could also trigger ammonia-induced brain swelling observed in the rainbow trout. Reductions in ATP production have also been reported in mammalian models of ammonia toxicity (Kosenko et al. 1994), which could potentially alter energy status and ion homeostasis by depriving ion pumps such as the Na^+/K^+-ATPase of ATP. Indeed, the present study implicates impaired ionoregulation as an underlying cause of this process, at least in fishes.

The 50% reduction in Na^+/K^+-ATPase activity that was observed with HEA would at least partially explain the increased Na^+ concentrations that were observed in

trout brain. However, a potential mechanism to explain these reductions is still lacking. One possibility is that the pump is impaired due to free-radical production during HEA. As described above, hyperammonemia causes oxidative stress in both teleosts (Ching et al. 2009; Sinha et al. 2014; Lisser 2016) and mammals (Kosenko et al. 2003a,b; Jayakumar et al. 2006a). A potential source of free radical generation could be NMDA receptor overactivation, which leads to elevated concentrations of intracellular Ca^{2+}, leading to mitochondrial dysfunction and ROS production (Kosenko et al. 2003a, b; Albrecht and Norenberg 2006; Jayakumar et al. 2006). Overproduction of ROS leads to lipid peroxidation, carbonylation of proteins, and damage to nucleic acids (Norenberg et al. 2007; Chen et al. 2008). As a result, enzymes, transport proteins and elements for the plasma membrane can be damaged, resulting in homeostatic disturbances in cells. Recent, evidence suggests that one potential target of free radicals is the α –subunit of the Na^+/K^+-ATPase (Gusarova et al. 2011).

The Na^+/K^+-ATPase is a ubiquitous plasma membrane protein, and is responsible for maintaining intracellular ion homeostasis by maintaining the electrochemical for Na^+ and K^+ gradients across the plasma membrane (Jamme et al. 1995; Li and Stys 2001; Lees et al. 1990; Lima et al. 2009). The pump is comprised of a α-subunit, which is the site of ATP hydrolysis and the translocation of Na^+ and K^+ across the plasma membrane, and a β-subunit, which stabilizes the pump by maintaining its polarity in the plasma membrane (Campbell 2000; Li and Langhans 2015). The impairment of Na^+/K^+-ATPase α-subunit by ROS would ultimately alter Na^+ and K^+ balance in the cell resulting in osmotic disturbances. Although information on ROS-induced ionic disturbances are limited, recent evidence demonstrates that H_2O_2, ROS and other free radicals ($O_2^{\cdot-}$, $HO^{\cdot}$)

appear to target and decrease the expression of α_1-subunit of the Na^+/K^+-ATPase in rat jejunum and cardiac myocytes (Orsenigo et al. 2007; Sag et al. 2013). It is therefore possible that similar ROS production plays a similar role in inhibiting Na^+/K^+-ATPase activities in the brain of the rainbow trout in response to hyperammonemia, contributing to Na^+ accumulation and ultimately brain edema due to the corresponding osmotic influx of water. A decrease Na^+/K^+-ATPase observed in ammonia-challenged rainbow trout could also lead to action potential prolongation (Sag et al. 2013), which would exacerbate any disturbances through overactivation of NMDA receptors and additional production of ROS.

CONCLUSION

The present study has demonstrated that ammonia-induced neurotoxicity in rainbow trout is reflected by significant alterations to brain Na^+ and K^+ balance due to an inhibition of the Na^+/K^+-ATPase, likely caused by ammonia-induced ROS production. Glutamine accumulation does not appear to directly lead to ammonia-induced brain swelling in rainbow trout, but a contribution through the stimulation of mitochondrial ROS via phosphate-activated glutaminase cannot be ruled out. However, ammonia-induced brain swelling is likely mediated by over-activation of NMDA receptors, which could potentially result in the subsequent ROS production known to occur during HEA exposure in fish. Nevertheless, the results of the present study implicate decreased Na^+/K^+-ATPase activity and subsequent imbalances in Na^+ and K^+ balance as one mechanism as the cause of ammonia-induced brain swelling in the rainbow trout, and this process likely occurs in response to ammonia-induced ROS production.

Table 2.1. Measured Ammonia Water Concentration for Rainbow Trout Exposed to High External Ammonia. Chart represents all experiments. Data shown represented as mean ± SEM.

Rainbow Trout (HEA)	**Time (h)**	**Actual Water Ammonia (mmol L^{-1})**
	4	1.02 ± 0.12
	6	1.13 ± 0.03
	12	1.27 ± 0.07
	24	1.09 ± 0.09
	48	1.13 ± 0.04
Rainbow Trout (MK801)	**Time (h)**	**Actual Water Ammonia (mmol L^{-1})**
	4	1.13 ± 0.21
	6	1.09 ± 0.12
	12	1.17 ± 0.04
	24	0.94 ± 0.02
	48	1.23 ± 0.07
Rainbow Trout (MSO)	**Time (h)**	**Actual Water Ammonia (mmol L^{-1})**
	4	1.11 ± 0.11
	6	1.09 ± 0.09
	12	0.97 ± 0.12
	24	1.04 ± 0.01
	48	1.24 ± 0.08

Table 2.2. Concentrations of glutamine in the brain of rainbow trout in the absence (control; *n* = 7), presence of HEA for 48 h, (T_{Amm} 1 mmol L^{-1}; *n* = 8) and (MSO Treated *n = 10*) and after 24 h recovery in ammonia-free well water (*n* = 5). Columns sharing the same letter are not significantly different from one another (One-way ANOVA followed by the Holm-Sidak post test $p<0.05$).

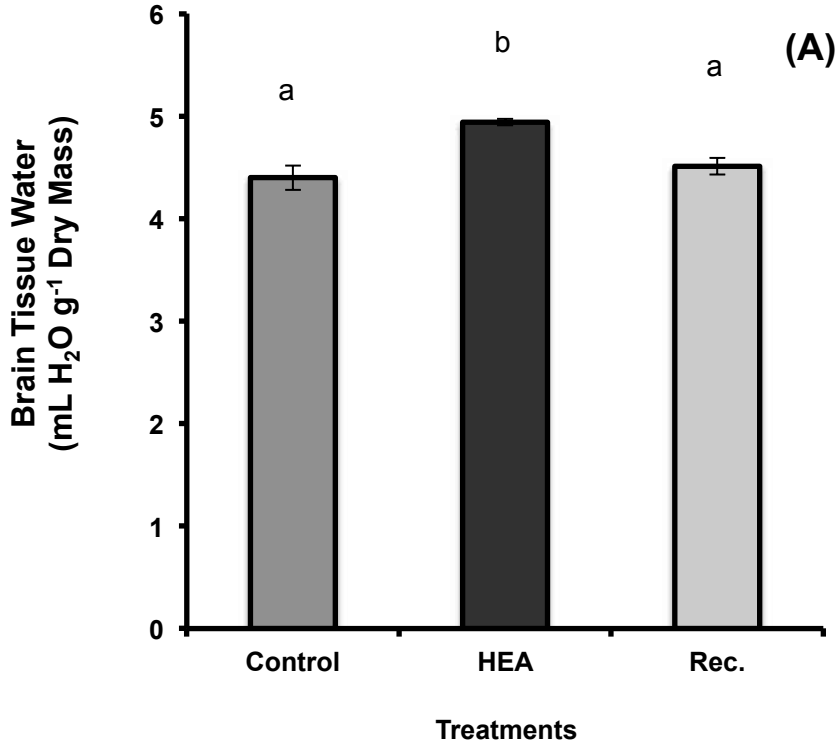
Rainbow Trout MSO Brain Glutamine	**Treatments**	**Brain Glutamine Measurements (µmol kg^{-1})**
	Control	3485.7 ± 55.7 (a)
	HEA	16094.5 ± 850.8 (b)
	MSO HEA	2127.1 ± 301.2 (a)
	HEA Recovery	5156.1 ± 581.2 (a)

Table 2.3. The Effects of MSO on Brain Na^+ in dry mass, Brain K^+ in dry mass and Plasma [TAmm] in Rainbow Trout Summary Chart. (Sham *n* = 8 and Saline *n* = 8) or exposed to 48 h HEA (T_{Amm} 1 mmol L^{-1}; Sham HEA *n* = 9, Saline HEA *n* = 13, and MSO HEA *n* = 12), and following recovery in ammonia-free well water (MSO Recovery *n* = 6) Data shown represented as mean ± SEM. Columns sharing the same letter are not significantly different from one another (One-way ANOVA followed by the Holm-Sidak post test $p<0.05$).

Rainbow Trout Brain Na^+, K^+ and Plasma $[T_{Amm}]$	**Treatments**	**$[Na^+]$ (µmol g^{-1} dry mass)**	**$[K^+]$ (µmol g^{-1} dry mass)**	**Plasma $[T_{Amm}]$ (µmol L^{-1})**
	Sham	377 ± 30 (a)	542 ± 53 (a)	133 ± 47 (a)
	Saline	386 ± 29 (a)	533 ± 31 (a)	204 ± 29 (a)
	Sham HEA	519 ± 18 (b)	556 ± 8 (a)	1464 ± 31 (b)
	Saline HEA	464 ± 20 (ab)	487 ± 21 (a)	1586 ± 40 (b)
	MSO HEA	544 ± 26 (b)	559 ± 26 (a)	1469 ± 44 (b)

Figure 2.1. (A) Brain tissue water content and (B) plasma ammonia concentration in rainbow trout held under control conditions in the absence (n = 10) or exposed to 48 h HEA (T_{Amm} 1 mmol L^{-1}; n = 11), and following recovery in ammonia-free water (n = 7). Data are presented as the mean ± SEM. Bars sharing the same letter are not significantly different from one another (One-way ANOVA followed by the Holm-Sidak post test $p<0.05$).

FIGURE 2.1.


(A)
Brain Tissue Water
(mL H_2O g^{-1} Dry Mass)
6
5
4
3
2
1
0
a
b
a
Control
HEA
Rec.
Treatments

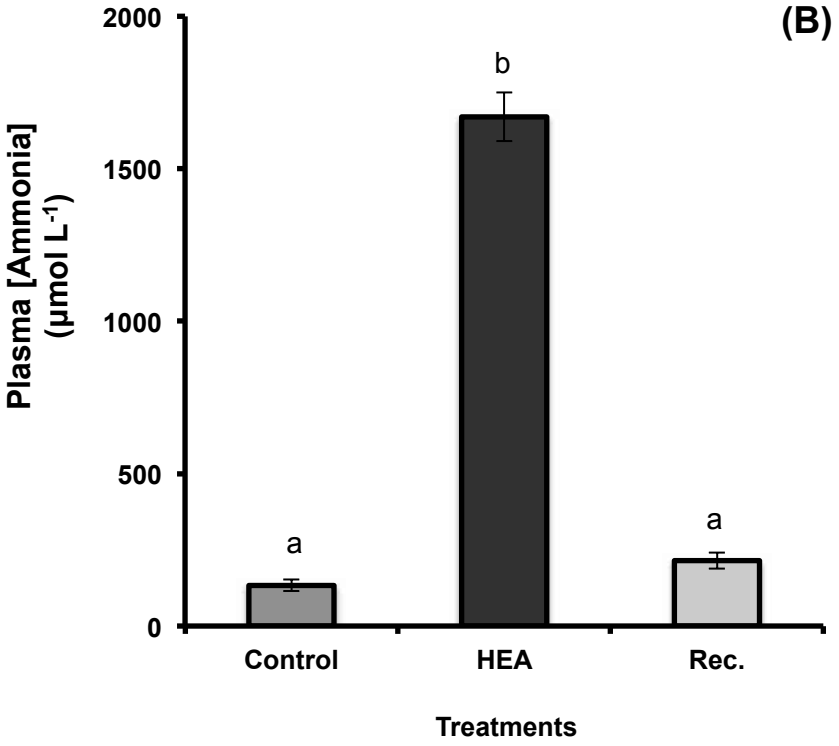

(B)
Plasma [Ammonia]
(µmol L^{-1})
2000
1500
1000
500
0
a
b
a
Control
HEA
Rec.
Treatments

Figure 2.2. Concentrations of (A) ammonia and (B) glutamine in the brain of rainbow trout in the absence (control; $n = 7$), presence of HEA for 48 h, (T_{Amm} 1 mmol L^{-1}; $n = 8$), and after 24 h recovery in ammonia-free well water ($n = 5$). Bars sharing the same letter are not significantly different from one another (One-way ANOVA followed by the Holm-Sidak post test $p<0.05$).

FIGURE 2.2.

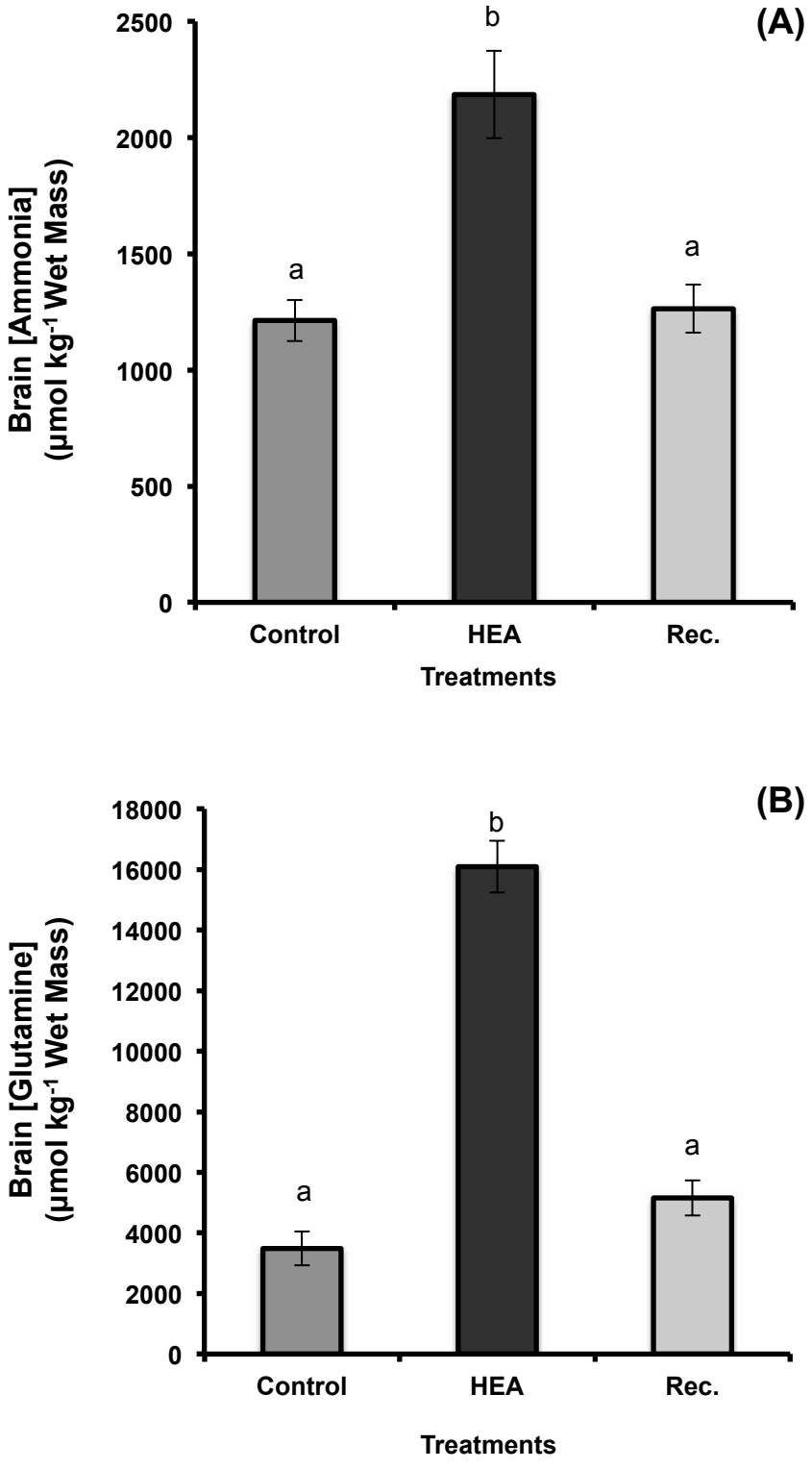
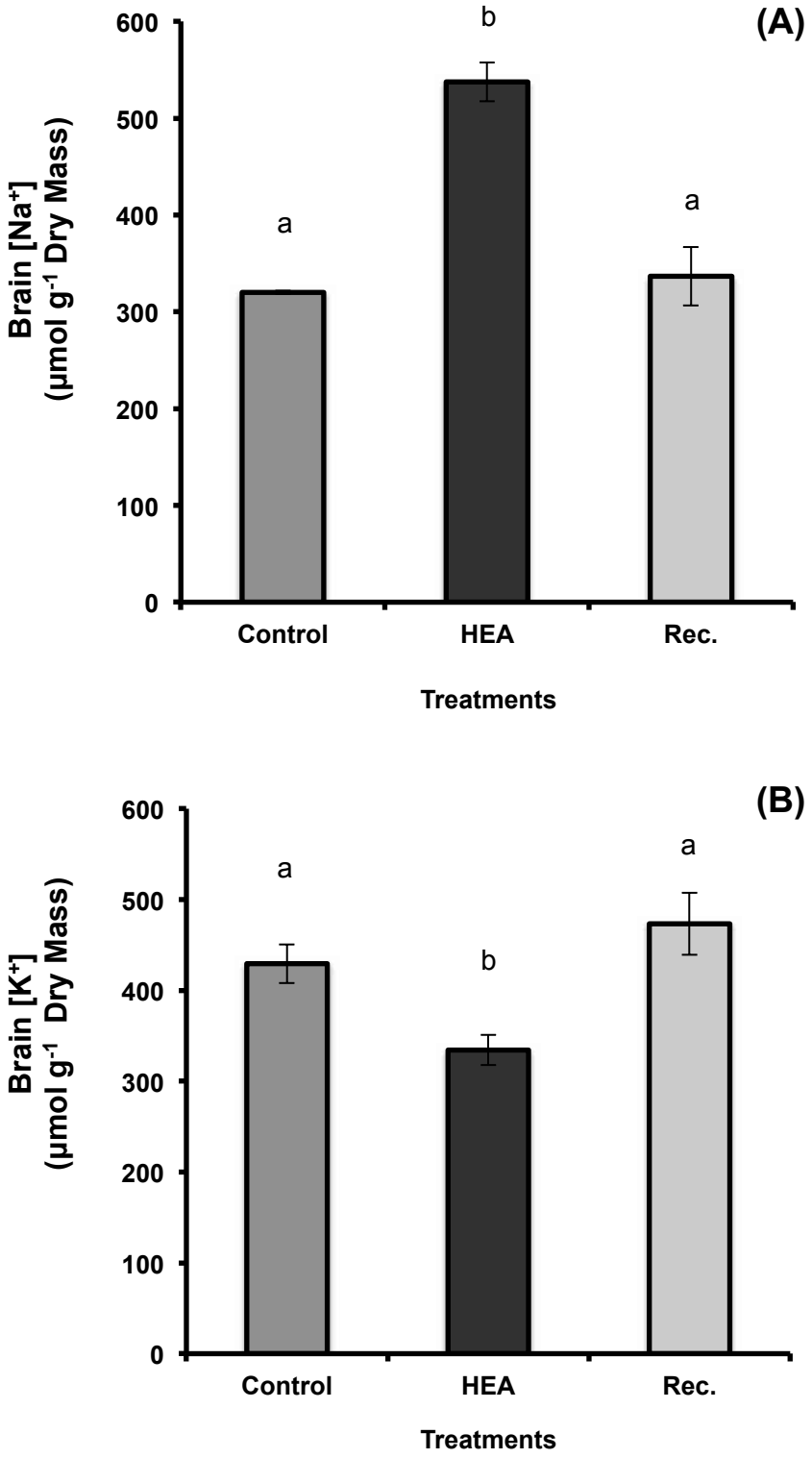

(A)
Brain [Ammonia] (µmol kg-1 Wet Mass)
2500
2000
1500
1000
500
0
a
b
a
Control
HEA
Rec.
Treatments
(B)
Brain [Glutamine] (µmol kg-1 Wet Mass)
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
a
b
a
Control
HEA
Rec.
Treatments

Figure 2.3. Effect of HEA exposure (1 mmol L^{-1}) on (A) dry brain Na^+ content, (B) dry K^+ content, (C) brain Na^+/K^+-ATPase activity in rainbow trout held under control conditions (*n* = 6-10), or exposed to HEA (T_{Amm} = 1 mmol L^{-1}) for 48 h (*n* = 10-11), and following recovery in ammonia-free well water (*n* = 5-7). Data are presented as mean ± SEM. Bars sharing the same letter are not significantly different (One-way ANOVA followed by the Holm-Sidak post test $p<0.05$).

FIGURE 2.3.


(A)
Brain [Na+] (µmol g-1 Dry Mass)
600
500
400
300
200
100
0
a
b
a
Control
HEA
Rec.
Treatments
(B)
Brain [K+] (µmol g-1 Dry Mass)
600
500
400
300
200
100
0
a
b
a
Control
HEA
Rec.
Treatments

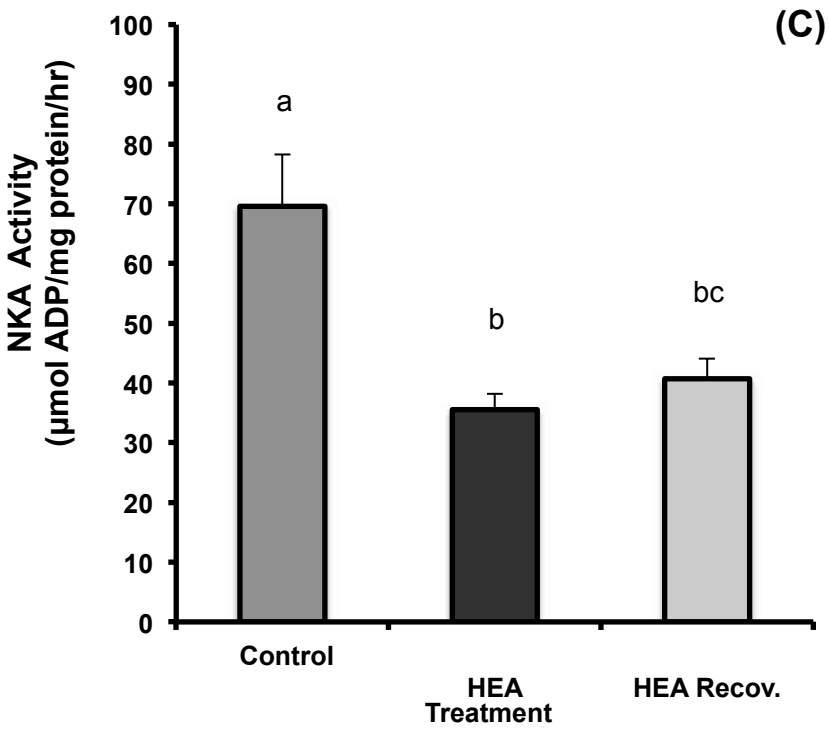

(C)
NKA Activity
(µmol ADP/mg protein/hr)
100
90
80
70
60
50
40
30
20
10
0
a
b
bc
Control
HEA
Treatment
HEA Recov.

Figure 2.4. The Effects of MSO on **(A)** Brain tissue water content measured in rainbow trout held under control conditions (Sham $n = 8$ and Saline $n = 8$) or exposed to 48 h HEA (T_{Amm} 1 mmol L^{-1}; Sham HEA $n = 9$, Saline HEA $n = 13$, and MSO HEA $n = 12$), and following recovery in ammonia-free well water (MSO Recovery $n = 6$). Data are presented as mean ± SEM. Bars sharing the same letter are not significantly different (One-way ANOVA followed by the Holm-Sidak post test $p<0.05$).

FIGURE 2.4.

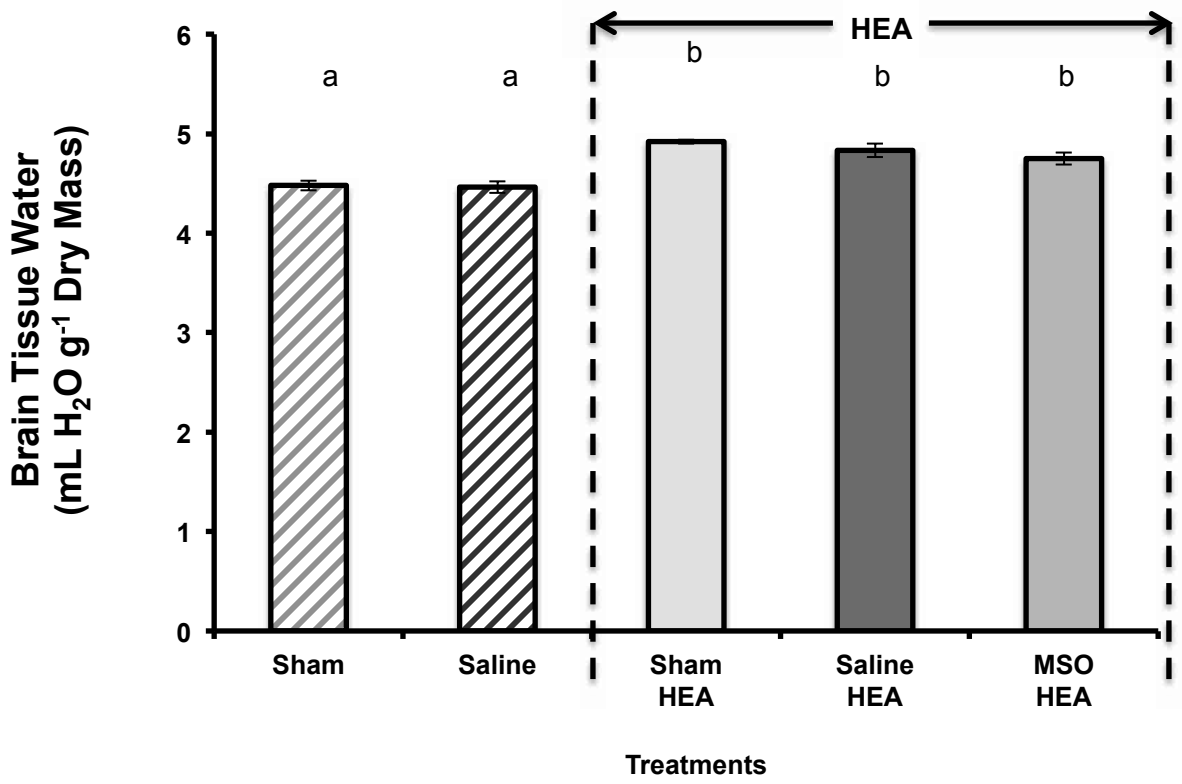
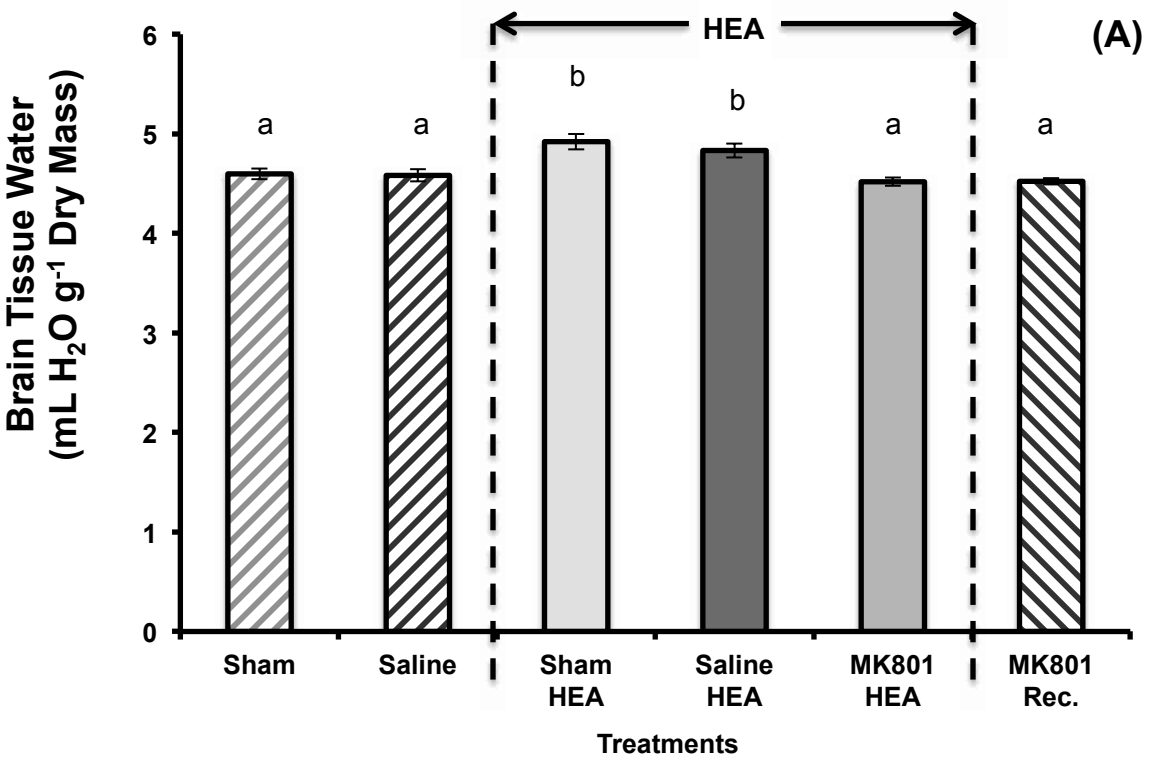

HEA
a
a
b
b
b
6
5
4
3
2
1
0
Brain Tissue Water
(mL H_2O g^{-1} Dry Mass)
Sham
Saline
Sham HEA
Saline HEA
MSO HEA
Treatments

Figure 2.5. Effect of MK801 administration on (A) brain tissue water content and (B) plasma T_{Amm} concentration measured in rainbow trout held under control conditions (Sham n = 11; Saline n = 13) or exposed to 48 h HEA (T_{Amm} 1 mmol L^{-1}; Sham HEA n = 9; Saline HEA n = 13; MK801 HEA n = 9), and following recovery in ammonia-free well water (n = 8). Data are presented as mean ± SEM. Bars sharing the same letter are not significantly different (One-way ANOVA followed by the Holm-Sidak post test $p<0.05$).

FIGURE 2.5.


HEA
(A)
Brain Tissue Water
(mL H_2O g^{-1} Dry Mass)
6
5
4
3
2
1
0
a
a
b
b
a
a
Sham
Saline
Sham HEA
Saline HEA
MK801 HEA
MK801 Rec.
Treatments

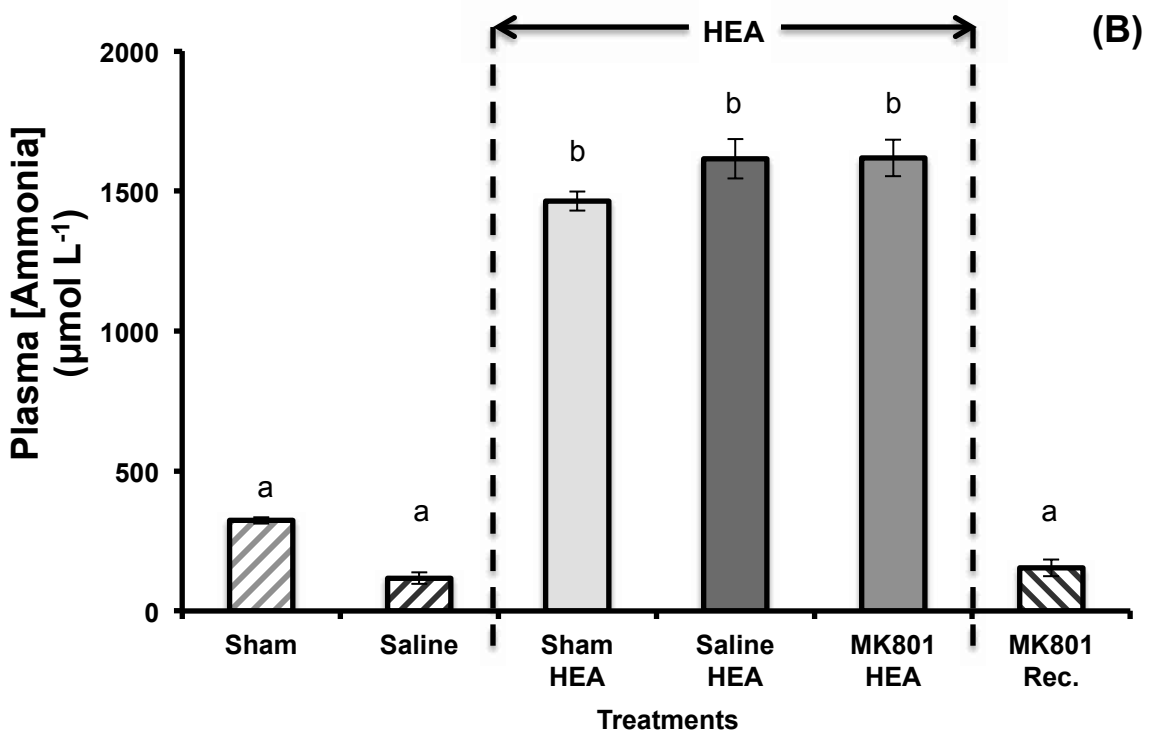
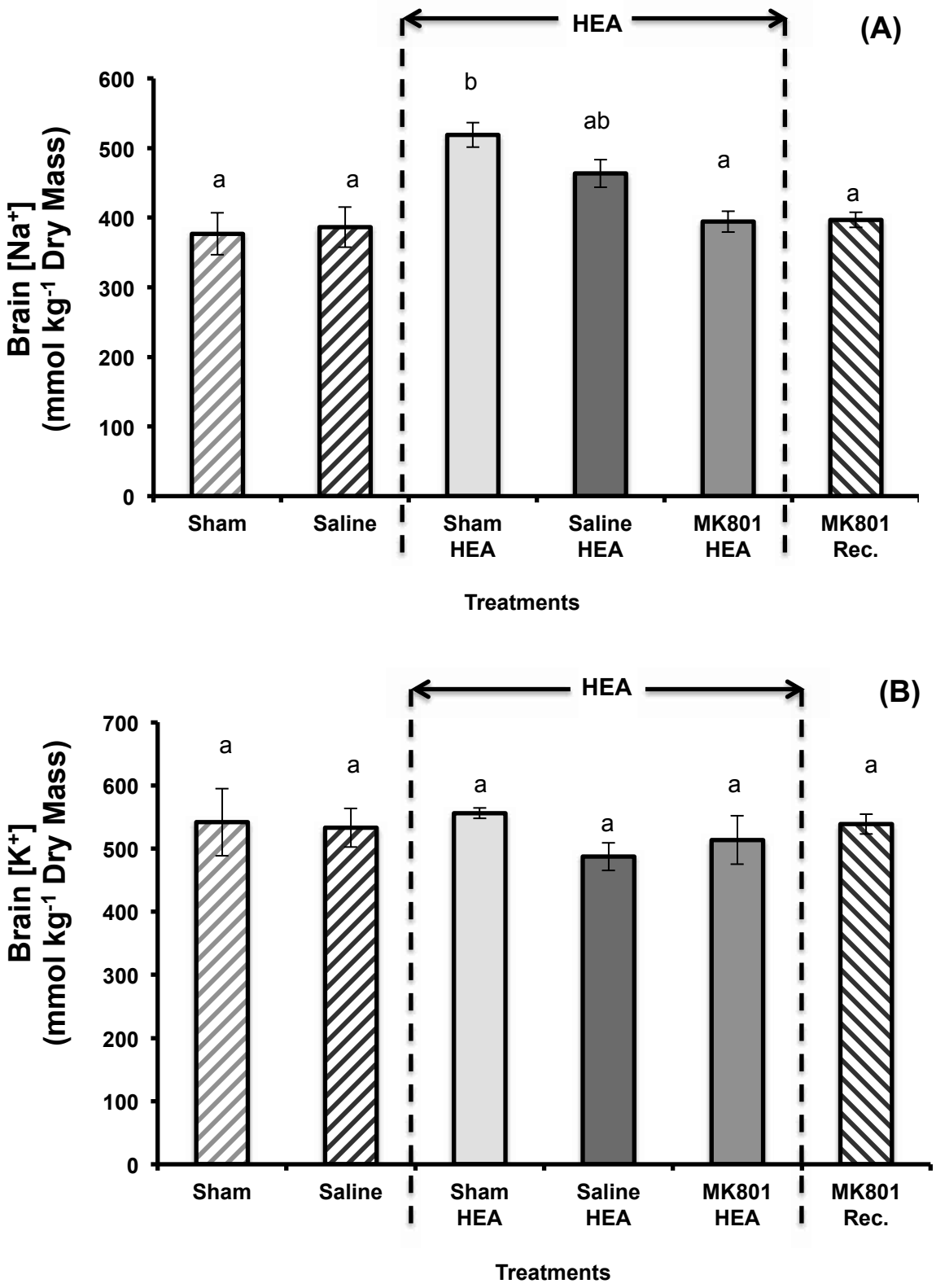

HEA
(B)
Plasma [Ammonia]
(µmol L^{-1})
2000
1500
1000
500
0
a
a
b
b
b
a
Sham
Saline
Sham HEA
Saline HEA
MK801 HEA
MK801 Rec.
Treatments

Figure 2.6. Effects of MK801 on **(A)** Brain sodium concentration dry mass and **(B)** Brain potassium content in dry mass measured in rainbow trout held under control conditions (Sham n = 8 and Saline n = 9) or exposed to 48 h HEA (T_{Amm} 1 mmol L^{-1}; Sham HEA n = 8, Saline HEA n = 8, and MK801 HEA n = 12), and following recovery in ammonia-free well water (MK801 Recovery n = 7). Data are presented as mean ± SEM. Bars sharing the same letter are not significantly different (One-way ANOVA followed by the Holm-Sidak post test $p<0.05$).

FIGURE 2.6.


HEA
(A)
Brain [Na+] (mmol kg-1 Dry Mass)
600
500
400
300
200
100
0
a
a
b
ab
a
a
Sham
Saline
Sham HEA
Saline HEA
MK801 HEA
MK801 Rec.
Treatments
HEA
(B)
Brain [K+] (mmol kg-1 Dry Mass)
700
600
500
400
300
200
100
0
a
a
a
a
a
a
Sham
Saline
Sham HEA
Saline HEA
MK801 HEA
MK801 Rec.
Treatments

Chapter 3

Neurotoxic Effects of Ammonia on the Brain of the Ammonia-Tolerant Goldfish (*Carassius auratus*)

INTRODUCTION

Elevated internal ammonia concentration, also termed hyperammonemia, in fish results in hyperventilation, hyperexcitability, convulsions and death (Ip et al. 2001; Felipo and Butterworth 2002). Although fish are more tolerant to ammonia compared to mammals, ammonia-tolerance is highly variable amongst different species of fishes. Nevertheless, the underlying mechanisms for ammonia-tolerance in fish remain unclear. In mammals, hyperammonemia is linked to hepatic encephalopathy (HE), which occurs due to liver disease, defects in the ornithine urea cycle (OUC), and exposure to hepatotoxins (Felipo and Butterworth 2002; Rama Rao et al. 2014). Severe defects to the liver impede the ability for the body to detoxify ammonia, consequently resulting in significant neurotoxicity. Overall, ammonia neurotoxicity results in glutamate excitotoxicity, seizures, coma, irreversible brain damaged and brain swelling (Felipo and Butterworth 2002; Walsh et al. 2007).

Despite extensive work by many research groups, the mechanism(s) of ammonia toxicity remains uncertain. However, a common characteristic of ammonia toxicity in mammals and fishes is brain edema, in which the brain takes up water and swells (Norenberg 1988; Brusilow 2002; Walsh et al. 2007; Butterworth 2008). Swelling is thought to take place in the astrocytes of the brain, rather than the neurons (Norenberg 1988; Swain et al. 1991). Astrocytes play a particularly important role in the glutamate-glutamine cycle, in which glutamate that is released into the synaptic cleft by pre-synaptic neurons is taken-up by Na^+: glutamate co-transporters and then combines with ammonia to generate glutamine, in an ATP dependent reaction catalyzed via intracellular glutamine synthetase (GS).

Traditionally, GS was thought to be important for the detoxification of ammonia in the brain and in other tissues However, in the last decade a number of researchers have suggested that ammonia-induced elevations in brain glutamine resulted in increased intracellular osmolarity in the astrocytes, which resulted in osmotic shifts leading to brain swelling in mammals and fishes (Brusilow 2002). However, the hypothesis that glutamine acts as an osmolyte has been challenged by Wilkie et al. (2015) who demonstrated that inhibition of glutamine synthetase using MSO did not mitigate ammonia-induced brain swelling in the goldfish (*Carassius auratus*). Moreover, it seems unlikely that the osmotic pressure generated by glutamine would be sufficiently high enough to produce the brain swelling (Rose et al. 2000; Rama Rao and Norenberg 2012; Ott and Vilstrup 2014) observed during high external ammonia (HEA) exposure in the goldfish (Wilkie et al. 2015). However, it is possible that other osmolytes/electrolytes contribute to ammonia-induced cell swelling.

Glutamate, rather than glutamine, has also been implicated as a cause of ammonia-induced excitotoxicity (Fan and Szerb 1993; Hermenegildo et al. 1996). Electrophysiological studies by Fan and Szerb (1993) using hippocampal slices, and more recently by Wilkie et al. (2011) using slices of goldfish telencephalon, have demonstrated that ammonia potentiates NMDA receptors currents, which can initiate glutamate excitotoxicity through over-activation of the receptors. A major consequence of NMDA receptors overactivation is the activation of proteases and lipases, and the generation of ROS, such as nitric oxide (NO), hydrogen peroxide (H_2O_2) and superoxide radicals (O_2^-) (Norenberg et al. 2006; Norenberg et al. 2009; Walsh et al. 2007; Wilkie at al. 2011). These reactive molecules are the byproducts of oxidative metabolism, which can severely

damage proteins and lipids in the brain, particularly ion transporters and the plasma membranes of astrocytes, potentially contributing to the manifestation of brain edema. However, a link between NMDA receptor activation and brain edema has not yet been established.

The goal of this present study is to determine if over-activation of NMDA receptors contributes to brain swelling in the ammonia tolerant goldfish. Accordingly, goldfish were pre-treated with NMDA receptor antagonist MK801 and exposed to HEA. Brain tissue water content and brain ion concentrations were then quantified to better understand what role, if any, NMDA receptors play in hyperammonemia-induced brain swelling in the goldfish. The possible role of glutamine in brain edema and brain ionic balance was also further investigated by inhibiting GS with MSO, a GS antagonist.

Under physiological conditions, electrolytes are maintained within a narrow range by homeostatic processes. However, imbalances of electrolytes such as Na^+ and K^+ would affect water distribution throughout the body and such disturbances may play a prominent role in the development of cerebral edema during HEA. In the past, a number of groups have demonstrated that experimentally induced ischemia in rats led to increases in dry Na^+ and decrease in dry K^+ content (Hossman 1980; Gotoh et al. 1985; Katzman et al. 1997). Additionally, previous *in vivo* studies on the ammonia-sensitive rainbow trout have revealed that HEA exposure resulted in significant ionic and metabolic alterations in the brain (Chapter 2). However, the mechanistic basis of ionic disturbances during ammonia-induced brain swelling in ammonia-tolerant fish remains poorly understood. Thus, the final goal of the present study was to further characterize the changes in Na^+ and K^+ balances during ammonia-induced brain swelling. Additionally, brain ionic

disturbances were characterized to determine if ammonia-induced inhibition of the Na^+/K^+-ATPase activity resulted in ionic disturbances that contributed to ammonia-induced brain swelling in the goldfish, as was demonstrated in the rainbow trout (Chapter 2).

METHODS AND MATERIALS

Experimental Animals and Holding

Goldfish (*Carassius auratus;* 25-50 g) were purchased from a commercial supplier (AQUAlity, Mississauga, Ontario) and transported to Wilfrid Laurier University Animal Care Facility, where they were held in 110 L tanks continuously receiving aerated well water (dissolved oxygen > 80% saturation) at 12 ± 1^{o}C and pH 7.8 ± 0.4. The animals were acclimated to the facility for a minimum of 2 weeks, during which time they were fed 3 times a week with commercially available fish pellets (2.0 mm Martin Profishent^{TM} Fish Food for Aquaculture, Elmira, Ontario). Prior to experiments, goldfish were fasted for 3-5 to eliminate the effects that feeding history might have on ammonia excretion and tolerance to build-ups of internal ammonia (Fromm, 1963; Wright and Wood, 1985; Wicks and Randall 2002). The fish were then placed in individual exposure containers (Volume = 3-3.5 L) for 12-24h, following careful examination for possible disease and for signs of distress before any experimental procedures began. All animal care and experiments conducted were approved by the Wilfrid Laurier University Animal Care Committee and followed the guidelines of the Canadian Council of Animal Care.

Experimental Set-Up

The experimental system comprised an angle iron frame, upon which was placed a large PVC tray, which held the individual fish holding containers into which an overhanging head tank drained via a flow-splitter and water lines that fed into each of the holding chambers at a rate of 0.5 to 1.0 L per minute. Water that drained out of each of the boxes into a lower reservoir was returned to the head tank using a submersible pump. To ensure that the relative proportions of NH_3 and NH_4^+ to which the fish were exposed

was constant, water pH was carefully regulated between 7.9-8.1 using a pH-stat system comprised of a GK2401C pH electrode (Radiometer, Copenhagen, Denmark) running from a PHM82 pH meter Radiometer connected to a TTT80 auto-titrator (Radiometer). When pH deviated from the set point, 0.5M HCl was added into the head tank by opening a solenoid valve (Cole Parmer Instruments Co., IL. USA), activated by the auto-titrator.

Experimental Protocols

Experiment 1: Effects of HEA Exposure on Brain Water Content, Ion Concentration and Na^+/K^+-ATPase Activity in Goldfish

To test the hypothesis that exposure to HEA results in brain swelling in fish, goldfish were transferred to individual dark holding chambers to which aerated well water was continuously flowing (dissolved oxygen > 80%), and allowed to acclimate overnight. The next morning ammonia exposure was initiated by cutting-off water flow to each holding chamber and to the to the entire recirculating system. An appropriate amount of 5 M NH_4Cl stock solution was then added to into each container, and to the entire system to reach the target concentration for 5 mmol L^{-1} total ammonia, and left for 1 h. Water pH in the system was monitored using the pH-stat set-up (above) and at regular intervals (0, 4, 6, 12, 24, 36, and 48 h) in the holding chambers using a handheld pH meter (Oakton Instruments, pH 11 Model, Vernon Hills, IL). At each sampling period, sub-sets of animals were euthanized with 1 g L^{-1} tricaine methanesulfonate buffered with 2.0 g L^{-1} $NaHCO_3$. Blood samples were collected by caudal puncture using a 1 mL-heparinized (7 mg heparin in 25 mL Cortland's saline to counteract coagulation) syringe and 21 gauge needle, transferred to 1.5 mL microcentrifuge tubes, and then centrifuged at 10,000 X *g* for 3 minutes. The plasma was then transferred to clean

microcentrifuge tubes, snap-frozen in liquid nitrogen (N_2) and stored at -80°C until later analysis of plasma ammonia concentration. The cranial cavity was then exposed and the whole intact brain (telencephalon, cerebellum, vagal lobes and optic tectum) carefully extracted, transferred to a pre-weighed 1.5 mL microcentrifuge tube, and dried to constant weight at 60^{o}C for the determination of brain water content. The brains of additional sub-sets of fish exposed to ammonia were collected in an identical manner, but snap frozen in liquid N_2 and stored at -80°C for later analysis of glutamine, ammonia, and Na^+-K^+-ATPase activity.

Experiment 2: The Effects of MK801 on Ammonia-Induced Brain Swelling and Ion Regulation

To determine if over-activation of NMDA-receptors contributes to ammonia-induced brain swelling, fish were administered the NMDA receptor antagonist MK801 and acclimated to their individual holding chambers for 24 h. Different groups of fish were then anaesthetized with an anaesthetic dose of 0.1 g L^{-1} tricaine methanesulfonate and 0.2 g L^{-1} $NaHCO_3$ and injected intraperitoneally (IP) with MK801 (5 mg kg^{-1}, dissolved in Cortland's saline; dose chosen based on previous experiments performed by Wilkie et al. (2011)), Cortland's saline (7.25 g - NaCl, 0.38 g - KCl, 0.23 g - $CaCl_2 \cdot 2H_2O$, 0.23 g - $MgSO_4 \cdot 7H_2O$, 0.41 g - $NaH_2PO_4 \cdot H_2O$, and 1.0 g - $C_6H_{12}O_6$) or sham treatment. In sham treated animals, anaesthetized fish were handled in an identical manner but not injected with solution. After 24 h recovery, the goldfish were exposed to ammonia (5 mmol L^{-1} HEA), and then sampled exactly as described for experiment 1. The MK801 treated fish were left to recover for 24h, and then exposed to 5 mmol L^{-1}

HEA for 48 h, and the tissues collected, sampled and processed as described above (*Experiment 1*).

Experiment 3: The Effects of MSO on Ammonia-Induced Brain Swelling

To determine if the accumulation of glutamine due to the over-activation of NMDA-receptors contributes to ammonia-induced brain swelling, fish were administered the glutamine synthetase inhibitor, MSO. As described for Experiment 2, the fish were transferred to their individual holding chambers the night before experiments. The next morning, subsets of fish were anesthetized and injected intraperitoneally (IP) with MSO (10 mg kg^{-1}, dissolved in Cortland's saline; dose chosen based on previous experiments performed by Sidhu (2009)), or Cortland's saline or subjected to a sham treatment (see above) and subjected HEA (5 mmol L^{-1} total ammonia) 24 h later, followed by brain and blood collection at 48 h of exposure. Brain tissue water was determined as described under Experiment 1. The brains from a sub-set of fish also administered MSO and subject to HEA were also preserved in liquid N_2, and preserved at -80^{o}C until later quantification of ammonia and glutamine.

Analytical Techniques

Water Ammonia Concentrations

Water ammonia concentrations were determined spectrophotometrically using the salicylate-hypochlorite assay at 650nm (Verdouw et al. 1978) using a 96 micro-well plate spectrophotometer (Spectramax 190, Molecular Devices, Sunnyvale, CA).

Brain Tissue Water Measurements

After brains were dried to constant mass, the measured decrease in wet tissue mass (converted to mL of H_2O) was divided by the original wet tissue mass (g) to yield

tissue water content per gram wet mass (mL H_2O g^{-1} wet mass). Brain water content was then expressed as ml H_2O/g dry mass by using the following equation:

$$\text{Water Content} = \frac{\text{ml } H_2O/\text{g wet mass}}{\text{g dry mass/ g wet mass}} \quad (1)$$

where brain water content is expressed in mL H_2O g^{-1} dry mass (Wilkie et al. 2015).

Brain Na^+/K^+ Quantification

Following determination of brain water content, the dried brains were digested in 6 volumes of 1 N HNO_3, and a sub-sample of the digest diluted 1000 times using deionized water, acidified with 1% 16 N HNO_3, and finally quantified using flame atomic absorption (AA) spectrophotometry (PinAAcle 900, Perkin Elmer, Woodbridge, Ontario). Samples processed for K^+ quantification were further treated with 0.1% cesium chloride to improve analytical resolution on the Flame AA. Dry brain tissue Na^+ and K^+ were expressed as μmol g^{-1} dry mass.

Plasma Ammonia Concentration Determination

Plasma ammonia concentrations were determined enzymatically (L-glutamate dehydrogenase; *Proteus sp.*; Sigma-Aldrich, G4387) with commercial reagents (AA0100, Sigma-Aldrich) and absorbance's determined using a plate spectrophotometer set to a wavelength of 340 nm. Quality control checks were performed using a commercial ammonia standard (A0978; 588μmol, Sigma-Aldrich).

Brain Glutamine and Ammonia Concentration Determination

Tissue Homogenization

Whole brain tissue was prepared for glutamine and ammonia analysis using a

bead mill homogenizer (Precelly ® 24 Bead Mill Homogenizer). Briefly, frozen brain samples were weighed, and then transferred to chilled 2 mL polypropylene centrifuge tubes, followed by the addition of 5 volumes of ice-cold 7% perchloric acid (PCA) containing 1 mmol L^{-1} ethylenediaminetetraacetic acid (EDTA). Three ceramic homogenizing beads were then added to each tube, which was then transferred to the bead mill homogenizer, and homogenized for two-15 seconds intervals, separated by a 30 second cooling interval. The homogenized samples were immediately transferred to a 4^{o}C refrigerated centrifuged, left for 10 minutes, centrifuged for 8 min at 12,000 X *g* and the supernatant drawn-off, then neutralized with 0.5 volumes of KOH (2N) to ensure pH was between 7-8. The neutralized supernatant was then used for the enzymatic quantification of ammonia as described above, or glutamine concentration. Glutamine quantification was determined using glutamine synthetase and hydroxylamine, which leads to the generation of γ-glutamylhydroxymate which forms a brown-yellow color complex in the presence Fe^{3+}, and is measured at 540nm on the plate spectrophotometer.

Brain Na^{+}/K^{+}/ATPase Activity Determination

Frozen brain samples were weighed, and transferred into 2.0 mL polypropylene centrifuge tubes, to which three ceramic beads and 25-times volume of ice-cold SEID (1X concentrate; 0.025 g sodium deoxycholate in 100 mL SEI; 250 mM sucrose, 10 mM Na_2EDTA, 50 mM imidazole, pH 7.5) were added. The tubes containing the brain tissue were then immediately transferred to the bead mill homogenizer (described above), and homogenized for two-15 seconds intervals. The resulting homogenates were then centrifuged for 8 minutes at 16,000 X *g* in a refrigerated centrifuge at 4^{o}C (Thermoscientific, Model Micro 21R), placed on ice, and the supernatant used to measure

Na^+/K^+-ATPase activity within 30 minutes.

Measurements of brain Na^+/K^+-ATPase activity were based on McCormick (1993), in which Na^+/K^+-ATPase activity was calculated as the difference between uninhibited total ATPase activities minus ouabain-inhibited ATPase activity. To measure total ATPase activity, brain tissue homogenate was added to an assay solution comprised of pyruvate kinase (375 units), lactate dehydrogenase (200 units), NADH (0.22 mM), phosphenolpyruvate (2.8 mM), and ATP (0.7 mM), in imidazole buffer (50 mM). An identical solution, but also containing ouabain (0.7 mM), was used to measure ouabain-inhibited ATPase activity. All assay mixtures were brought to room temperature (25^{o}C) immediately prior to enzyme activity determinations.

Total ATPase and ouabain-inhibited ATPase activity were determined using 96-well microplates that were placed on a dry ice pack during assay preparation containing 2.5μL of brain tissue homogenate in each well (in quadruplicate). Assay mixture, with or without ouabain, was then added to each well, at which time the microplate was placed into the temperature-controlled microplate spectrophotometer (25^{o}C), and ATPase activity was determined based on the rate of NADH disappearance (oxidation) measured at 340 nm over 10 minutes. An NADH standard curve (0-40 nmoles/well) was used to determine the extinction coefficient of NADH at 340 nm, and the protein concentration of each sample was determined using the Bradford assay (Bradford 1976). Wet weight Na^+/K^+-ATPase activity was calculated as the difference of activity in the presence and absence of ouabain, which was divided by the protein content of each sample to yield the specific Na^+/K^+-ATPase activity in μmol ADPmg protein^{-1}h^{-1}.

Statistical Analysis

All data are expressed as the mean ± standard error of mean (SEM). Differences between treatments were analyzed using one-way analysis of variance (ANOVA) to determine significance and variation between means, followed by a Holm-Sidak post-hoc test. All significant differences were determined at the $p < 0.05$ level using GraphPad InStat,Version 3 (GraphPad Software, Inc., San Diego, CA, USA).

RESULTS

Effects of HEA Exposure on Brain Water Content, Ion Concentration and Na^+/K^+-ATPase Activity in Goldfish

Brain water content was 15 % greater in goldfish exposed to nominal total ammonia (T_{Amm}) concentration of 5 mmol L^{-1} for 48 h, compared to the control (non-exposed) group in which brain water content averaged 4.76 ± 0.044 mL H_2O g^{-1} dry mass (**Figure 3.1A**). However, the HEA-induced increase in brain water content was completely reversible, as brain water content averaged 4.63 ± 0.06 mL H_2O g^{-1} dry mass after 24 recovery in (nominally) ammonia-free water (**Figure 3.1A**).

The increases in brain water content, were accompanied by by marked increases of plasma and brain tissue ammonia concentration ($[T_{Amm}]$), which increased approximately 10-fold from 136 ± 19 to 1365 ± 86 μmol L^{-1}. After 24 h recovery in ammonia-free water plasma ammonia concentrations decreased from 1366 ± 87 μmol L^{-1} to approximately 202 ± 20 μmol L^{-1} (**Figure 3.1B**).

Similarly, brain $[T_{Amm}]$ increased by approximately 140% from 1338 ± 130 μmol L^{-1} to 3200 ± 314 μmol L^{-1} (**Figure 3.2A**). Glutamine concentrations increased markedly, from control concentrations of 2766. ± 196 to 14609 ± 717 μmol g^{-1} wet mass following 48 h HEA, respectively (**Figure 3.2B**). However, the increase in glutamine was also reversible, decreasing to approximately 3340 ± 153 μmol g^{-1} wet mass following the 24 h depuration period (**Figure 3.2B**).

HEA ammonia also resulted in pronounced disturbances to brain Na^+ content, which increased by approximately 1.4-fold, from 343 ± 18 to 491 ± 12 μmol g^{-1} dry mass, before returning to control levels of 337 ± 14 μmol g^{-1} dry mass after 24 h of depuration

in ammonia-free water (**Figure 3.3A**). In contrast, brain K^+ content in HEA exposed goldfish decreased from 619 ± 24 to 558 ± 20 μmol g^{-1} dry mass. However, goldfish brain K^+ concentration rebounded back towards control levels, to 633 ± 13 μmol g^{-1} dry mass following 24 h recovery in ammonia-free water (**Figure 3.3B**). Despite the alterations in dry brain Na^+ and K^+ concentration with HEA exposure, Na^+/K^+-ATPase activity was only slightly lower in the fish following HEA 48 h exposure. There was no significant difference from the control animals following 24 h recovery in ammonia-free water (**Figure 3.3C**).

The Inhibition of GS Effects on Brain Tissue Water Content

The inhibition of GS by MSO injection did not brain increases in brain water content in the in the goldfish, which increased approximately 17% compared to the sham-treated and saline-injected animals (**Figure 3.4**). Brain water content increased to a similar extent as the MSO-injected in the sham and saline-injected fish exposed to HEA (**Figure 3.4)**. However, brain water content in MSO-treated fish was restored to pre-exposure control values of 4.74 mL H_2O g^{-1} dry mass following recovery in ammonia-free water (**Figure 3.4)**.

As expected, plasma [T_{Amm}] increased approximately 10-fold in exposed treated groups from control treated groups (**Table 3.2)** returning to pre-exposure values within 24 h of recovery in ammonia-free water (**Table 3.2)**.

Brain Na^+ accumulation was significant in HEA exposed-treated groups increasing approximately 1.3-times. Brain K^+ concentrations displayed an inverse effect. HEA treated groups displayed decrease in brain K^+ content compared to control treated groups (**Table 3.2**).

Effects of MK801 on Brain Tissue Water Content and Brain Ion Content

The injection of MK801 (5mg kg^{-1}) did not prevent significant accumulation of water in the brain of the goldfish during HEA exposure. In goldfish injected with MK801, brain water content of 48 h HEA exposure resulted in brain tissue water contents that were approximately 12% greater than the values measured in the non-exposed sham and saline injected animals (**Figure 3.4A**). However, amongst the groups exposed to HEA, the increase in brain water content in the MK801 treated animals was significantly less than observed in the sham, but not the saline control animals (**Figure 3.4A**).

Brain tissue water content in the MK801-injected group recovering from HEA exposure, was similar to control values, near 4.63 ± 0.06 mL H_2O g^{-1} dry mass after 24 h (**Figure 3.5A**).

Exposure to a nominal concentration of 5 mmol L^{-1} for 48 h resulted in a 10 to 15-fold increase in plasma [T_{Amm}] from pre-exposure value. Control treated fish groups all displayed significant increases in plasma [T_{Amm}] compared to HEA treated fish groups. The increase in brain water content was directly correlated to increases in plasma [T_{Amm}]. Plasma [T_{Amm}] was significantly lower in MK801 recovery group following 24 h recovery in ammonia-free water (**Figure 3.5B**).

Treatment of fish with MK801 blocked the increase in brain Na^+ content that was characteristic of HEA exposure in non-injected fish (Compare **Figure 3.3A** to **Figure 3.6A**).

Similarly, the reduction in brain K^+ content that characterized exposure of untreated animals to HEA (Compare **Figure 3.3B** to **Figure 3.5B**) was also prevented by MK801 administration. In the MK801-injected fish, brain K^+ content of the fish exposed

to HEA was not significantly different the saline-injected and sham-treated fish held under control (non-exposed) conditions. However, the saline-injected fish exposed to HEA underwent a significant 22 % decrease in brain K^+ content following 48 h HEA, from 620 ± 37 to 485 ± 24 μmol g^{-1} dry mass (**Figure 3.6B**). Although brain K^+ trended downwards in the sham treated fish exposed to HEA, the levels were not significantly different from the non-exposed control animals (**Figure 3.6B**).

DISCUSSION

Ammonia-tolerance in the goldfish

Compared to mammals, fish have greater tolerance to ammonia. Normally, plasma ammonia concentrations in mammals are 100 μmol L^{-1} or less and brain ammonia concentrations range between 200 and 800 μmol kg^{-1}, but they experience ammonia-induced neurotoxicity when respective plasma [T_{Amm}] and brain [T_{Amm}] concentrations exceed of 200 μmol L^{-1} and 1000-2000 μmol kg^{-1} (Swain et al. 1992; Mans et al. 1994; Kosenko et al. 1994). In contrast, goldfish readily survive plasma and brain [T_{Amm}]s that can be more 3-5 times higher than these values (**Figure 3.1**). Other ammonia tolerant fishes, including the goldfish' close relative the crucian carp (*Carassius carassius*) exhibit similar tolerance (Wilkie et al. 2015). Plasma [T_{Amm}] in the swamp eel (*Monopterus albus*) has been known to reach as high as 3540 μmol L^{-1}, with no visible signs of experiencing ammonia toxicity (Ip et al. 2004b). The air-breathing mudskippers (*Periophthalmodon schlosseri* and *Boleophthalmus boddaerti*) exposed to sub-lethal concentrations of HEA (100 and 8 mmol L^{-1}, respectively) had brain [T_{Amm}] concentrations near 18000 and 14500 μmol kg^{-1}, respectively (Ip et al. 2005). In comparison, plasma and brain [T_{Amm}]s in the ammonia-sensitive rainbow trout (*Oncorhynchus mykiss*) exposed to 1 mmol L^{-1} HEA, increased significantly to 1670 μmol L^{-1} and 2185 μmol kg^{-1}, respectively (Chapter 2). Similar to the goldfish, these increases were readily reversible in the ammonia-sensitive rainbow trout. While goldfish are ammonia tolerant, the aforementioned examples also illustrate that ammonia tolerance is not uniform amongst fish species, which suggests that underlying

mechanism(s) of ammonia tolerance may reflect different adaptations of the central nervous system (CNS) to this neurotoxin.

It was notable that the increases of plasma [T_{Amm}] and brain [T_{Amm}] in the goldfish paralleled brain tissue water content (**Figure 3.1**), as they did in previous work on the ammonia-sensitive rainbow trout (Chapter 2). In the present study, goldfish brain tissue water content increased by approximately 15 %, which was less than reported in previous studies, in which brain water content increased by 25-35% in goldfish and crucian carp exposed to much higher concentrations of HEA (Wilkie et al. 2015). A number of additional factors including the duration of ammonia exposure, external pH, during exposure, water chemistry and differences in the amount of ammonia taken-up by the animals may also explain some of the variation between studies.

Nevertheless, in mammals, the magnitude of ammonia-induced brain swelling is considerably less than observed in fishes exposed to sub-lethal concentration of ammonia in the water. For instance, the administration of the hepatotoxin thioacetamide (TAA), used to induce acute liver failure (ALF) in rats, resulted in pronounced brain edema characterized by only a 2.9% increase in brain water content which paralleled a 3-fold increase in brain [T_{Amm}] to 6 μmol g^{-1} wet mass (Rama Rao et al. 2010). Similar techniques used to measure brain water content used by Master et al. (1999) indicated that similar increases in brain water content took place in rats infused with ammonium acetate ($NH_4C_2H_3O_2$), resulting in an increase in brain water content from 79.97 ± 0.04 % brain H_2O (379 ± 14 g wet mass) to 81.11 ± 0.13 brain H_2O % (379 ±15 g wet mass). These changes are equivalent to brain H_2O contents (mL H_2O g^{-1} dry mass) of 4.03 and 4.29 mL H2O g^{-1} dry mass, which represents a 6.5% increase in brain water content,

which is less then reported for rainbow trout (Chapter 2), goldfish and crucian carp (Master et al. 1999; Wilkie et al. 2011; Wilkie et al. 2014). These data indicate that the degree of ammonia-induced swelling in fish is much greater than that experienced by mammals.

In the present study, brain swelling in the goldfish was readily reversible with no obvious evidence of damage or adverse behavioral effects. In the mammalian brain, brain edema results in swelling, increased intracranial pressure, herniation and often coma or death because of limited volume of the cranial cavity (Desjardins et al. 2014). Cyprinid fishes on the other hand, including the goldfish and common carp (*Cyprinius carpio*), have cranial with a much larger relative volume, allowing them to accommodate changes in brain water swelling to a greater extent, apparently without any lasting damage (Van der Linden et al. 2001).

Understanding the role of glutamine in the ammonia-tolerant goldfish

The ability for goldfish to readily reverse hyperammonemic symptoms with no visible signs of pathological damage proves to be a significant characteristic in ammonia tolerance amongst fish. During hyperammonemia, brain glutamine concentrations have been observed to increase in both mammals and fish (Dabrowska and Wlasow, 1986; Cooper and Plum 1989; Mommsen and Walsh, 1992; Peng et al., 1998; Brusilow 2002; Butterworth 2002; Randall 2002; Veauvy et al. 2005; Albrecht and Norenberg 2006; Walsh et al. 2007). In mammals, brain glutamine accumulation has been traditionally regarded as a mechanism for detoxifying ammonia (Brusilow 2002; Butterworth 2002). However, a number of researchers have suggested that glutamine accumulation may be the cause of astrocytic swelling (Brusilow 2002). During hyperammonemia, there appears

to be strong correlations between glutamine concentrations and ammonia-induced brain swelling in mammals (Brusilow 2002; Tofteng et al. 2006; Albrecht and Norenberg 2006; Walsh et al. 2007). Indeed, glutamine concentrations substantially increased in the brain of goldfish by and were accompanied by a 15% increase in brain tissue water content, following 48 h HEA exposure. However, administering MSO to the goldfish prior to HEA exposure did not attenuate ammonia-induced increases in the brain water content in goldfish, despite preventing glutamine from increasing in the brain. Although brain swelling does not appear to be directly caused by intracellular glutamine, the group of fish treated with MSO still exhibited significantly higher Na^+ and lower K^+ in the brain. This conclusion is further supported by the lack of effect that MSO administration had on brain ion content in response to HEA exposure. As in the saline and sham-injected fishes, HEA exposure resulted in similar increase in dry brain Na^+ and a decrease in K^+, Taken together, the results of the present experiments and Lisser (2016) suggest that brain swelling is initially caused by ionic disturbances caused by oxidative and nitrosative stress, which lead to changes in intracellular osmolarity that result in intracellular water uptake and brain edema.

Recently, it has been proposed that the increased production of glutamine could indirectly result in oxidative stress and nitrosative stress during ammonia toxicity in mammalian models (Kosenko et al. 2003; Norenberg 2003; Albrectht and Norenberg et al. 2006; Rama Rao et al. 2014). In a model proposed by Albrecht and Norenberg (2006) glutamine acts as a "Trojan Horse" which imports ammonia into the mitochondria of cells. In the "Trojan Horse" model, the excess glutamine is taken up by the mitochondria and converted to glutamate and NH_3 by phosphate-activated glutaminase (PAG), which

can be blocked by cylosporin. The presence of NH_3 triggers the induction of mitochondrial permeability transition (MPT) pore, which ultimately interferes with mitochondrial potential and leads to the generation of free radicals, and astrocyte swelling (Norenberg 2003; Albrectht and Norenberg et al. 2006). The present study argues against this hypothesis of ammonia toxicity in the both goldfish and trout (Chapter 2), however, blocking GS mediated glutamine synthesis with MSO still leads to osmotic and ionic disturbances and the brain swelling characteristic of ammonia toxicity in vertebrates.

The effects of MK801, an NMDA receptor inhibitor on the ammonia-tolerant goldfish

The over-activation of NMDA receptors by ammonia leads to significant increases of intracellular Ca^{2+} and possibly significant generation of ROS by activating superoxide constitutive nitric oxide synthetase (cNOS), which generates NO and free radicals (Nakamura et al. 1989; Mayer et al. 1990; Kramer and Sharp 1997; Kosenko 1998, 1999; Cocco et al. 1999; Jayakumar et al. 2009). Although, MK801 did not prevent significant swelling in the goldfish, it did appear to offers some protection against brain edema. The degree of swelling in MK801 treated fish, though not significantly reduced, was trending downward compared to sham and saline exposed groups, and swelling was reversed in the MK801 treated fish recovering from HEA. Additionally, the brains of MK801 treated fish did not experience significant increases of Na^+ during HEA, in contrast to the saline injected and sham fish exposed to HEA. In fact, these findings further suggest Na^+ accumulation within cells may trigger cell swelling in the fish brain. Further support for this hypothesis was that dry brain K^+ concentration decreased, which

lends further support to the hypothesis that ammonia interferes with ionoregulatory processes in the brain.

It appears a common characteristic in some ammonia-tolerant fish is the ability to demonstrate high neural tolerance in the presence of ammonia (Wilkie et al. 2011; Lutz and Nilsson 2004; Bickler and Buck 2007), which could explain why MK801 did not attenuate brain swelling in the goldfish. Previously, MK801 treatment has been shown to enhance survival rate in ammonia-sensitive rainbow trout exposed to HEA, but had no effect on survival duration in the ammonia-tolerant goldfish (Wilkie et al. 2011). Furthermore, Wilkie et al. (2011) revealed that goldfish NMDA receptors are sensitive to ammonia and that acute ammonia exposure resulted in reverse potentiation of NMDA receptor currents in vitro (Wilkie et al. 2011). Lastly, NR1 subunit located on the NMDA receptor abundance was significantly reduced by 40-60% in ammonia-exposed goldfish (Wilkie et al. 2011). Nevertheless, the weight of evidence suggests that goldfish may have the ability avoid ammonia-induced glutamate excitotoxicity by down-regulating NMDA receptor (Wilkie et al. 2011).

Ionoregulation and disturbances during HEA

Ammonia-induced increases of brain tissue water were accompanied by elevations of dry brain Na^+ accumulation, which is suggestive of a shift of Na^+ into the intracellular space of the cells during HEA exposure. As observed in rainbow trout exposed to HEA (Chapter 2), the increase in dry brain Na^+ also paralleled increases in brain and plasma [T_{Amm}] and brain glutamine following 48 h HEA. During experimentally induced ischemia, rats experienced significant brain swelling, loss of dry brain K^+ and increased dry brain Na^+, following 12-48 h middle cerebral artery (MCA)

occlusion (Gotoh et al. 1985). Similarly, Hossman et al. (1977) demonstrated that complete cerebral ischemia in cats resulted in a Na^+ gain of 139 mmol kg^{-1} dry mass, accompanied by a 64 mmol kg^{-1} dry mass loss of K^+. Although the pathophysiology of ischemia differs from ammonia-induced swelling, similar ionic disturbances were observed at a whole brain level, as described in this study for goldfish. The rapid loss of brain ion-homeostasis during HEA following drastic increases of dry brain Na^+ therefore suggests that ionic disturbances might be a contributor to brain swelling in the goldfish.

A possible mechanism for brain swelling could involve ROS-mediated inhibition of the Na^+/K^+-ATPase pump. The production of ammonia-induced ROS may be a possible explanation for the disturbances of cell volume and ionic homeostasis. When overproduced ROS can damage essential proteins, lipids and nucleic acid required in maintaining an ionic and osmotic gradient thus, affecting astrocytic membrane integrity. There is now evidence in fish that HEA exposure results in ROS generation in the brain, which results in the oxidation of lipids and the carbonylation of proteins (Lisser 2016). Moreover, ROS production has been known to target the alpha sub-unit of the Na^+/K^+-ATPase, which is the catalytic subunit of the pump (Orsenigo et al. 2007; Sag et al. 2013). Recent evidence showed that overproduction of ROS, H_2O_2, and other free radicals can decrease the expression of α_1-subunit of the Na^+/K^+-ATPase in rat jejunum and cardiac myocytes (Orsenigo et al. 2007; Sag et al. 2013). While these findings are not specific to the regions of the brain, it is possible that in response to ammonia toxicity, ROS may play a similar role in inhibiting Na^+/K^+-ATPase activities in the brain of the goldfish. While the Na^+/K^+-ATPase was not significantly reduced following HEA exposure, its activity was trending downwards following 48 h of ammonia exposure, and

may have decreased further had the exposure period been longer. Moreover, the slight increase seen in Na^+/K^+-ATPase activity following recovery in ammonia-free water does suggest that the pump was inhibited by ammonia exposure. Furthermore, the lack of significant recovery may indicate that protein channels were possibly damaged by ROS. However, the mechanism(s) that can explain these reductions is still not fully understood. Indeed, a similar, persistent reduction in Na^+/K^+-ATPase activity was also observed in the trout during recovery from exposure to 1 mmol L^{-1} ammonia (Chapter 2).

During hyperammonemia, there was a less pronounced response of Na^+/K^+-ATPase activity in the ammonia-tolerant goldfish compared to the ammonia-sensitive rainbow trout (Figure 3C and Chapter 2). It has been shown that the ammonia-tolerant goldfish demonstrate high antioxidant capacity (Lushchak et al. 2001; Lisser et al. 2016). The ability for goldfish to increase antioxidant enzyme activities during ammonia toxicity may act as a defense mechanism, limiting cellular oxidative damage (Lushchak 2006; Lisser et al. 2016) and increase neural tolerance (Wilkie et al. 2011). These findings may explain why goldfish have a higher tolerance to ammonia compared to the trout. Additionally, evolved antioxidant defenses may also explain why the effects on Na^+/K^+-ATPase were less pronounced in goldfish compared to the rainbow trout (Chapter 2).

CONCLUSION

The present study demonstrated that goldfish exhibit increased glutamine formation during ammonia-induced neurotoxicity; however, brain swelling still persists during the absence of glutamine formation. While brain glutamine accumulation does not appear to be the direct cause of ammonia-induced brain swelling in goldfish, these findings suggest that glutamine-mediated oxidative and nitrosative stress may be a responsible for brain swelling. Ammonia-induced brain was not prevented in goldfish by pre-treated with MK801. A key adaptation demonstrated by goldfish may the ability to prevent neuronal depolarization during ammonia toxicity, which could explain why goldfish were unaffected by MK801 (Wilkie et al. 2011). Additionally, ammonia-induced neurotoxicity resulted in significant alterations of brain Na^+ and K^+. Though, Na^+/K^+-ATPase activity remained unaffected during high external ammonia exposure, suggesting that additional mechanisms may be responsible for ionic disturbances in the ammonia-tolerant goldfish.

Table 3.1. Measured Ammonia Water Concentration for Goldfish Exposed to High External Ammonia. Chart represents all experiments. Data shown represented as mean ± SEM.

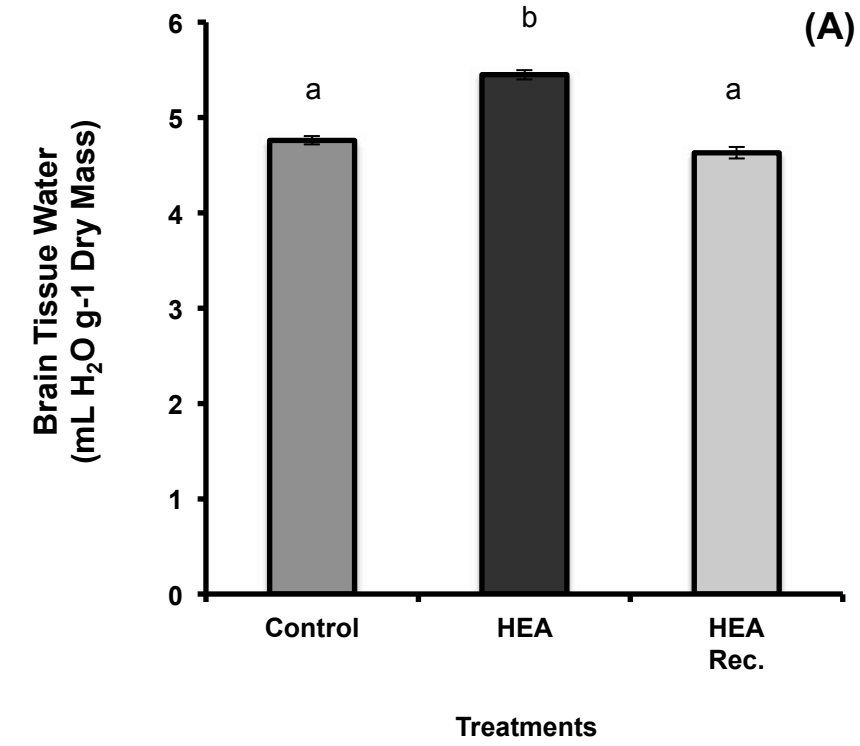
Goldfish (HEA)	**Time (h)**	**Actual Water Ammonia ($mmol\ L^{-1}$)**
	4	4.74 ± 0.12
	6	5.21 ± 0.24
	12	5.17 ± 0.09
	24	5.32 ± 0.17
	48	5.42 ± 0.04
Goldfish (MK801)	**Time (h)**	**Actual Water Ammonia ($mmol\ L^{-1}$)**
	4	5.57 ± 0.49
	6	5.24 ± 0.31
	12	Not Observed
	24	5.43 ± 0.32
	48	5.73 ± 0.17
Goldfish (MSO)	**Time (h)**	**Actual Water Ammonia ($mmol\ L^{-1}$)**
	4	4.00 ± 0.03
	6	4.89 ± 0.19
	12	5.20 ± 0.31
	24	5.31 ± 0.41
	48	5.81 ± 0.03

Table 3.2. The Effects of MSO on Brain Na^+ in dry mass, Brain K^+ in dry mass and Plasma [TAmm] in Goldfish Summary Chart. (Sham *n* = 11 and Saline *n* = 11) or exposed to 48 h HEA (T_{Amm} 5 mmol L^{-1}; Sham HEA *n* = 6, Saline HEA *n* = 6, and MSO HEA *n* = 11), and following recovery in ammonia-free well water (MSO Recovery *n* = 6) Data shown represented as mean ± SEM. Columns sharing the same letter are not significantly different (One-way ANOVA followed by the Holm-Sidak post test $p<0.05$).

Goldfish Brain Na^+, K^+ and Plasma [T_{Amm}]	**Treatments**	**[Na^+] (µmol g^{-1} dry mass)**	**[K^+] (µmol g^{-1} dry mass)**	**Plasma [T_{Amm}] (µmol L^{-1})**
	Sham	381 ± 16 (a)	596 ± 33 (a)	96 ± 24 (a)
	Saline	385 ± 12 (a)	613 ± 11 (a)	161 ± 11(a)
	Sham HEA	509 ± 64 (a)	517 ± 71 (ab)	1194 ± 64 (b)
	Saline HEA	508 ± 58 (a)	462 ± 26 (b)	1063 ± 105 (b)
	MSO HEA	518 ± 35 (ab)	505 ± 16 (b)	1266 ± 111 (b)
	MSO HEA Recovery	393 ± 7 (ab)	637 ± 8 (a)	168 ± 31 (a)

Figure 3.1. (A) Brain tissue water content and (B) Plasma ammonia concentration measured in goldfish held under control conditions (n = 13) or exposed to 48 h HEA (T_{Amm} 5 mmol L^{-1}; n = 19), and following recovery in ammonia-free well water (n = 6). Data are presented as mean ± SEM. Bars sharing the same letter are not significantly different (One-way ANOVA followed by the Holm-Sidak post test $p<0.05$).

FIGURE 3.1.


(A)
Brain Tissue Water
(mL H_2O g-1 Dry Mass)
6
5
4
3
2
1
0
a
b
a
Control
HEA
HEA
Rec.
Treatments

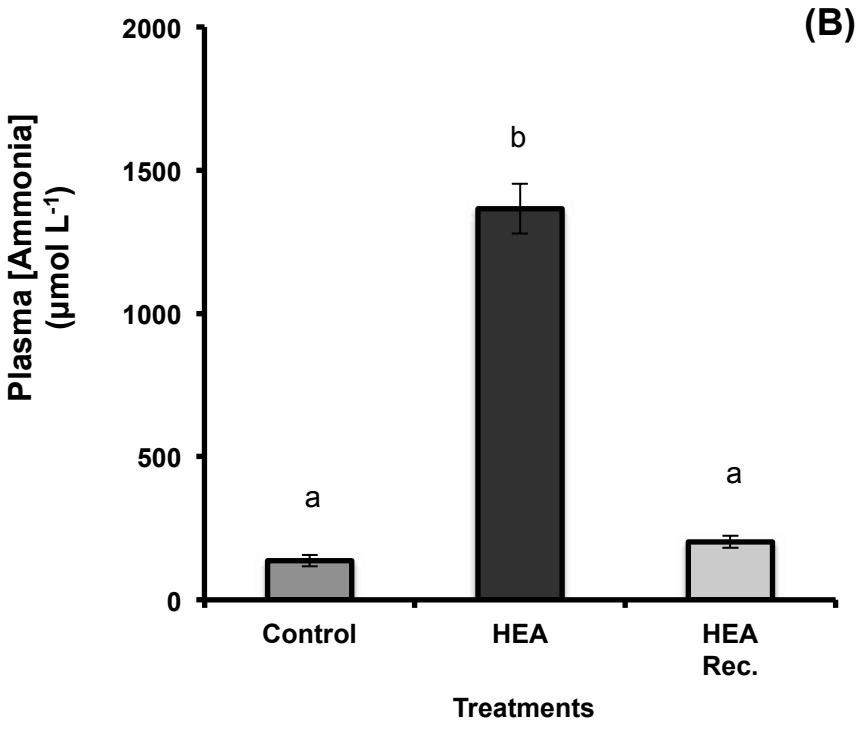

(B)
Plasma [Ammonia]
(µmol L^{-1})
2000
1500
1000
500
0
a
b
a
Control
HEA
HEA
Rec.
Treatments

Figure 3.2. Concentrations of (A) ammonia and (B) glutamine in the brain of goldfish in the absence (control; $n = 7$), presence of HEA for 48 h, (T_{Amm} 5 mmol L^{-1}; $n = 18$), and after 24 h recovery in ammonia-free well water ($n = 6$). Data are presented as mean ± SEM. Bars sharing the same letter are not significantly different (One-way ANOVA followed by the Holm-Sidak post test $p<0.05$).

FIGURE 3.2.

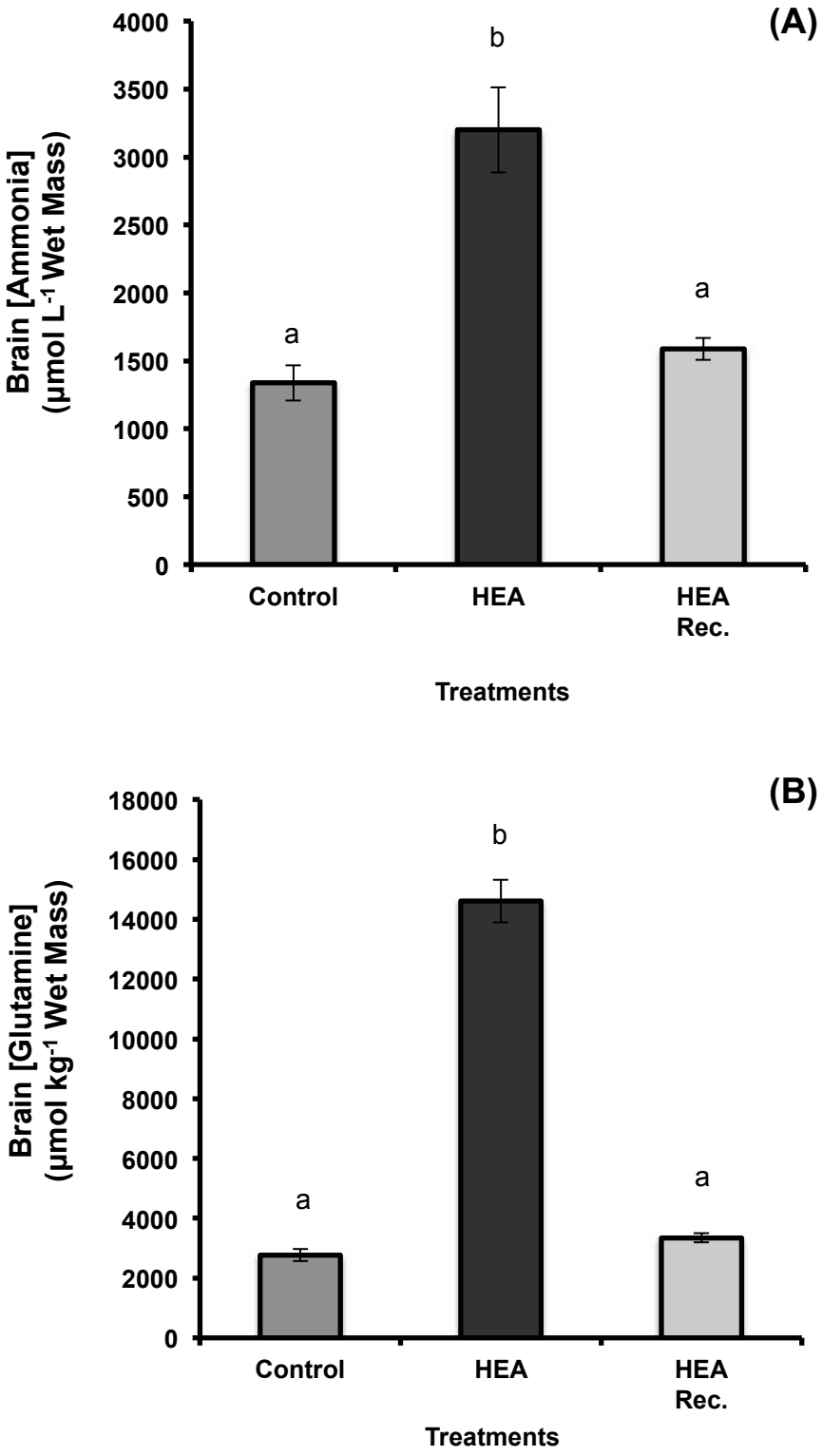
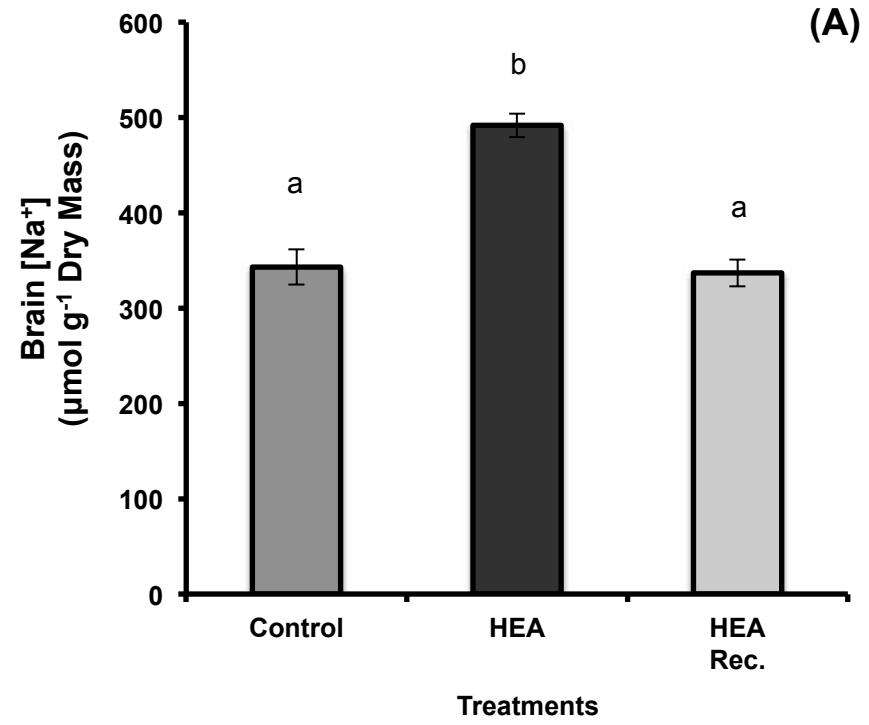
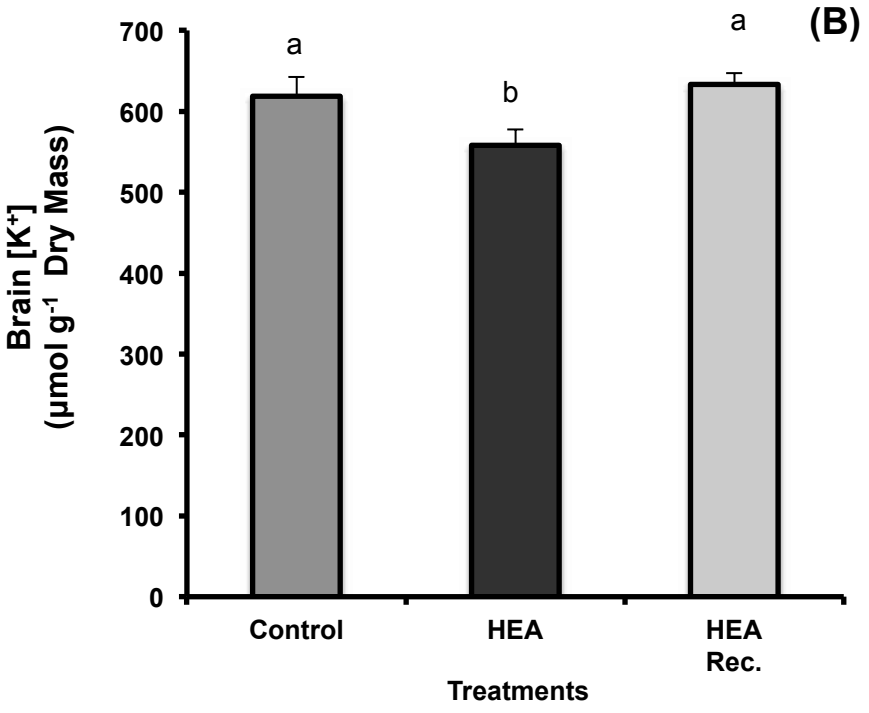

(A)
Brain [Ammonia] (µmol L-1 Wet Mass)
4000
3500
3000
2500
2000
1500
1000
500
0
a
b
a
Control
HEA
HEA Rec.
Treatments
(B)
Brain [Glutamine] (µmol kg-1 Wet Mass)
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
a
b
a
Control
HEA
HEA Rec.
Treatments

Figure 3.3. Effect of HEA exposure (5 mmol L^{-1}) on (A) dry brain Na^+ content, (B) dry K^+ content, (C) brain Na^+/K^+-ATPase activity in goldfish held under control conditions (*n* = 11), or exposed to HEA (T_{Amm} = 5 mmol L^{-1}) for 48 h (*n* = 19), and following recovery in ammonia-free well water (*n* = 6). Data are presented as mean ± SEM. Bars sharing the same letter are not significantly different (One-way ANOVA followed by the Holm-Sidak post test $p<0.05$).

FIGURE 3.3.


(A)
600
500
400
300
200
100
0
Brain [Na+] (µmol g-1 Dry Mass)
a
b
a
Control
HEA
HEA Rec.
Treatments


(B)
700
600
500
400
300
200
100
0
Brain [K+] (µmol g-1 Dry Mass)
a
b
a
Control
HEA
HEA Rec.
Treatments

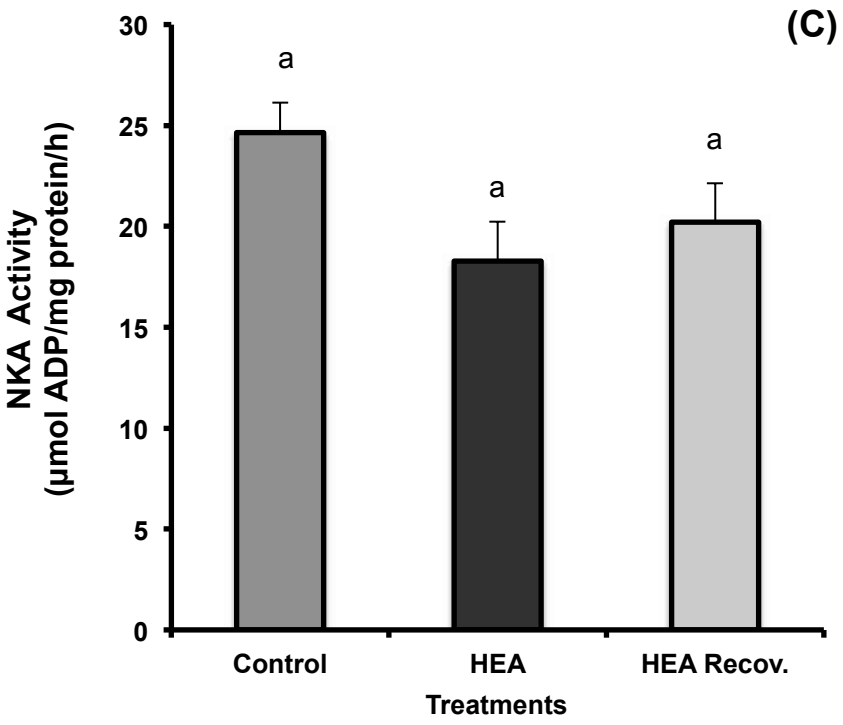

(C)
30
25
20
15
10
5
0
a
a
a
NKA Activity
(µmol ADP/mg protein/h)
Control
HEA
HEA Recov.
Treatments

Figure 3.4. The Effects of MSO on Brain tissue water content measured in goldfish held under control conditions (Sham n = 11 and Saline n = 11) or exposed to 48 h HEA (T_{Amm} 5 mmol L^{-1}; Sham HEA n = 6, Saline HEA n = 6, and MSO HEA n = 11), and following recovery in ammonia-free well water (MSO Recovery n = 6). Data are presented as mean ± SEM. Bars sharing the same letter are not significantly different (One-way ANOVA followed by the Holm-Sidak post test $p<0.05$).

FIGURE 3.4.

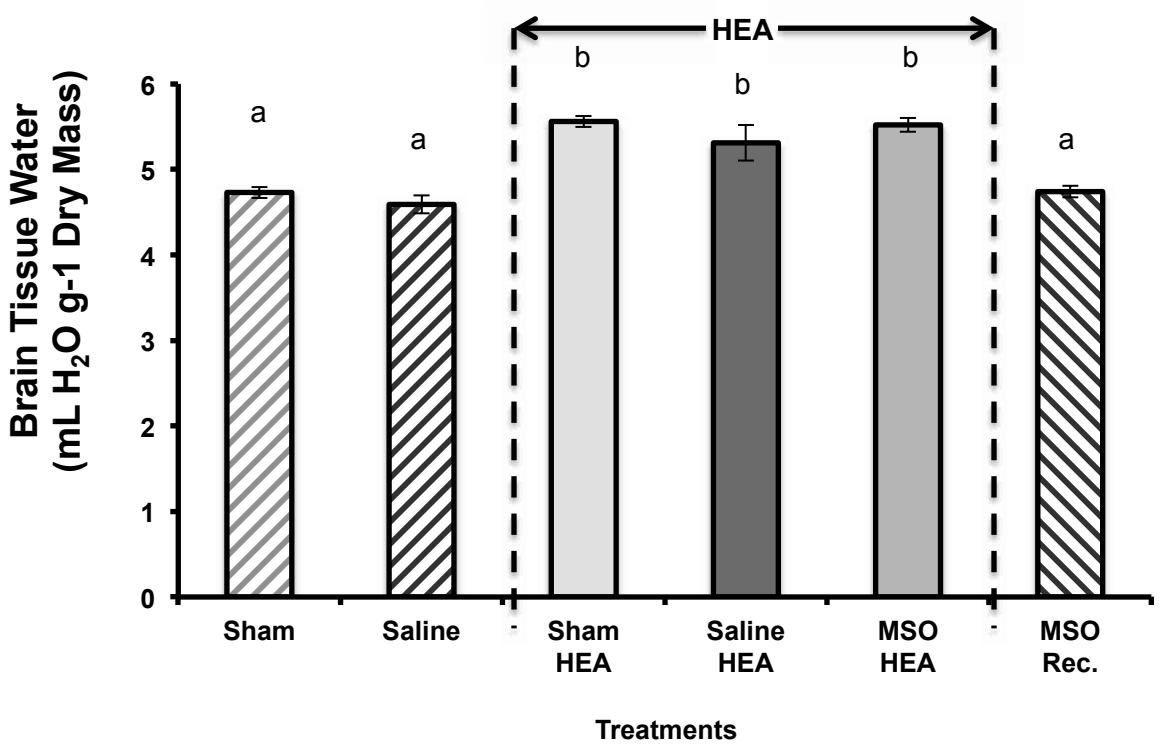

HEA
Brain Tissue Water
(mL H_2O g-1 Dry Mass)
6
5
4
3
2
1
0
a
a
b
b
b
a
Sham
Saline
Sham HEA
Saline HEA
MSO HEA
MSO Rec.
Treatments

Figure 3.5. Effect of MK801 administration on (A) brain tissue water content and (B) plasma T_{Amm} concentration measured in goldfish held under control conditions (Sham *n* = 8; Saline *n* = 9) or exposed to 48 h HEA (T_{Amm} 5 mmol L^{-1}; Sham HEA *n* = 8; Saline HEA *n* = 8; MK801 HEA *n* = 12), and following recovery in ammonia-free well water (*n* = 7). Data are presented as mean ± SEM. Bars sharing the same letter are not significantly different (One-way ANOVA followed by the Holm-Sidak post test $p<0.05$).

FIGURE 3.5.

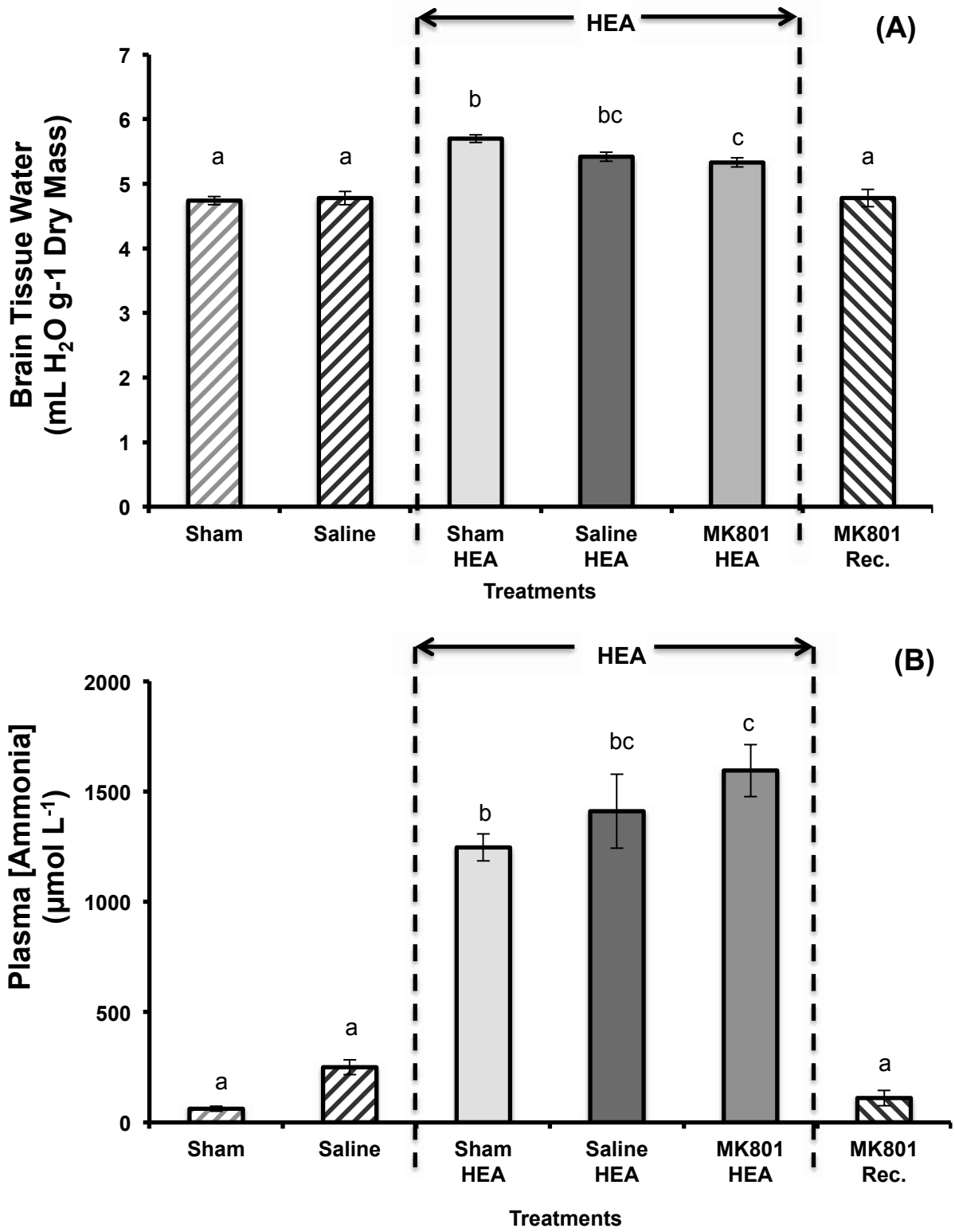
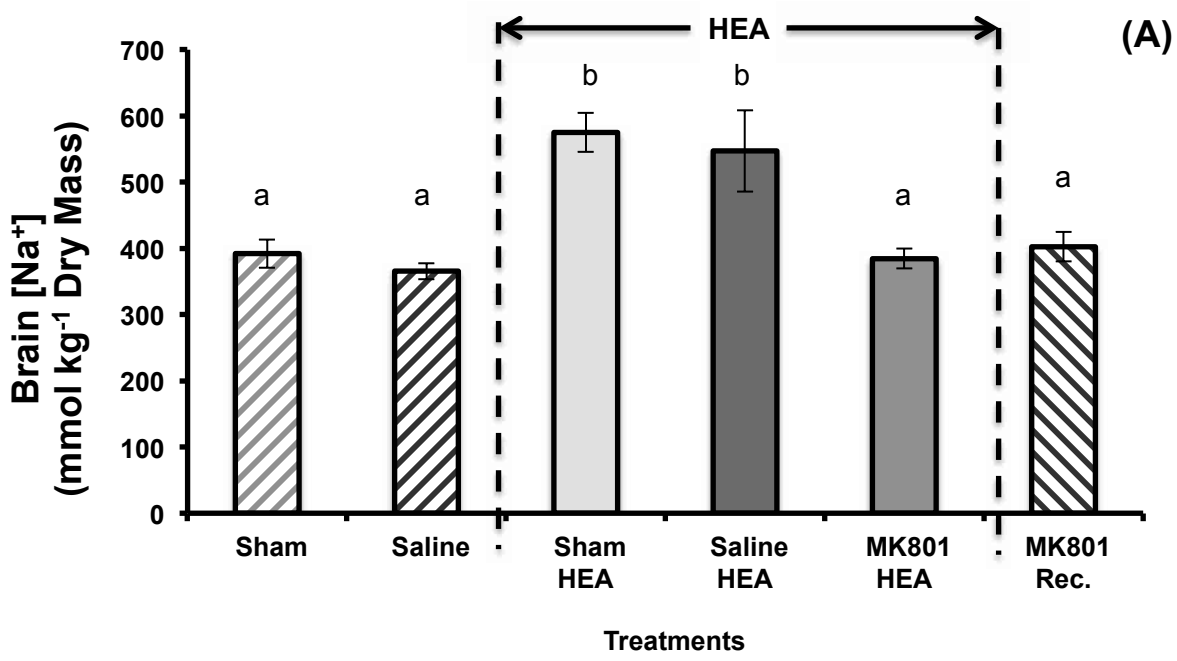
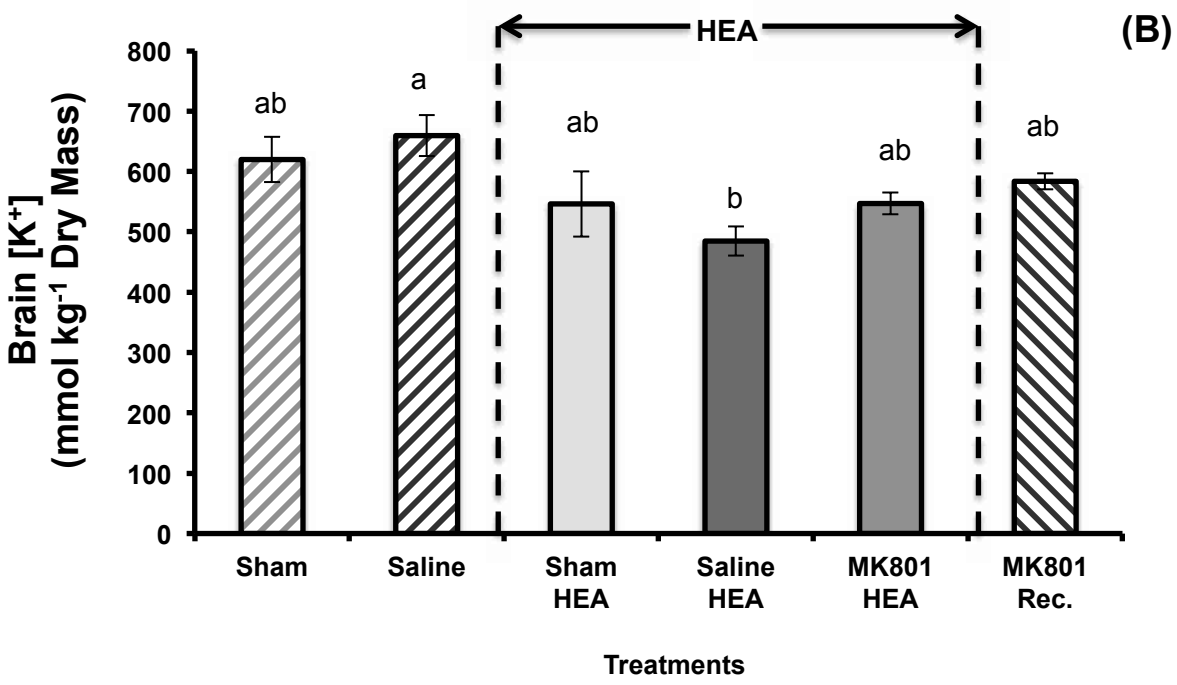

HEA
(A)
Brain Tissue Water
(mL H_2O g-1 Dry Mass)
7
6
5
4
3
2
1
0
a
a
b
bc
c
a
Sham
Saline
Sham HEA
Saline HEA
MK801 HEA
MK801 Rec.
Treatments
HEA
(B)
Plasma [Ammonia]
(µmol L^{-1})
2000
1500
1000
500
0
a
a
b
bc
c
a
Sham
Saline
Sham HEA
Saline HEA
MK801 HEA
MK801 Rec.
Treatments

Figure 3.6. Effects of MK801 on (A) Brain sodium concentration dry mass and (B) Brain potassium content in dry mass measured in goldfish held under control conditions (Sham $n = 8$ and Saline $n = 9$) or exposed to 48 h HEA (T_{Amm} 5 mmol L^{-1}; Sham HEA $n = 8$, Saline HEA $n = 8$, and MK801 HEA $n = 12$), and following recovery in ammonia-free well water (MK801 Recovery $n = 7$). Data are presented as mean ± SEM. Bars sharing the same letter are not significantly different (One-way ANOVA followed by the Holm-Sidak post test $p<0.05$).

FIGURE 3.6.


(A)
HEA
Brain [Na+] (mmol kg-1 Dry Mass)
700
600
500
400
300
200
100
0
a
a
b
b
a
a
Sham
Saline
Sham HEA
Saline HEA
MK801 HEA
MK801 Rec.
Treatments


(B)
HEA
Brain [K+] (mmol kg-1 Dry Mass)
800
700
600
500
400
300
200
100
0
ab
a
ab
b
ab
ab
Sham
Saline
Sham HEA
Saline HEA
MK801 HEA
MK801 Rec.
Treatments

Chapter 4

An Integrative Approach to Investigate the Pathogenesis of Ammonia-induced Neurotoxicity in Rainbow Trout (*Oncorhynchus mykiss*) and Goldfish (*Carassius auratus*)

The effects of ammonia toxicity on brain water content and ion homeostasis in the fish brain

The objective of my thesis was to better characterize the mechanism(s) that causes ammonia-induced brain swelling in fishes. Two species of fish, ammonia-sensitive rainbow trout (*Oncorhynchus mykiss*) and ammonia-tolerant goldfish (*Carassius auratus*) were studied using *in vivo* approaches.

Unlike mammals, goldfish and rainbow trout experienced reversible brain swelling following HEA exposure, without any noticeable changes in their behavior or health. In mammals, multiple layers of connective tissues protect the brain and spinal cord. Three layers of meninges, which consist of dura mater, archanoid mater, and pia mater (Nabeshima et al. 2004), provide the brain and spinal cord with a cushion-like protection. However, the additional layer means less room and limits for mammalian brain swelling capacity (Nilsson 2001). The compromise of "padding" makes mammals more sensitive in the presence of brain related injuries or diseases. In comparison, the meninges in fish are comprised of, ectomeninx, intermeningeal, and endomeninx (Wang et al. 1994). Where the ectomeninx is considered to be the counterpart of the dura mater in mammals and endomeninx is the counterpart of the pia and arachnoid mater in mammals (Momose et al 1988; Wang et al. 1995). Moreover, fish may have evolved strategies such as, larger cranial cavities to tolerate internal ammonia elevations during feeding bouts and following exhaustive exercise. Larger cranial cavities would reduce the risk of brain damage arising from increases in intracranial pressure. Additionally, in aquatic environments the water surrounding the fish may act as a natural "shock

absorber", providing support, buoyancy and "padding" for the brain and body of the fish (Kardong 2001).

Variation in the ammonia-tolerance of different fish species

Ammonia toxicity in fish is primarily attributed to the un-ionized NH_3 form, which is highly diffusible across the gills, allowing it to rapidly build-up in the blood (Wilkie et al. 2002). Upon entry, it is unclear how ammonia causes brain swelling. However, in the present study it is evident that ammonia resulted in significant ionic disturbances. These changes were accompanied by a decrease in Na^+/K^+-ATPase. Additionally increases in internal brain ammonia led to a decrease in Na^+/K^+-ATPase protein activity in rainbow trout but not in goldfish. The observed suggests that ammonia-induced oxidative stress may have altered, damaged or decreased Na^+/K^+-ATPase protein abundance thus affecting V_{max} and ion homeostasis.

The inhibition of N-Methyl-D-aspartate receptors (NMDA receptors) with (dizocilpine) MK801 attenuated brain swelling in rainbow trout but not goldfish during HEA, suggesting that more ammonia-sensitive fishes have greater susceptibility to the excitotoxic effects of ammonia. Wilkie et al. (2011) demonstrated that MK801 treatment prior to HEA, prolonged rainbow trout survival during exposure to the toxic concentrations of ammonia, but did not enhance goldfish survival time, suggesting that goldfish receptor may be resistant to ammonia-induced excitotoxicity. This would make sense because goldfish and closely related crucian carp (*Carassius carassius*) have evolved different strategies to overcome anoxia and ammonia toxicity, both of which target NMDA receptors and cause brain edema (Walsh et al. 2007; Wilkie et al. 2008; Wilkie et al. 2011; Wilkie et al. 2015). A common adaptation demonstrated in most

ammonia-tolerant fishes and goldfish exposed to HEA is a high neural tolerance to ammonia (Wilkie et al. 2011). Although *in vitro* experiments revealed that goldfish NMDA receptors are sensitive to ammonia, they also demonstrated, long term *in vivo* exposure to HEA resulted in a 40-60% reduction in the abundance of the NR1 subunit of the receptor, suggesting that there is a downregulation of NMDA receptors to protect against ammonia toxicity and perhaps brain swelling (Wilkie et al. 2011). The present study demonstrated that MK801 prevented brain swelling and ionic disturbances during HEA exposure in rainbow trout, which further implicates the NMDA receptors role in ammonia toxicity response of fishes, including brain swelling.

Another possible source of NMDA receptor over-excitation is due to ammonia's inhibitory effect on the Na^+: glutamate co-transporters EAAT-1 and EAAT-2, which regulates glutamate concentration in the synaptic cleft. Such inhibition could cause increased concentration of synaptic glutamate (Norenberg et al. 2005; Felipo and Butterworth 2002), resulting in over-activation of NMDA receptors, leading to the increases in intracellular Ca^{2+} that consequently causES neuronal swelling and death (Hermengildo et al. 1996). Additionally, HEA and anoxic conditions have been observed to cause NMDA receptor depolarization. Because of the similarities in mechanisms of toxicity, it is hypothesized that anoxia-tolerant species should also be tolerant to high concentrations of ammonia. Glutamate excitotoxicity results in the over-activation of NMDA receptors following the production of reactive oxygen species (ROS) and reactive nitrogen species (NO; Kosenko *et. al* 1994; Albrecht and Norenberg 2006), resulting in oxidative stress. Another possible explanation for goldfish high tolerability to ammonia is that they may increase their antioxidant activities to limit oxidative damages to the brain.

Indeed, studies have demonstrated that goldfish have a very high capacity to detoxify ROS due to the presence of high activities of antioxidant enzymes in their brains (Luschak et al. 2001), which allows them to be more resistant to ROS production during post-anoxic/hypoxic exposures, limiting cellular oxidative damage (Lushchak 2006; Lisser et al. 2016) and increase neural tolerance (Wilkie et al. 2011). These findings may explain why goldfish have a higher tolerance to ammonia compared to the trout.

I also tested the hypothesis that brain swelling is a result of intracellular accumulation of glutamine via astrocyte cells. However, the findings in this study do not support the hypothesis that elevated intracellular glutamine accumulation results in astrocytic osmotic disturbances. However, we cannot completely dismiss the fact that glutamine may act as an osmolyte. Though glutamine may not act as an osmolyte during HEA, brain swelling may be mediated by ROS and RONS caused by cellular accumulation of glutamine during ammonia toxicity (Albrecht and Norenberg 2006). These findings were consistent with the work of Sanderson et al. (2010), who reported that brain ammonia levels remain unaffected following MSO treatment and HEA exposure.

A model for ammonia-induced brain edema in fishes

In order for ammonia to enter the brain, it has to pass the blood brain barrier (BBB). The BBB structure consists of two cell layers, the capillary endothelium and the endfeets of astrocyte cells (Ott and Vilstrup 2014). A unique feature of the brain endothelium is that it contains the highest concentration of mitochondria than other endothelium in the body (Ott and Vilstrup 2014). The high density of mitochondria is required to meet the energy demands for the large number of membrane proteins, which

mediate the transport of ions and organic molecules (Ott and Vilstrup 2014). Astrocyte cells form the additional layer around the endothelial cells, which protect the neurons and provide structural support (Ott and Vilstrup 2014). However, as atrial ammonia concentrations elevate the brain becomes more susceptible to ammonia uptake (Sorensen and Keidling 2007). Ammonia may passively diffuse across the BBB in the form of NH_3; however, NH_4^+ may be actively transported by competing with K^+ dependent ion channels.

It is evident that ammonia triggers brain swelling in mammals and fish, adversely altering numerous metabolic pathways. Based on my finding with previous work done on the neurophysiology of ammonia toxicity in fishes (Refer to References). I proposed that acute ammonia toxicity is the result of the over-activation of NMDA receptors triggering oxidative stress, which leads to the disruption of ion protein channels thus, interfering with ionic homeostasis in fish. In the presence of ammonia, NMDA receptors located on neurons and astrocytes are more susceptible to activation resulting in marked increases of intracellular Ca^{2+} and Na^+. Ammonia depolarizes the post-synaptic membrane causing the removal of Mg^{2+} block, which leads to the overstimulation of NMDA receptors leading extracellular glutamate in the synaptic cleft (Fan and Szerb 1993). Glutamate levels rise sharply resulting in overly excited cells, initiating the excessive opening of pores, which allow large influxes of substances that are normally regulated to enter the cell. One of these substances is Na^+; increase Na^+ entering the cell accompanies water, consequentially resulting in subsequent swelling of the brain. Additionally, glutamate released from the pre-synaptic neuron promotes the overstimulation of post-synaptic NMDA receptors promoting increase intracellular Ca^{2+}. Increases in intracellular Ca^{2+}

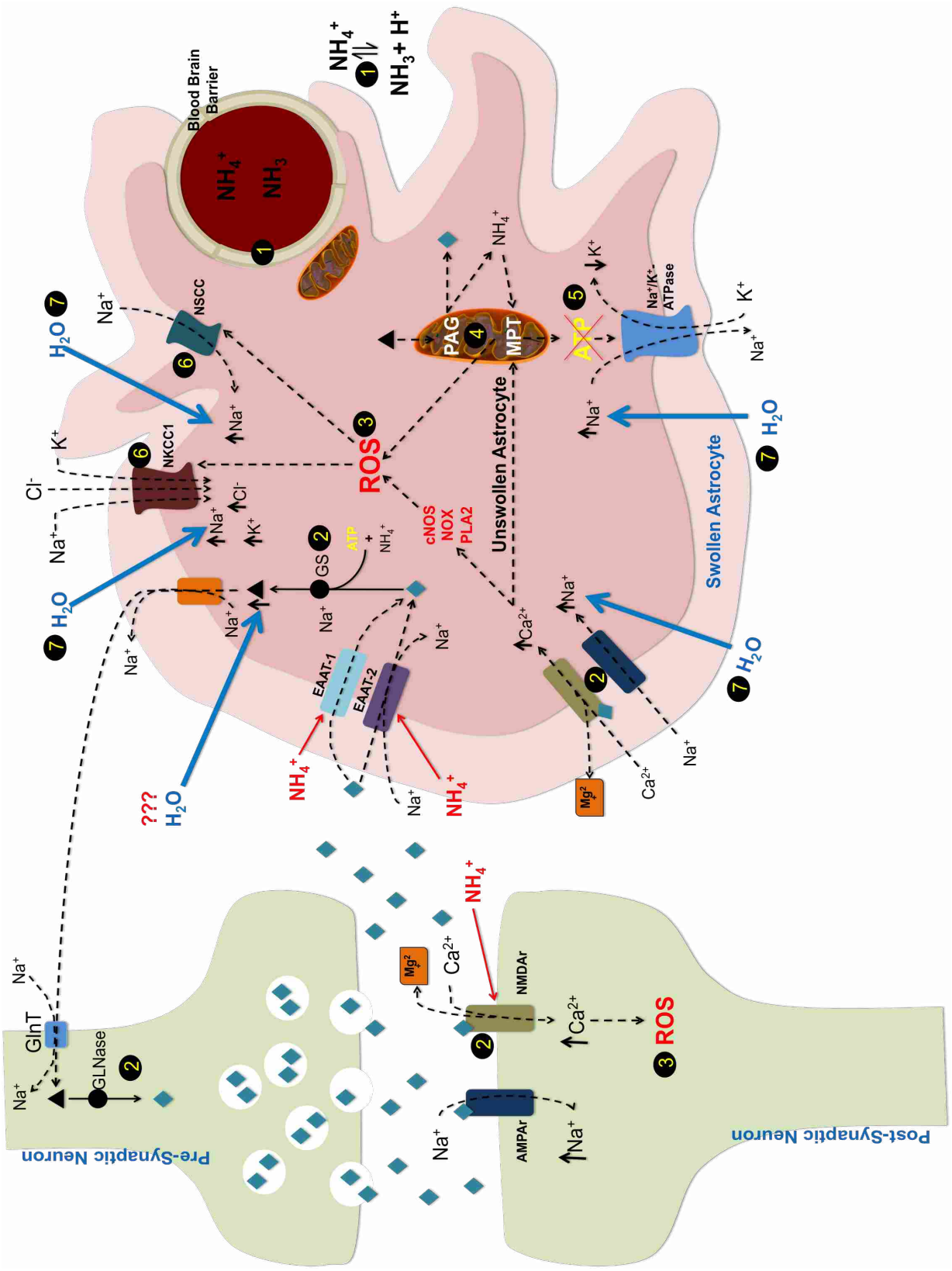
concentration stimulate the production of ROS and NO in neurons and astrocytes (Schliess et al. 2002). The generation of ROS represent a possible key event for the induction of the various downstream events in ammonia neurotoxicity including the initiation of mitochondrial permeability transition (MPT; Rama Rao et al. 2005), MAP Kinase (MAPK), and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB,) causing further free-radical production and injury, lipid peroxidation, protein damage, RNA/DNA oxidation, marked astrocytic swelling, and necrosis/apoptosis (Norenberg et al. 2009; Sinha et al. 2014; Lisser 2016). The enhanced production of ROS during ammonia neurotoxicity instigates lipid peroxidation, oxidation of proteins, enzyme inhibition, and damage to nucleic acid, involved in maintaining ionic and osmotic homeostasis. Overproduction of ROS may impair of Na^+/K^+-ATPase α-subunit, which ultimately alter Na^+ and K^+ balance in the cell resulting in osmotic disturbances. All of which combined may be a source for astrocytic swelling.

Ammonia causes astrocyte cells to become more susceptible to depolarization. Bender and Norenberg (1998) observed that there was an increase in extracellular K^+ followed by a decrease in K^+ uptake by the astrocyte cells. Perhaps, due to the close resemblance of NH_4^+ to K^+, NH_4^+ may be competing with K^+ and effect NKCC1 cotransporters, Na^+/K^+-ATPase and Na^+/K^+-ATPasae-induced signal by endogenous ouabain. Thus, eliciting the production of oxidative (ROS) and nitrosative (RONS) stress acting on NKCC1. ROS and RONS agent triggers astrocytic swelling by slowly acting on NKCCI, resulting in an ionic disturbance (Hertz et al. 2015). Future studies should focus on NH_4^+ effects on these ion channels. The close resemblance of K^+ to NH_4^+ may also explain the rapid depletion of ATP during ammonia toxicity (Kosenko et al. 1994). The

rapid depletion of ATP pumps would starve Na^+/K^+-ATPase leading to the loss of membrane integrity by disrupting the transmembrane ion gradient in neurons and astrocytes.

Figure 4.1. Proposed Model of Ammonia-Induced Astrocyte Swelling: (1) (Entrance of Ammonia): Ammonia enters the brain via blood brain barrier in the form of NH_3 because brain pH is lower than arterial pH, excess protons in the CNS converts NH_3 to NH_4^+. NH_4^+ becomes trapped and acts on the CNS functions. (2) (Glutamate Excitotoxcity): NH_3 and NH_4^+ target neurons and astrocytes making them more susceptible to depolarization. Glutamate is the major excitatory neurotransmitter in the CNS binding onto AMPA receptors and NMDA receptors. Depolarization leads to the removal of Mg^{2+}, overstimulating NMDA receptors leading to an influx of Na^+ and Ca^{2+}. Furthermore, uncontrolled release of glutamate results in ATP depletion in astrocyte via ATP-Dependent GS conversion to GLN. (3) (Production of ROS): Increase intracellular Ca^{2+} can lead to the overproduction of ROS in neurons and astrocytes. (4) (Bioengergertic Failure): ROS induces mitochondrial permeability transition impairing ATP production in astrocytes. (5) (Ionic Homeostasis Disturbances): ATP depletion results in the failure of Na^+/K^+-ATPase pump, which leads to the collapse of the ionic gradient. As a result, Na^+ ions accompanied by Cl^- move along its concentration gradient into the cell. (6) (ROS-mediated Ionic Homeostasis Disturbances): ROS directly affects alpha 1 subunit of Na^+/K^+-ATPase, NKCC-1 and NSCC resulting in further loss of ion homeostasis. (7) (Astrocytic Swelling): Water accompanies the influx of ions via osmosis resulting in astrocytic swelling.

FIGURE 1.


Blood Brain Barrier
NH_4^+
NH_3
NH_4^+ NH_3 + H^+
Unswollen Astrocyte
Swollen Astrocyte
ROS
cNOS
NOX
PLA2
PAG
MPT
ATP
GS
NSCC
NKCC1
Na+/K+-ATPase
EAAT-1
EAAT-2
GlnT
GLNase
NMDAr
AMPAr
Pre-Synaptic Neuron
Post-Synaptic Neuron
H_2O
???

Future directions

Very little is known about the relative cranium size and variability amongst the different species of fish. But, future studies should carefully measure and examine the cranial volume in more species of fish. These observations would prove extremely useful for studying ammonia and anoxia tolerance variability between the different species of fish. Additionally, future studies should focus on a side-by-side comparison of ammonia-tolerant and ammonia-sensitive fish brain in response to an equivalent concentration of internal ammonia. This study would provide additional information between the different mechanistic responses to ammonia toxicity between ammonia-tolerant and ammonia-sensitive fish.

During hyperammonemia, there may be a strong connection between the large concentrations of mitochondria in the endothelium and high arterial concentrations of ammonia. Recently, studies demonstrate that high level of ammonia concentrations results in the induction of the mitochondrial permeability transition (MPT), which leads to additional ROS production and bioenergetics failure. Perhaps, the high density of mitochondria located around the BBB could amplify the production of ROS, which can lead to ROS-mediated swelling of astrocyte cells. Therefore, the understanding the downstream signaling pathway of mitochondrial-mediated oxidative stress in fish could prove useful in determining the underlying mechanism.

It is not known if the NMDA receptor is involved in the ammonia toxicity response of other fishes, particularly those with high ammonia tolerance, which can widely vary among different species. For instance, the swamp eel (*Monopterus albus*) can survive in concentrations of ammonia greater than 75 mmol L^{-1} and accumulate brain

[T_{Amm}] to concentrations as high as 4.5 mmol kg^{-1} (Ip et al. 2013), much higher than in the goldfish. Future studies are needed to determine if the brains of extremely ammonia tolerant fishes such as the swamp eel are more resistant to ammonia induced swelling and ionic disturbances than goldfish and other fishes. Additionally, along with studies aimed at further elucidating how inhibition of the NMDA receptors in these more ammonia-tolerant fish alters their neurophysiological responses to ammonia.

The down-regulation of Na^+/K^+-ATPase activity has been previously demonstrated in the ammonia-tolerant *M. albus* (Chen et al. 2013). Additionally, recent studies revealed that ROS might target and inhibit Na^+/K^+-ATPase activity. Thus, further investigation of Na^+/K^+-ATPase mRNA expression in rainbow trout and goldfish would prove useful in determining the pathology of ammonia toxicity. Additionally, future studies should focus on the functional studies of Na^+ channel density and activity to elucidate the role of reduced Na^+/K^+-ATPase activity observed in rainbow trout exposed to 48 h HEA.

Lastly, ammonia toxicity results in glutamine accumulation by ways of glutamine synthetase and activation of NMDA receptors. Synergistic protection with MK801, and memantine an NMDA receptors inhibitor and MSO a GS inhibitor, should be the next approach for future studies in ammonia-tolerant and ammonia-sensitive fish. The information generated from both species could prove extremely useful in the development of new neuroprotective strategies.

Environmental & Clinical Significance

Elevated levels of external ammonia stemming from anthropogenic sources such as, sewage effluents, agricultural run-off, overfeeding and overcrowding can be a concern

for aquatic environments. Hyperammonemia may occur in fish following bouts of feeding and vigorous exercise (Wick and Randall, 2002a; Randal and Tsui 2002). Studies have reported that fed fish experience plasma ammonia concentrations [T_{Amm}] spikes, similar to those associated with death resulting from high external ammonia (HEA; Randal and Tsui 2002; Wicks and Randall 2002a). High external ammonia (HEA) has been demonstrated to have adverse neurological effects in aquatic organisms. Thus, using ammonia-sensitive and ammonia-tolerant fish for this study provide as a useful and beneficial model for studying ammonia toxicity. The key to ammonia tolerance of fish appears to be located in the CNS. The wide variety of studies involving ammonia toxicity demonstrates that the CNS in fish is more resilient and superiorly adapted to ammonia toxicity compared to mammals. Fish demonstrate the ability to tolerate a much higher plasma and brain [T_{Amm}] compared to mammals. Some species of fish have the ability to withstand millimolar increases in plasma and brain ammonia concentrations (Randall and Tsui 2002; Ip et al. 2004a; Wilkie et al. 1999, 2011). Thus, by understanding adaptive responses in neurons and astrocytes of fish, we can develop new protective strategies for other organisms that are more susceptible to ammonia.

The mechanism(s) of brain swelling in mammals let alone fish is poorly understood. Brain swelling arises from the accumulation of excessive fluid in the intracellular spaces of brain cells and/or fluid build-up in the ventricles of the brain (Chesler 2003). In mammals, the excess fluid exerts outward pressure on the brain tissue, pressing against the skull leading to coma, brain damage and death. A condition referred as hepatic encephalopathy, which can arise from trauma, stroke, the accumulation of toxic substances in the body, and liver damage or failure. Hepatic encephalopathy takes

place when brain swelling occurs due to liver damage or failure. In this condition, the liver is unable to convert ammonia to urea, resulting in accumulation ammonia in the bloodstream, which is termed hyperammonemia. Ammonia targets the astrocytes, which play a crucial role in promoting the growth, development and nourishment of neurons in the nervous system. Excess ammonia is associated with cumulative increases in the intracellular fluid volume of the astrocytes. Because the cellular composition of the brain is mainly astrocytes, swelling of these cells causes the brain to swell. However, little else is known about the pathological processes that underlie HE and the actual mechanism(s) of ammonia-induced brain swelling remain unclear. In mammals, increased ammonia in the blood can arise from liver failure, alcohol abuse, inborn genetic error of ornithine-urea cycle, prehepatic "portal" hypertension, resulting in cerebral edema and death (Felipo and Butterworth, 2002; Wilkie et al. 2011). Hyperammonemia is a serious condition that often results in brain damage. Current treatments involved are dietary changes to maintaining blood ammonia concentration, urea cycle disorder drug treatments, hemodialysis and ultimately liver transplant (Feldman et al. 2014). Ultimately, the only permanent treatment for HE patients is a liver. The development of successful neuroprotective drugs to protect the CNS from ammonia-induced damages would be extremely beneficial. In the present study, using fish to understand the effectiveness and response to different pharmacological agents can be valuable in developing innovative therapies for patients suffering from hepatic encephalopathy.

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