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Physiological & Cellular Mechanisms of Ammonia Tolerance in the Goldfish (Carassius auratus)

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by

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ABSTRACT

Ammonia may increase in the blood and tissues of fishes following feeding or exposure to high concentrations of environmental ammonia arising from the degradation of organic matter in aquatic environments, sewage effluent, and run-off from landfills. Ammonia may be detoxified using the enzyme glutamine synthetase (GS), which catalyzes the conversion of ammonia to glutamine. It has been suggested, however that build-ups of glutamine within the astrocytes of the brain can cause brain swelling by increasing intracellular osmolarity and water uptake by the cells. Using *in vivo* and *in vitro* approaches, the goal of this thesis was to determine if: (i) exposure to high external ammonia (HEA) caused brain swelling in ammonia-sensitive trout (*Oncorhynchus mykiss*) compared to ammonia-tolerant goldfish (*Carassius auratus*), and (ii) glutamine accumulation was the underlying cause of brain swelling. Exposure of goldfish and trout to HEA led to an increase in plasma and brain ammonia concentration, and consequently an increase in brain tissue water content in both species, which was consistent with brain swelling. The threshold external ammonia concentration for initiating brain swelling was 5-fold greater in the goldfish than in the trout, which was consistent with the greater ammonia tolerance of goldfish. Brain water content was restored to control levels following recovery in ammonia-free water in both species. Administration of the GS inhibitor methionine sulfoximine (MSO) to each species prior to HEA caused a reduction in GS activity and glutamine accumulation but did not lead to the attenuation of brain swelling. Experiments using trout and goldfish brain cell lines demonstrated that MSO exposure did not increase cell viability, but the osmoprotectant amino acid taurine did increase cell viability of rainbow trout brain cell lines. In conclusion, increased internal

ammonia does cause water accumulation in the brain, but this is not the result of increased glutamine production.

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List of Abbreviations

AB	Alamar Blue
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid
ATP	adenosine triphosphate
CFDA-AM	5-carboxy-fluorescein diacetate acetoxymethyl ester
CNS	central nervous system
CSF	cerebrospinal fluid
DAPI	4', 6-diamidino-2-phenylindole
DCF-DA	5-(6-) carboxy-2', 7' dichlorodihydrofluorescein diacetate
DTT	dithiothreitol
EAAT	excitatory amino acid transporter
EDTA	ethylenediaminetetraacetic acid
FBS	Fetal Bovine Serum
FITC	fluorescein isothiocyanate
GDH	glutamate dehydrogenase
GFAP	glial fibrillary acidic protein
GFB3C	goldfish cerebellum cell line
GLN	glutamine
GLNase	glutaminase
GS	Glutamine synthetase
GLU	glutamate
HBSS	Hank's Buffered Saline Solution
HE	hepatic encephalopathy
HEA	high external ammonia
^{3}H-OMG	3-O-methyl[3H]-D-glucose
IC_{50}	Inhibition concentration causing 50% of effect
ICF	intracellular fluid
ILCM	interlamellar cell mass
L15	Leibovitz 15 growth medium

L15 ex	Simplified version of Leibovitz L-15, used as exposure media
MPT	mitochondrial permeability transition
MSO	methionine sulfoximine
MS222	tricaine methanesulfonate
NADPH	nicotinamide adenine dinucleotide phosphate
NF	neuronal filament
NMDA	N-Methyl-D-Aspartate
NO	nitric oxide
NOS	nitric oxide synthase
PBS	Phosphate Buffered Saline
PCA	perchloric acid
RFU	relative fluorescent units
ROS	reactive oxygen species
RTB	rainbow trout brain cell line
SOD	superoxide dismutase
T_{Amm}	total ammonia
TBARs	thiobarbituric acid reactive substances
TCA	tricarboxylic acid / citric acid cycle

Chapter 1

General Introduction

INTRODUCTION

Ammonia production and excretion

Excess protein undergoes proteolysis into smaller peptides and amino acids. The majority of amino acids are then converted via transaminase enzymes into the amino acid glutamate, which is then oxidatively deaminated in the liver via the enzyme glutamate dehydrogenase (GDH), generating NH_3 and α-ketoglutarate (Mommsen and Walsh 1991; Wright 1995), the latter which is a C-skeleton that can be used for energy production (Schenone *et al.* 1982). Because this process leads to the generation of highly toxic ammonia, animals must have a means to either readily excrete NH_3 or convert it to alternate less toxic nitrogenous waste products such as urea (Wright 1995).

Ammonia exists as either un-ionized NH_3 or the ionized cation NH_4^+, and the sum of these two species of ammonia is referred to as the total ammonia (T_{Amm}) concentration. The relationship is described by the following equation:

$$NH_3 + H_2O \leftrightarrow NH_4^+ + OH^-$$

At a physiological pH of 7.8 in the blood of fish (Wilkie and Wood 1991), the majority of T_{Amm} exists as NH_4^+ as a result of the high pK of ammonia (9.5 at 15ºC; Cameron and Heisler 1983). Un-ionized NH_3 is most toxic to fishes because it readily crosses the lipid bilayer of the membranes that comprise the gill epithelium (Wilkie 1997; Ip *et al*. 2001; Eddy 2005).

Being a building block of amino acids and nucleic acids, ammonia may be beneficial to organisms, but excessive concentrations can have toxic effects (Campbell 1973; Wood 2001). Most fishes excrete ammonia in the form of NH_3

because it is highly diffusible, and soluble in water (Wood 1993; Wright 1995; Wilkie 2002). However, semi-terrestrial and terrestrial vertebrates, such as amphibians and mammals, convert ammonia to less toxic urea, a process which requires less water to dilute ammonia to non-toxic levels (Wright 1995). Animals that live in very dry environments, such as reptiles, convert ammonia to uric acid, which requires little water to be excreted and can be stored in the body for long periods of time due to its low mass and toxicity (Wright 1995; Wright and Wood 2009).

The mechanism of ammonia excretion in fish is controversial, but it appears to rely on the presence of rhesus (Rh) glycoproteins in the gills, which provide channels for ammonia to move by passive diffusion from water into the blood down NH_3 partial pressure gradients (see Weihrauch *et al*. 2009; Wright and Wood 2009 for reviews). When water ammonia concentration or pH is elevated, the internal ammonia concentrations undergo marked increases due to the reduction or elimination of the NH_3 diffusion gradient (Schenone *et al*. 1982; Wright and Wood 1985; Wilkie and Wood 1991; Knoph and Thorud 1996; Ip *et al*. 2001; Ip *et al*. 2004a, 2004b; Wilkie *et al*. 2011). Some aquatic organisms, however, have mechanisms to transport ammonia against inwardly directed gradients when exposed to high environmental ammonia (Wilson *et al.* 1994; Randall *et al*. 1999) while others are able to convert ammonia to urea, which is less toxic (Mommsen and Walsh 1989; Randall *et al*. 1989).

Ammonia toxicity in mammals and aquatic organisms

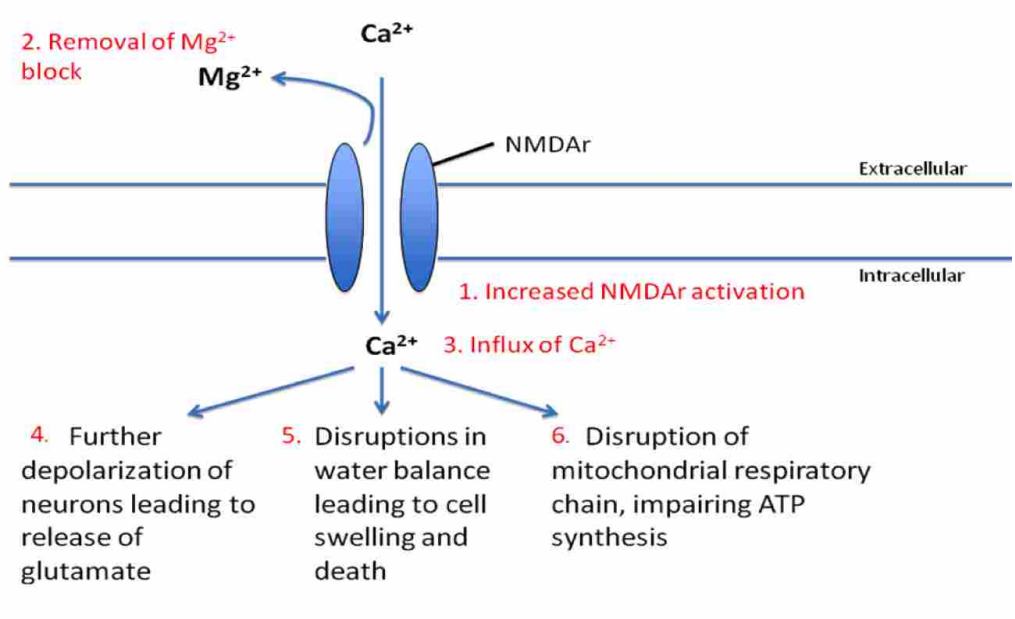
The mechanism by which ammonia toxicity occurs in mammals is poorly understood. Increased ammonia (hyperammonemia) can arise from liver disease due to ethanol

abuse, viral infections, autoimmune disease or inherited deficiencies in urea production (Ip *et al.* 2001; Felipo and Butterworth 2002; Randall and Tsui 2002) and can lead to brain edema, coma, and, in severe cases, death.

The brain and spinal cord make-up the central nervous system (CNS) in vertebrates, which is comprised of two main classes of cells: neurons and glia cells. Glial cells generally outnumber the neurons in higher vertebrates (Allen and Barres, 2009) but the relative amounts of each are not well established in fishes. Nevertheless, they provide structural support, metabolic support and electrical insulation for nerves (Kandel *et al.* 2000), and are most prone to the multi-faceted neurophysiological effects of increased ammonia. These disturbances include: glutamate excitotoxicity, the generation of reactive oxygen species, and the swelling of astrocytes (specialized glial cells) (Fan and Szerb 1993; Hermenegildo *et al*. 1996; Brusilow 2002; Felipo and Butterworth 2002; Kosenko *et al*. 2003).

Glutamate excitotoxicity is caused by over-activation of NMDA receptors in the CNS. The NMDA receptors are ionotropic glutamatergic receptors that are activated by glutamate, an amino acid that is also the most abundant excitatory neurotransmitter in the CNS and important for numerous processes such as learning and memory (Wenthold *et al.* 2003). NMDA receptors primarily act as Ca^{2+} channels when activated by the agonist NMDA or by glutamate (Wenthold *et al.* 2003). When over-activated, the NMDA receptor facilitates the movement of Na^{+} and large amounts of Ca^{2+} ions into the intracellular space of the cell (Figure 1.1), activating protein kinases and lipases, which interfere with cell function.

Figure 1.1: Proposed Mechanism of Neuronal Excitotoxicity Caused by Hyperammonemia. Excitotoxicity is a process by which over-activation of the NMDA receptor causes the opening of ion channels allowing an influx of Ca^{2+}. 1) The increase in NMDA receptor activation occurs due to an increase in glutamate. 2) Depolarization leads to the removal of the Mg^{2+} block. 3) The removal of the Mg^{2+} block allows for an influx of Ca^{2+}. This influx causes: 4) further depolarization of the neuron, 5) disruptions in the osmotic balance of the neuron, leading to cellular swelling and potentially cell death as well as 6) disruptions in the mitochondrial respiratory chain, impeding ATP production.


2. Removal of Mg^{2+} block
Ca^{2+}
Mg^{2+}
NMDAr
Extracellular
Intracellular
1. Increased NMDAr activation
Ca^{2+}
3. Influx of Ca^{2+}
4. Further depolarization of neurons leading to release of glutamate
5. Disruptions in water balance leading to cell swelling and death
6. Disruption of mitochondrial respiratory chain, impairing ATP synthesis

It is unclear how ammonia over-activates the NMDA receptor but one possibility is that it depolarizes neurons promoting the release of Mg^{2+} (Fan and Szerb 1993), which normally prevents Ca^{2+} from entering the channel under resting conditions (Wenthold *et al*. 2003). Another possibility is that ammonia exacerbates excitotoxicity by causing increased synaptic glutamate due to the inhibitory effects of ammonia on the Na^{+}: glutamate co-transporters EAAT-1 and EAAT-2, that normally remove glutamate and regulate its concentration in the synaptic cleft (Norenberg *et al.* 2005; Felipo and Butterworth 2002). Both processes lead to over-stimulation of the NMDA receptor leading to increased intracellular calcium, causing excitotoxicity and neuronal death (Hermenegildo *et al.* 1996). In mammals, NMDA receptor antagonists, such as dizocilpine (MK-801), ketamine, butanol, and ethanol can protect against ammonia toxicity by preventing NMDA receptor over-activation (Hermenegildo *et al*. 1996). The most protective agents are MK-801 and ketamine, which are non-competitive channel blockers of the NMDA channel that appear to protect the brain by reducing the influx of Ca^{2+} ions, thereby preventing excitotoxicity (Hermengildo *et al.* 1996).

The influx of calcium caused by the over-activation of NMDA receptors may also lead to increased production of the chemical messenger nitric oxide (NO) (Kosenko *et al*. 2003). A number of researchers have argued that as NO diffuses into astrocytes it also inhibits glutamine synthetase, which is thought to detoxify ammonia by catalyzing the formation of less toxic glutamine from ammonia and glutamate (Itzhak and Norenberg 1994; Rao *et al.* 1995; Rao *et al.* 1997). Although glutamine production is normally considered as a means to protecting against ammonia toxicity,

some researchers have suggested that increased glutamine production in astrocytes (astroglial cells) causes brain swelling by increasing intracellular osmolarity, leading to increased water uptake by the cells, and further damage or death (Haussinger *et al*. 1994; Norenberg 1998; Brusilow 2002).

The increased calcium in the brain resulting from glutamate excitotoxicity can also cause increased reactive oxygen species (ROS) and NO production in neurons and astrocytes (Schliess *et al.* 2002; Kosenko *et al.* 2003). Although NO mediates intracellular signaling, if present in excess quantities it can severely damage cells and cause cell death. Peroxides (H_2O_2) and ROS, including free radicals that consist of superoxide ions (O^{2-}) (Moyes and Schulte 2006), are often created as a by-product of oxidative metabolism, which takes place in the mitochondria. ROS react with macromolecules like lipids, proteins, carbohydrates and nucleic acids altering their function. As a result, both ROS and NO arising from oxidative stress can lead to cellular damage (Monfort *et al.* 2002; Lodish *et al.* 2003). Free radicals and ammonia may also induce the presence of mitochondrial permeability transition (MPT) pores in astrocytes, which could also lead to cell swelling (Rao *et al.* 2005). The MPT is a Ca^{2+}-dependent process, which is associated with oxidative stress. During oxidative stress, mitochondrial permeability is increased by the opening of the MPT pore, causing the mitochondrial inner membrane to collapse and the mitochondrial matrix to swell (Gunter and Pfeiffer 1990).

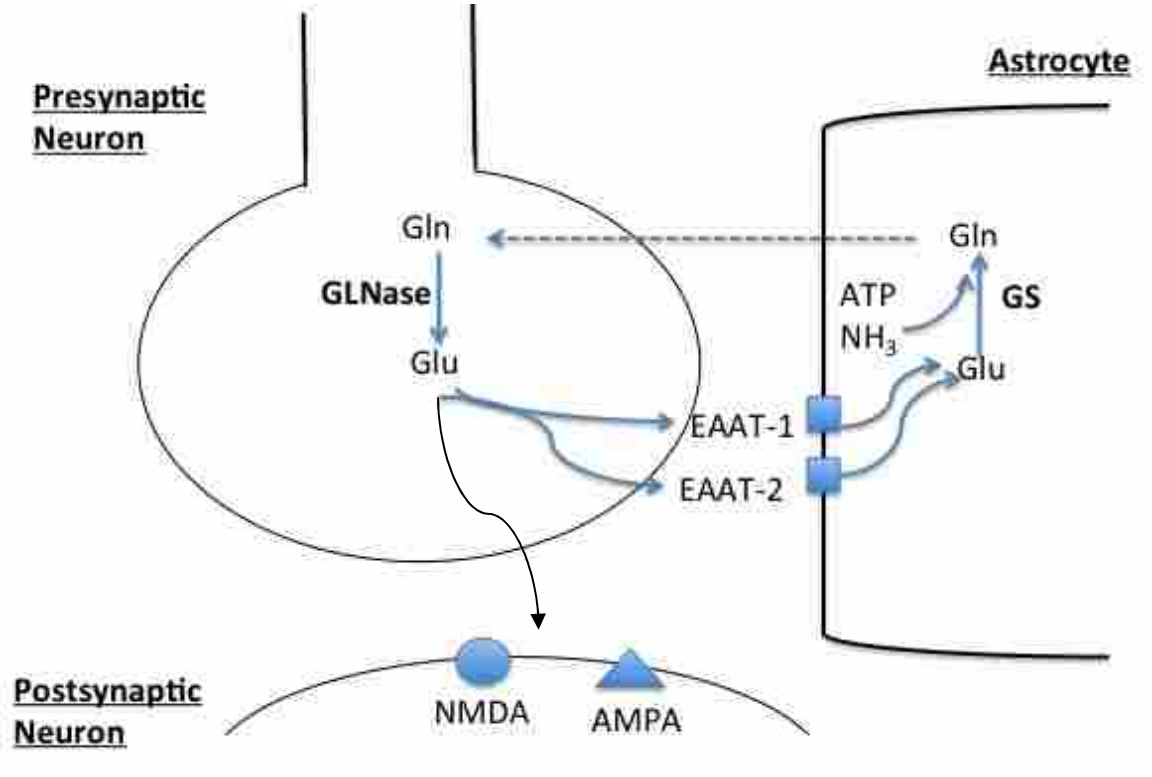
During hyperammonemia, there is only swelling of brain astrocytes rather than of neurons. The damaged astrocytes resemble Alzheimer Type II astrocytes, which are characterized by swollen cells, with distinct enlarged and pale nuclei

(Norenberg and Bender 1994). It has been suggested that the swelling may be a consequence of intra-astrocytic glutamine accumulation (Brusilow 2002; Albrecht and Norenberg 2006). A normal astrocyte recycles glutamate neurotransmitter from the synaptic cleft and, using glutamine synthetase, converts glutamate to glutamine by the addition of ammonia at the expense of ATP (Walsh *et al.* 2007). The glutamine is then transported back to the pre-synaptic neuron where it is processed into glutamate and sent to synaptic vesicles for synaptic release. This is referred to as the Glutamate-Glutamine cycle (Figure 1.2; Walsh *et al.* 2007).

Ammonia toxicity in anoxia-tolerant goldfish

Goldfish (*Carassius auratus*) are considered to be one of the most anoxia-tolerant species known because of their ability to reduce their ATP consumption in order to conserve energy (Walsh *et al.* 2007). Goldfish, and the closely related crucian carp (*Carassius carassius*), encounter low-oxygen environments during winter months when ponds are covered with ice and snow, which prevents oxygen replenishment from the atmosphere and oxygen generation since photosynthesis by plants and algae is reduced because sunlight penetration of the water column is blocked. (Lutz *et al*. 2003; Vornanen and Paajanan 2006). Reductions in western painted turtle (*Chrysemys picta*) ATP demand are brought about by "channel arrest" (Hochachka 1986; Bickler and Buck 2007), which decreases membrane ion-permeability, lowering the energetic demands of ion pumps such as the Na^+/K^+-ATPase and Ca^{2+}-ATPase. Similarly, channel arrest may also contribute to ATP conservation in goldfish in which decreases in NMDA receptor activity were observed in brain slices exposed to anoxia conditions (Wilkie *et al*. 2008).

Figure 1.2: Glutamate-Glutamine Cycling. Glutamate (Glu) is released from pre-synaptic neuron, and diffuses across the synaptic cleft where it activates glutamate receptors (NMDA and AMPA) leading to depolarization of post-synaptic neurons. The process is normally terminated when the glutamate is taken up by adjacent astrocytes via the Na^+:glutamate co-transporters EAAT-1 and EAAT-2. Within the astrocyte, the glutamate combines with ammonia (NH_3) and is converted to glutamine (Gln) in a reaction catalyzed by glutamine synthetase (GS). The Gln is then exported from the astrocyte, and taken up by the presynaptic neuron and converted back to Glu completing the cycle. Adapted from Walsh *et al.* 2007.


Astrocyte
Presynaptic Neuron
Gln
GLNase
Glu
Gln
ATP
NH3
GS
Glu
EAAT-1
EAAT-2
Postsynaptic Neuron
NMDA
AMPA

Anoxia-tolerant turtles, crucian carp and goldfish maintain basal ATP using anaerobic glycolysis, which is maintained by high stores of liver glycogen (Lutz *et al*. 2003). Goldfish also have high constituent concentrations of antioxidant enzymes in their brains and liver (Lushchak *et al*. 2001), which allow them to be resistant to post-anoxia/hypoxia increases in ROS production that is a universal response of different organisms when oxygenation is restored to tissues after periods of O_2 deprivation (Walsh *et al.* 2007). Moreover, the brains of anoxia-tolerant crucian carp, and likely goldfish, do not appear to swell with anoxia/hypoxia (Van der Linden *et al*. 2001). In more hypoxia/anoxia-sensitive vertebrates, such swelling is thought to arise in neurons as a result of insufficient ATP supply for ion transporters, leading to increases in intracellular Na^+ and Ca^{2+} that cause neuronal death in mammals (Lutz *et al*. 2003; Bickler and Buck 2007). Due to the similarities in the mechanisms of toxicity, it is believed that anoxia-tolerant species should also be tolerant to high ammonia. Walsh *et al*. (2007) have suggested that fish tolerant to hypoxic environments are also able to tolerate high environmental ammonia since both ammonia and anoxia cause the NMDA receptor to depolarize. If the mechanism used to tolerate both insults is the same, then goldfish are an ideal model to study the effects of elevated ammonia on the CNS.

The possibility of ammonia-induced brain swelling in fishes has only been examined in the gulf toadfish (*Opsanus beta*), a fish that can detoxify ammonia by converting it to urea using the ornithine urea cycle (Veauvy 2007). Veauvy *et al*. (2005) demonstrated that ammonia exposure had no effect on the hydration status of the gulf toadfish brain. With the exception of this study, fish brain swelling in

response to high environmental ammonia (HEA) has not been examined. Because of their high ammonia tolerance and large cranium (Van der Linden *et al*. 2001), the fish belonging to the genus *Carassius* provide an excellent model to determine if the brains of ammonia tolerant fishes are more resistant to swelling than those of more sensitive species such as the rainbow trout (*Oncorhynchus mykiss*). Because the brains of fishes are generally easy to remove from the cranium, or brain case, and less complicated than a mammalian brain because they lack a complex cerebral cortex (Nabeshima *et al.* 2004), fishes could also be an excellent model for studying the underlying mechanisms of brain swelling during hyperammonemia.

Hypotheses & Objectives

Using a combination of whole animal and cell culture techniques, the goal of my thesis was to determine the underlying mechanisms that lead to brain swelling during periods of hyperammonemia in the goldfish and rainbow trout. Accordingly, this thesis tested the following hypotheses:

Hypothesis 1: Goldfish survival at high external ammonia is related to the ability of these fish to recover from ammonia-induced brain swelling more quickly than rainbow trout.

Hypothesis 2: Increased intracellular glutamine is the cause of brain swelling in both goldfish and the trout.

To test these hypotheses, various *in vivo* and *in vitro* experiments were carried out. Goldfish and rainbow trout were exposed to HEA and brain water content was measured (Hypothesis 1). Some fish were placed in recovery to deduce if goldfish were able to recover from brain swelling more quickly than rainbow trout. Brain ammonia was also quantified to determine the ammonia tolerance of goldfish brain compared to the rainbow trout brain. *In vitro* studies were also conducted by exposing goldfish and rainbow trout brain cell lines to various concentrations of ammonia to determine if cell lines provided a proxy model system that could be used to learn more about the underlying mechanisms of ammonia toxicity and tolerance in fishes. Accordingly, viability assays and cell morphological analyses were conducted to better understand how the CNS of the goldfish and rainbow trout responded to hyperammonemia.

To learn more about the underlying mechanisms of brain swelling, goldfish and rainbow trout were exposed to HEA after being injected with the glutamine synthetase antagonist methionine sulfoximine (MSO) following which brain water content, ammonia and glutamine content, and glutamine synthetase activity were measured (Hypothesis 2). *In vitro* experiments were conducted to further test the hypothesis that glutamine accumulation was the underlying cause of brain swelling in the fish brain. In these experiments goldfish and rainbow trout brain cell lines were pre-exposed to MSO and then exposed to HEA to test whether MSO is able to increase the viability of the cultured cells. Brain cell lines were also exposed to ammonia in the presence of taurine, an osmoprotectant amino acid to determine if ammonia-toxicity was in fact due to increases in intracellular osmotic pressure

(Saransaari and Oja 2000). Although *in vivo* responses to ammonia and anoxia have been studied, few studies have been conducted *in vitro* to confirm *in vivo* results and to directly examine the modes of ammonia tolerance and toxicity in goldfish brain tissue. In this study, we were able to complement our *in vitro* findings at a cellular level to determine how ammonia affects neuronal physiology, growth, and survival.

Chapter 2

Glutamine Accumulation Involvement in Water Accumulation by the Brain of Trout or Goldfish During High External Ammonia Exposure

ABSTRACT

Hyperammonemia can cause fatal brain swelling due to fluid shifts into the astrocytes but the underlying mechanisms remain unresolved. A number of studies have suggested that increases in the concentration of glutamine within astrocytes increases intracellular osmolarity, leading to water uptake by the cells and brain swelling. The overarching goal of this study was to test this hypothesis by exposing ammonia-sensitive rainbow trout (*Oncorhynchus mykiss*) and ammonia-tolerant goldfish (*Carassius auratus*) to high external ammonia (HEA). These fish are readily available and the simplicity of their brain makes them ideal for determining if ammonia exposure leads to water uptake by astrocytes, which provide structural and metabolic support to neurons in the brain. The specific objectives of this study were to deduce whether glutamine synthetase (GS) is the primary mechanism by which ammonia is detoxified in the brain, and whether an increase in brain glutamine causes increased water content in the brain. These questions were investigated by exposing the trout to 1 $mmolL^{-1}$ total ammonia (T_{Amm}), and the goldfish to a range of T_{Amm} concentrations between 1 and 5 $mmolL^{-1}$ T_{Amm}. Brain water content increased by 28% in trout exposed to HEA and by 30% in the goldfish, and the effects were reversed after the fish were returned to ammonia-free water. Significant swelling did not occur below T_{Amm} concentrations of 2 $mmolL^{-1}$ in the goldfish, whereas exposure to a T_{Amm} concentration of 1 $mmolL^{-1}$ was sufficient to cause significant swelling in the trout brain. In both species, swelling persisted even after glutamine production was reduced by more than 75 % following injection of the GS antagonist methionine sulfoximine (MSO), which lowered GS activity by greater than 90 %. These

experiments indicate that ammonia causes reversible brain swelling in both the goldfish and trout, but that ammonia-sensitive trout are more sensitive to such disturbances. Moreover, brain swelling does not appear to be related to an increase in brain glutamine in either rainbow trout or goldfish during exposure to HEA.

INTRODUCTION

Fishes live in aquatic environments where they mainly excrete ammonia down passive NH_3 diffusion gradients across the gills (Wilkie 2002; Evans *et al*. 2005; Weihrauch *et al*. 2009). This makes them particularly vulnerable to external ammonia, which can increase in aquatic environments as a result of the natural decomposition of organic material, agricultural run-off arising from animal wastes and the use of fertilizers, municipal wastes, leachate from landfill sites, or as a result of over-crowding in aquaculture facilities (Environment Canada and Health Canada 2001; Eddy 2005). Ammonia exists as either un-ionized NH_3, or as ionized NH_4^+. Due to its high pK, ~9.5 at 15^{o}C, the majority of ammonia exits as NH_4^+at physiological pH (pH 7.8) (Cameron and Heisler 1983) and in circumneutral pH water (pH 7-8; Emerson *et al.* 1975). Thus, under conditions of HEA, blood-water NH_3 gradients are reduced or reversed, which leads to build-up of ammonia in the blood and tissues of the fish (Wilkie 1997; Randall and Tsui 2002; Ip *et al*. 2004b; Weihrauch *et al*. 2009). Much of the excess ammonia is then enzymatically converted into less toxic glutamine via glutamine synthetase using glutamate as a substrate (Walsh *et al.* 2007) via the following reaction:

$$NH_3 + \text{glutamate} + ATP \underset{GS}{\leftrightarrows} \text{glutamine} + ADP + Pi$$

Ammonia mainly exerts its toxic effects on the nervous system, and many of its neurophysiological disturbances are similar to those seen with anoxia or hypoxia (see Nilsson 2001; Bickler and Buck 2007; Walsh *et al.* 2007 for reviews), including ATP deficits (Kosenko *et al*. 2003), over-activation of NMDA receptors (Felipo and Butterworth 2002; Walsh *et al.* 2007), generation of reactive oxygen species (ROS)

(Jayakumar *et al.* 2004), and brain swelling (Brusilow 2002; Albrecht and Norenberg 2006). The mechanism(s) by which ammonia exposure causes brain swelling remain(s) poorly understood, especially given the important ramifications that this could have for treating hyperammonemia in clinical settings (Butterworth, 2001; Brusilow 2002; Albrecht and Norenberg 2006). It is well established that hyperammonemia causes astrocytes to swell, but the proposed mechanisms resulting in this disorder are controversial (e.g.: Blei *et al*. 1994; Brusilow 2002; Veauvy *et al*. 2005; Albrecht and Norenberg 2006).

Astrocytes are support cells involved in the maintenance of the blood brain barrier, and provide metabolic support to neurons (Kettenmann and Ransom 1995). During hyperammonemia, excess ammonia is thought to be taken-up by astrocytes and converted to glutamine via GS. Greater intracellular fluid (ICF) glutamine has been proposed to cause astrocytic swelling by increasing ICF osmolarity, which promotes water uptake by increasing the osmotic gradient between the cerebrospinal fluid and ICF (Willard-Mack *et al*. 1996; Brusilow 2002; Albrecht and Norenberg 2006; Walsh *et al*. 2007).

Glutamine synthesis is normally thought to protect the vertebrate brain against ammonia toxicity because it lowers the ammonia levels in the body (Norenberg *et al.* 2009). GS is not only present in the brain, but also in other organs including the liver (Cooper and Plum 1987; Walsh *et al*. 2007). In some fishes, GS activity increases in response to ammonia exposure or following feeding (Ip *et al*. 2001; Wicks and Randall 2002), but normally GS activity is sufficiently high to eliminate the need for any increase in enzyme amount or activity (Sanderson *et al*. 2010). As a consequence,

brain glutamine levels are usually increased following the internal accumulation of ammonia (Wicks and Randall 2002; Veauvy *et al*. 2005; Sanderson *et al.* 2010). It remains unclear if such increases lead to increased water uptake by the brain, however.

The overarching goal of this study was to test the hypothesis that glutamine synthesis contributes to ammonia toxicity by causing increased water uptake by the brain in ammonia-sensitive rainbow trout (*Oncorhynchus mykiss*) and in ammonia-tolerant goldfish (*Carassius auratus*; Wilkie *et al*. 2011) exposed to HEA. The water content of the whole brain was then determined and used as an indicator of brain swelling in both goldfish and trout. To assess the role of glutamine accumulation in causing brain swelling, the GS inhibitor MSO was administered to fish 24 h prior to ammonia exposure. It was hypothesized that MSO treatment would reduce glutamine accumulation during ammonia exposure and therefore water accumulation in the brain. MSO administration has been shown to attenuate glutamine accumulation and brain edema in rats injected with toxic doses of ammonia (Willard-Mack *et al*. 1996), and it also has been shown to attenuate glutamine accumulation in fish exposed to ammonia (Veauvy *et al*. 2005; Sanderson *et al*. 2010). In this study we compared ammonia tolerant goldfish to ammonia sensitive rainbow trout to gain insight on the unique abilities employed by goldfish to withstand HEA conditions.

MATERIAL AND METHODS

Experimental Animals and Set-Up

Goldfish (*C. auratus*, 15-30 g, 10-15 cm), purchased from Aleong International (Mississauga, Ont. Canada), and rainbow trout (*O. mykiss*, 80-120 g, 20-25 cm), purchased from Rainbow Springs Trout hatchery (Thamesford, Ont., Canada) were held separately in 500L tanks receiving aerated, Wilfrid Laurier University well water on a flow-through basis (pH ~ 8.0; 80-100% dissolved oxygen; temperature 10-13^{o}C). The fish were held under a 12h light: 12h dark photoperiod and fed 3 times per week with ground floating pellets (Corey Feed Mills, Fredericton, NB). The animals were held in the lab for a minimum of two weeks before experiments commenced. Prior to experiments fish were starved for 48h. All experiments and fish husbandry were approved by the Wilfrid Laurier University Animal Care Committee, and followed Canadian Council of Animal Care guidelines.

Exposure of fish to ammonia was conducted in a 70 L re-circulating system to which the appropriate amounts of NH_4Cl (254134, Sigma-Aldrich) were added to produce the desired concentration of total ammonia. The system comprised a head tank, which drained into a flow-splitter that directed water into twelve individual holding chambers positioned on a tray that in-turn emptied into a lower reservoir. Water from the reservoir was subsequently pumped to the head tank using a submersible pump. The water in the system was partially replenished daily, with the appropriate amount of ammonia and water needed to replace the small volumes of water lost due to evaporation from the system and water sampling. Water pH was controlled in the head tank using a system comprised of an auto-titrator (TTT80,

Radiometer, Copenhagen, Denmark) connected to a pH meter (PHM82, Radiometer) fitted with a pH electrode (GK2401C, Radiometer). When the pH deviated from a set point of pH 8.0, 1M HCl was added to the head tank in a drop-wise fashion via a solenoid valve (Cole Parmer Instruments Co., IL. USA), connected to the auto-titrator.

Experimental Protocol

Experiment 1 – Effects of HEA on Blood Ammonia and Brain Water Content

The first experimental series quantified how HEA exposure altered plasma ammonia and

brain water content in goldfish and rainbow trout. The goldfish were exposed to three nominal concentrations of ammonia (1 $mmolL^{-1}$, 2.5 $mmolL^{-1}$, 5 $mmolL^{-1}$), while ammonia-sensitive rainbow trout were exposed to 1 $mmolL^{-1}$ ammonia only.

For each trial, fish (n = 72 to 96) were transferred to individual darkened 3L containers and acclimated overnight. The next day they were exposed to various concentrations of NH_4Cl in re-circulating well water (pH 8; dissolved oxygen >95%) for three days. Fish were sampled after 72h, and following 48h recovery in ammonia-free water. Control fish were held in identical darkened containers receiving nominally ammonia free well water for 72h prior to sampling. At each sampling period, the fish were anaesthetized (0.1gL^{-1} MS222) and euthanized with a lethal dose (1.0gL^{-1}) of tricaine methanesulfonate (MS222; Syndel Labs, Qualicum Beach, BC, Canada), each formulation buffered with 2 parts sodium bicarbonate. After the fish were unresponsive following a tail pinch, they were blotted dry using a paper towel.

The masses and lengths of each fish were measured and recorded, and blood was then collected by caudal puncture using a heparinized 28G needle with 3 ml disposable syringe for goldfish and 18G needle with 1ml syringe for trout. The blood was then centrifuged for 3 minutes at 10,000 *g* and the plasma (12.5 µL) was transferred to a different centrifuge tube (500 µL). The plasma samples were frozen at -80^{o}C, and the plasma later used for ammonia analysis.

Following blood sampling, the whole intact brains were carefully dissected out of the cranium of the trout or goldfish, and transferred to a pre-weighed 1.5 mL centrifuge tube, which was weighed again to calculate the initial wet mass of the tissue. The brains were then dried to constant mass in an oven at 65°C to determine the dry tissue weight , which was used to calculate the wet tissue water content (in %), based on the percent decrease in tissue weight. To verify that the nominal (target) ammonia concentration matched the measured values, water samples (10mL) were collected at 0, 6, 12, 24, 36, 48, 72h and frozen at -20^{o}C for later analysis of ammonia concentration (Table 2.1).

Experiment 2 – Effects of Glutamine Accumulation on Brain Water Content During HEA

To determine if glutamine accumulation in the brain contributed to ammonia-induced changes in brain water content, anaesthetized fish (0.1 gL^{-1} tricaine methanesulfonate buffered with 0.2 gL^{-1} $NaHCO_3$) were injected intraperitoneally (IP) with a 10 $mgkg^{-1}$ dose of the GS antagonist MSO, or an equivalent volume of Cortland's saline (e.g.

Veauvy *et al*. 2005; Sanderson *et al*. 2010). After 24 h, the fish were then exposed to a nominal concentration of 5mM ammonia for goldfish (actual ammonia concentration = 5.32 ± 0.04 $mmolL^{-1}$; Table 2.2), and $1mmolL^{-1}$ for rainbow trout (1.06 ± 0.02 $1mmolL^{-1}$; Table 2.2) in the re-circulation system. Fish were sampled after 24 h (n=6), 72 h (n=6) or following 48h recovery (n=6), at which times the fish were anaesthetized, removed from their tanks, and the brain collected for water content determination as described above. Plasma was also collected, and snap-frozen in liquid nitrogen, and saved for later analysis of plasma ammonia as described above.

Table 2.1: Measured Ammonia Water Concentrations for Goldfish and Rainbow Trout Exposed to High External Ammonia (Experiment 1)

Organism	Nominal Water Ammonia ($mmolL^{-1}$)	Actual Water Ammonia ($mmolL^{-1}$)
Experiment 1		
Goldfish	Control	0.25 ± 0.16
	1	1.02 ± 0.08
	2.5	2.17 ± 0.15
	5	5.08 ± 0.12
	Recovery	0.30 ± 0.09
Rainbow Trout	Control	-0.01 ± 0.00
	1	0.93 ± 0.00
	Recovery	0.09 ± 0.01

Data shown represented as mean ± SEM (N=20 to 40).

Table 2.2: Measured Ammonia Water Concentrations for Goldfish and Rainbow Trout Exposed to High External Ammonia (Experiment 2)

Organism	Nominal Water Ammonia ($mmolL^{-1}$)	Actual Water Ammonia ($mmolL^{-1}$)
Experiment 2		
Goldfish	Control	0.01 ± 0.02
	5	5.32 ± 0.04
	Recovery	0.05 ± 0.00
Rainbow Trout	Control	0.03 ± 0.02
	1	1.06 ± 0.02
	Recovery	0.01 ± 0.00

Data shown represented as mean ± SEM (N= 20 to 40).

An additional group of fish (trout and goldfish), also exposed to HEA, were sampled at the same time, except that the brains were collected and snap-frozen in liquid nitrogen, and subsequently stored in -80^{o}C for later analysis of brain ammonia and glutamine concentration. In this series of experiments the goldfish were exposed to a nominal external ammonia concentration of 5 mmolL^{-1} (5.24 ± 0.00 mmolL^{-1}; Table 2.3) and rainbow trout were exposed to 1 mmolL^{-1} ammonia (1.03 ± 0.00 mmolL^{-1}; Table 2.3) in the re-circulating system. The night before experiments, the fish were transferred to their respective holding containers in the recirculating system, and allowed to acclimate overnight. The next day, the fish were injected with MSO. They were either held under control (no ammonia) conditions, or sampled after 24h (n=6), 72h (n=6), or after a 48h recovery period, following 72h ammonia exposure. As an additional measure to ensure that MSO had the desired effect, GS activity was also measured on this subset of brain samples. Liver samples were also collected, and snap-frozen in liquid nitrogen for determination of liver glutamine concentration and GS activity.

Table 2.3: Measured Ammonia Water Concentrations for Goldfish and Rainbow Trout Exposed to High External Ammonia (Experiment 2b)

Organism	Nominal Water Ammonia ($mmolL^{-1}$)	Actual Water Ammonia ($mmolL^{-1}$)
Experiment 3		
Goldfish	5	5.24 ± 0.01
	Recovery	0.06 ± 0.02
Rainbow Trout	1	1.03 ± 0.00
	Recovery	0.02 ± 0.00

Data shown represented as mean ± SEM (N= 20 to 40).

Analytical techniques

Brain water determination

Brain swelling was measured by extracting the brain carefully (using fine tweezers and scissors) and placing it in pre-weighed centrifuge tubes (1.5 mL). The centrifuge tubes were weighed with freshly extracted brains and then placed in an oven at 65^{o}C. The weight of the bullet tubes were measured every 24 hours until the weight stabilized and the final weights were measured. Brain water content was calculated using the following equation:

$$\text{Brain water (ml } H_2O\text{/g dry mass)} = \frac{\text{ml } H_2O\text{/g wet mass}}{\text{g dry mass/g wet mass}}$$

Water ammonia determination

Water ammonia concentrations were determined colorimetrically using the salicylate-hypochlorite method, and nitroprusside as a catalyst (Verdouw *et al.*1978). In the presence of ammonia salicylate and hypochlorite produced a blue indophenol, which was quantified at a specific absorbance of 650nm on a SpectraMax 190 plate spectrophotometer (Molecular Devices, Sunnyvale, California), after being incubated in the dark for 90 minutes (Verdouw *et al*. 1978; Refer to Appendix B).

Plasma and brain ammonia determination

Brain tissue was prepared for ammonia and glutamine analysis by grinding the tissue to a fine powder under liquid nitrogen, and transferring the powder to 4 volumes of

7% perchloric acid (PCA) containing 1 mmolL^{-1} ethylenediaminetetraacetic acid (EDTA). The mixture was vortexed and placed on ice for 5-10 minutes, and centrifuged (Microcentrifuge 5415D, Eppendorf, Canada) for 5 min at 10,000*g* at 4^{o}C. The resulting supernatant was then neutralized with 0.5 volumes of KOH (2M), and vortexed. The sample was then centrifuged for 5min at 10,000*g* at 4^{o}C, and the resulting supernatant used to determine ammonia and glutamine as described below.

Plasma and brain ammonia concentrations were measured enzymatically (glutamate dehydrogenase) using untreated plasma or neutralized extracts of the homogenized brain tissue, and a commercial assay kit (AA0100, Sigma-Aldrich). Ammonia concentrations in the blood and tissue samples were based on differences in the initial and final absorbance of the assay mixture at 340nm using the plate spectrophotometer. With this protocol, the absorbance decreases in proportion to the amount of ammonia in the sample due to the oxidation of NADPH to $NADP^{+}$ as ammonia combines with alpha-ketoglutarate to produce glutamate in the presence of GDH (Mondzac *et al.* 1965). Commercial ammonia standard (A0978; 588 μM, Sigma-Aldrich) was included in each series as a quality control check.

Brain and liver GS activity was assayed based on Shankar and Anderson (1985) in which GS activity was based on the formation of γ-glutamylhydroxymate in the presence of glutamate and hydroxylamine. Pyruvate generated as a result of this reaction consequently reacts with hydroxylamine and ferric chloride reagent to form a brown-yellow compound which was quantified colorometrically at 540nm using the plate spectrophotometer (Vorhaben *et al.* 1979). Frozen brain/liver were weighed and ice-cold homogenization buffer (4 times the weight of the tissues in grams) was

added. The liver was homogenized and supernatant was used to measure GS activity (in $\mu molg^{-1}min^{-1}$) (Refer to Appendix D).

Glutamine concentration was measured using GS to promote the reaction between glutamate and ammonia to form glutamine (Mecke, 1985). Before conducting the assay the tissue of interest was homogenized in homogenization buffer [10mmolL^{-1} K_2HPO_4, 10 mmolL^{-1} HEPES, 0.5 mmolL^{-1} EDTA, 1 mmolL^{-1} DTT, and glycerol] using a hand-held tissue homogenizer (PowerGen 125, Fisher Scientific) while being kept on ice. The samples were then centrifuged (Microcentrifuge 5415D, Eppendorf, Canada) at 10,000g for 1 minute at 4^{o}C, and the supernatant was used for the glutamine assay. The assay was similar to the GS assay, in that it was based on the generation of γ-glutamyl hydroxymate, which was measured at 540nm with the plate spectrophotometer (Refer to Appendix E).

Statistical Analysis

Data were expressed as the mean ± standard error of mean (SEM). Data were analyzed using one-way analysis of variance (ANOVA), and where significant variation between means was indicated, followed by a Tukey-Kramer post-hoc test. In cases where there was significant variation amongst the standard deviations for a given data set, statistical significance was determined using a non-parametric ANOVA, followed by Dunn's post-test. All significant differences were determined at the $P<0.05$ level, using GraphPad InStat, Version 3.02 (GraphPad Software, Inc., San Diego, CA, USA).

RESULTS

Experiment 1 – Effects of HEA on Blood Ammonia and Brain Water Content

The actual mean ammonia concentration to which the rainbow trout were exposed was 0.93 ± 0.003 $mmolL^{-1}$ total ammonia at a nominal ammonia concentration of 1 $mmolL^{-1}$, while goldfish were exposed to mean ammonia concentrations of 1.02 ± 0.08 $mmolL^{-1}$, 2.17 ± 0.15 $mmolL^{-1}$, and 5.08 ± 0.12 $mmolL^{-1}$ at nominal ammonia concentrations of 1, 2.5, and 5 $mmolL^{-1}$ respectively (Table 2.1).

Goldfish were able to survive at nominal ammonia concentrations of 1, 2.5 and 5 $mmolL^{-1}$ for the entire exposure period. However, 15% mortality was seen in the more ammonia-sensitive rainbow trout during 1 $mmolL^{-1}$ ammonia exposure.

Increased external ammonia caused a dose-dependent increase in plasma ammonia in goldfish as external ammonia concentrations were increased from 1 to 5 $mmolL^{-1}$ (Fig. 2.1). At the highest concentration of ammonia, plasma ammonia increased by 8 times from pre-exposure values of approximately 300 $\mu molL^{-1}$ (Fig. 2.1A). Plasma ammonia concentrations in rainbow trout, on the other hand, increased by approximately 6 times during exposure to 1 $mmolL^{-1}$ ammonia (Fig. 2.1B). After recovery for two days in ammonia-free water, plasma ammonia in both species had returned to control levels (Fig. 2.1 A, B).

Brain water content also increased in relation to nominal external ammonia, and in proportion to changes in plasma ammonia. Brain water content did not change significantly in goldfish exposed to 1 $mmolL^{-1}$ total ammonia, but it did significantly increase by 20 % and by 30 % at 2.5 and 5 $mmolL^{-1}$ total ammonia, respectively (Fig.

Figure 2.1: Effects of High External Ammonia (HEA) on Plasma Ammonia Concentration in Goldfish and Rainbow trout.

(A) Plasma ammonia concentration of goldfish exposed to high water ammonia for 3 days and following 2 days recovery in ammonia free water. (B) Plasma ammonia concentration of rainbow trout exposed to high water ammonia for 3 days followed by 2 days recovery in ammonia free water. Asterisks represent significant ($p<0.05$) changes in plasma ammonia between control and ammonia treatment groups. Data presented as the mean ± SEM. N=6-8 for goldfish, and N=5 for rainbow trout for each treatment.

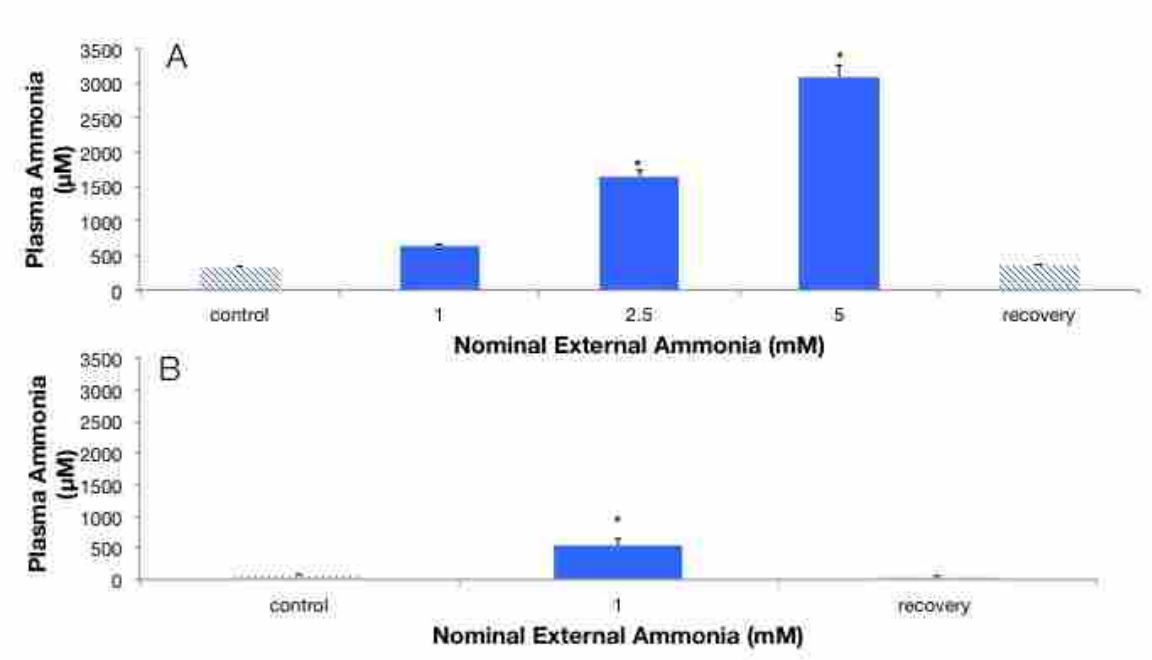
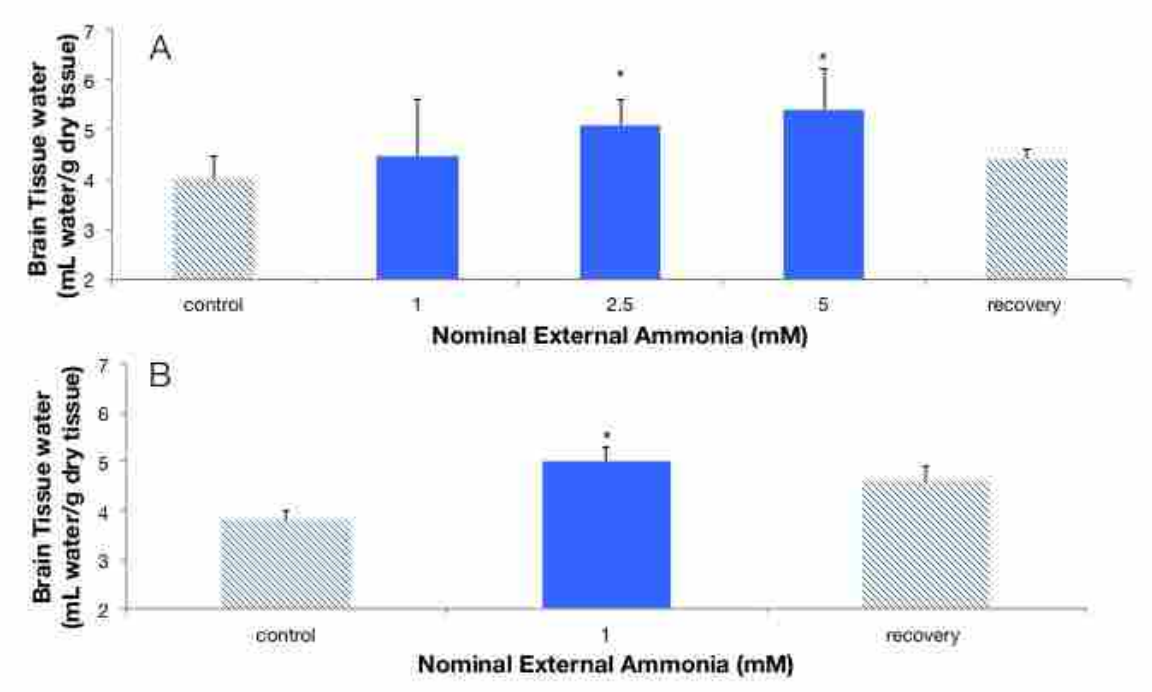

A
Plasma Ammonia (μM)
3500
3000
2500
2000
1500
1000
500
0
control
1
2.5
5
recovery
Nominal External Ammonia (mM)
B
Plasma Ammonia (μM)
3500
3000
2500
2000
1500
1000
500
0
control
1
recovery
Nominal External Ammonia (mM)

Figure 2.2: Effects of High External Ammonia (HEA) on Brain Tissue Water in Goldfish and Rainbow trout.

Changes in brain tissue water content in (A) goldfish exposed to 1, 2.5, and 5mM nominal ammonia and (B) rainbow trout exposed to 1mM ammonia. Asterisks represent significant ($p<0.01$) change in brain tissue water content between control and high ammonia groups. Data presented as the mean ± SEM. N= 6-8 for goldfish and N=5 for rainbow trout for each treatment.


A
Brain Tissue water (mL water/g dry tissue)
7
6
5
4
3
2
control
1
2.5
5
recovery
Nominal External Ammonia (mM)
B
Brain Tissue water (mL water/g dry tissue)
7
6
5
4
3
2
control
1
recovery
Nominal External Ammonia (mM)

2.2A). At 1 mmolL^{-1} total ammonia, rainbow trout brain tissue water increased by 28% (Fig. 2.2B). Brain water content and plasma ammonia was restored to control levels in both goldfish and rainbow trout following 24 h recovery in nominally ammonia-free water (Figs. 2.1 and 2.2).

Experiment 2 – Effects of Glutamine Accumulation on Brain Water Content During HEA

As with plasma ammonia, goldfish brain ammonia concentrations increased by approximately two-fold when exposed to nominal concentrations of 5 mmolL^{-1} ammonia for 24 h and 72h (Fig. 2.3A). After recovery in nominally ammonia-free water, brain ammonia returned to control levels (Fig. 2.3A). Rainbow trout brain ammonia concentrations increased by 0.7 times after exposure to 1 mmolL^{-1} ammonia for 24h and 72h (Fig 2.3B). Trout brain ammonia concentration returned to control levels after the recovery period. When injected with MSO (10 mgkg^{-1}), goldfish brain ammonia did not significantly increase during the ammonia exposure after 24h or 72h (Fig. 2.3A). However, the MSO injections did not significantly attenuate the HEA induced increase in brain ammonia of rainbow trout (Fig. 2.3B).

Goldfish brain glutamine increased by 3 times during exposure to ammonia for 24h and by almost 5-fold after 72h (Fig. 2.4A). Following recovery in ammonia-free water, the brain glutamine levels returned to control levels. Similarly, rainbow trout brain glutamine increased by 3 times when exposed to ammonia for 24h and 4.5 times when exposed for 72h (Fig. 2.4B). However after recovery in ammonia-free water, brain glutamine levels returned to control levels. In both goldfish and rainbow

Figure 2.3: Effects of MSO on Brain Ammonia Accumulation in Goldfish and Rainbow trout Exposed to High External Ammonia (HEA).

Brain ammonia concentration was quantified in (A) goldfish or (B) rainbow trout injected (IP) with saline or methionine sulfoximine (MSO; 10mg kg^{-1}) and exposed to HEA or held under control conditions. Daggers denote significant differences ($p<0.05$) from the respective pre-ammonia exposure values in the saline injected or MSO injected fish. Asterisks denote significance differences ($p<0.05$) between the saline injected and MSO injected fish at each treatment interval. All values are presented as mean ± SEM. N=5-8 for goldfish and rainbow trout for each treatment.

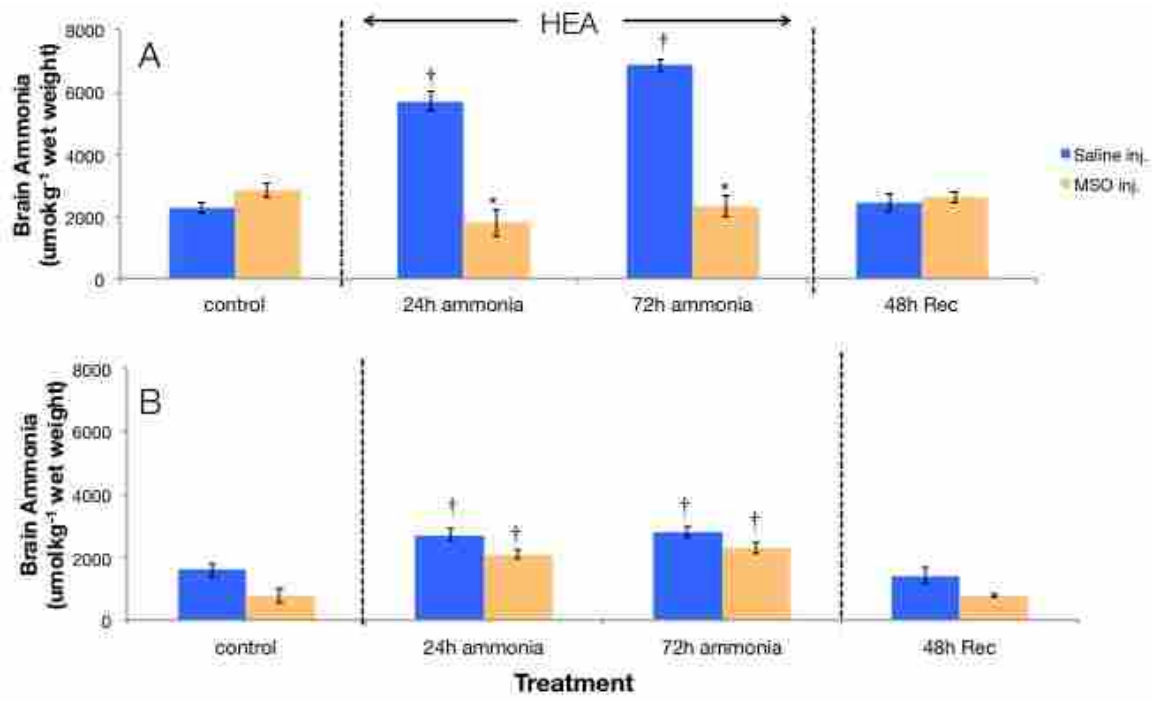
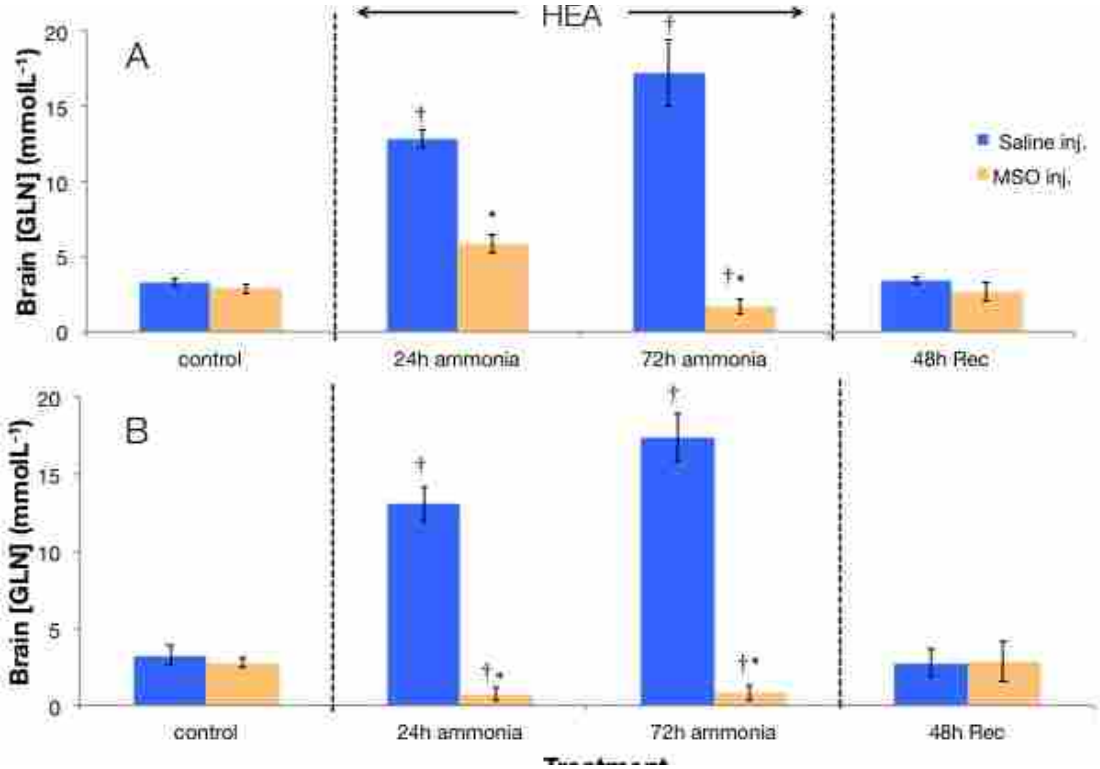

HEA
A
B
Brain Ammonia (umolkg-1 wet weight)
8000
6000
4000
2000
0
control
24h ammonia
72h ammonia
48h Rec
Saline inj.
MSO inj.
Treatment

Figure 2.4: Effects of MSO on Brain Glutamine Accumulation in Goldfish and Rainbow trout Exposed to High External Ammonia (HEA).

Fish were saline or MSO injected (10mg kg^{-1}), (A) goldfish (B) rainbow trout. Dagger denotes significant differences ($p<0.05$) from the respective pre-ammonia exposure values in the saline injected or MSO injected fish. Asterisks denote significance ($p<0.05$) between the saline injected and MSO injected fish at each treatment interval. All values are presented as mean ± SEM. N=5-8 for goldfish and rainbow trout for each treatment.


HEA
A
B
Brain [GLN] (mmolL⁻¹)
20
15
10
5
0
Saline inj.
MSO inj.
control
24h ammonia
72h ammonia
48h Rec

trout, pre-injection of MSO prevented glutamine from increasing during ammonia exposure. By 72h, the respective glutamine concentrations in the brain of both goldfish and trout injected with MSO were reduced by greater than 80 % and 90 %, compared to the saline-injected fish (Fig. 2.4).

Goldfish liver glutamine levels increased by approximately 4 times when exposed to HEA for 24h and by 3 times after 72h exposure, but to control levels following recovery ammonia-free water (Fig. 2.5A). Rainbow trout liver glutamine increased by 2.6 times when exposed to ammonia for 24h and by 3 times when exposed for 72h (Fig. 2.5B). Trout liver glutamine returned to control levels following recovery. When compared to control saline injected fish MSO-treated goldfish and trout both experienced an increase in liver glutamine during HEA exposure (Fig. 2.5).

Brain glutamine synthetase activity was not significantly altered in saline-injected goldfish and trout exposed to ammonia, but it was inhibited by 90% for both MSO-treated goldfish and rainbow trout (Fig. 2.6). This observation likely explained the significant suppression of glutamine accumulation that was observed when these fish were exposed to ammonia (Fig. 2.4).

In goldfish, liver glutamine synthetase increased by 1.3 times when fish were exposed to HEA for 24h and by 1.5 fold when exposed for 72h. Post- recovery, the liver glutamine synthetase activity returned to control levels (Fig. 2.7A). There was a 30% decrease in liver glutamine synthetase activity in goldfish that were administered MSO when compared to saline injected group. In the saline injected rainbow trout, liver glutamine synthetase increased by 1.6 times when the fish were exposed to HEA

Figure 2.5: Effects of MSO on Liver Glutamine Accumulation in Goldfish and Rainbow trout Exposed to High External Ammonia (HEA).

Fish were either saline injected or MSO injected (10mg kg^{-1}), (A) goldfish (B) rainbow trout. Dagger denotes significant differences ($p<0.05$) from the respective pre-ammonia exposure values in the saline injected or MSO injected fish. Asterisks denote significance ($p<0.05$) between the saline injected and MSO injected fish at each treatment interval. All values are presented as mean ± SEM. N=5-8 for goldfish for rainbow trout for each treatment.

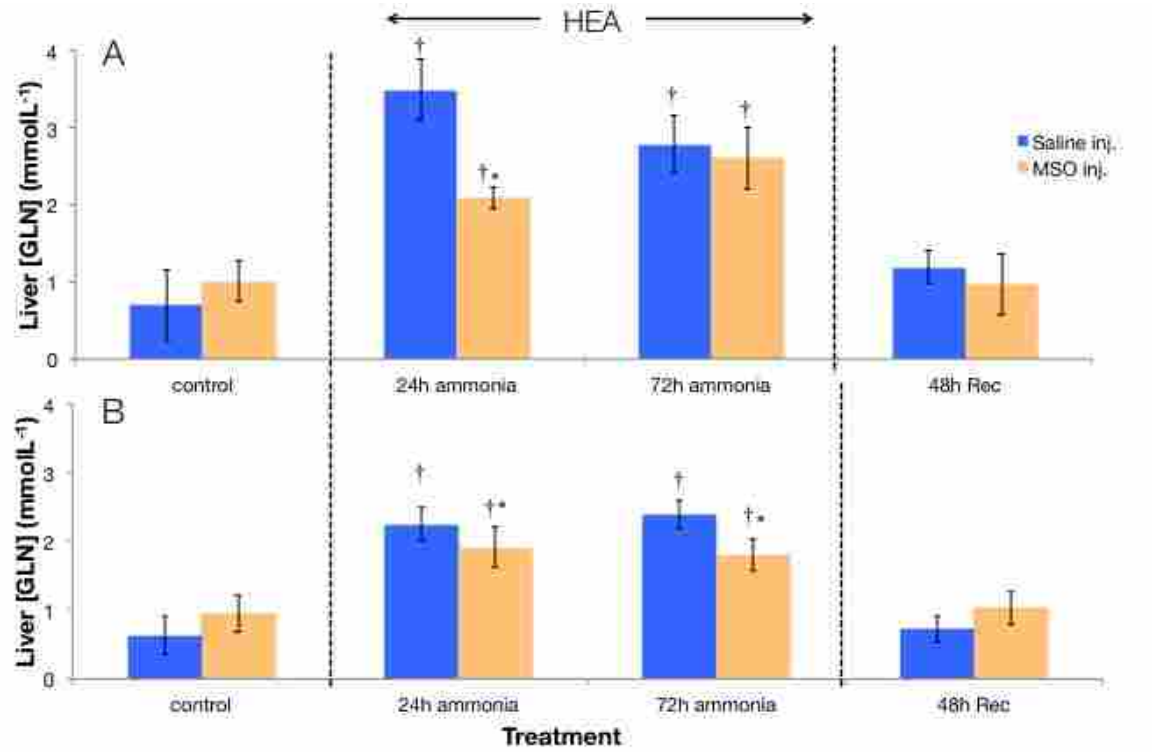

HEA
A
B
Liver [GLN] (mmolL-1)
Saline inj.
MSO inj.
control
24h ammonia
72h ammonia
48h Rec
Treatment

Figure 2.6: Effects of MSO Brain Glutamine Synthetase (GS) Activity in Goldfish and Rainbow trout Exposed to High External Ammonia (HEA).

Brain GS activity was measured in saline injected or MSO injected (10mg kg^{-1}), (A) goldfish or (B) rainbow trout. Asterisks denotes significance ($p<0.05$) between the saline injected and MSO injected fish at each treatment interval. All values are presented as mean ± SEM. N=5-8 for goldfish and rainbow trout for each treatment.

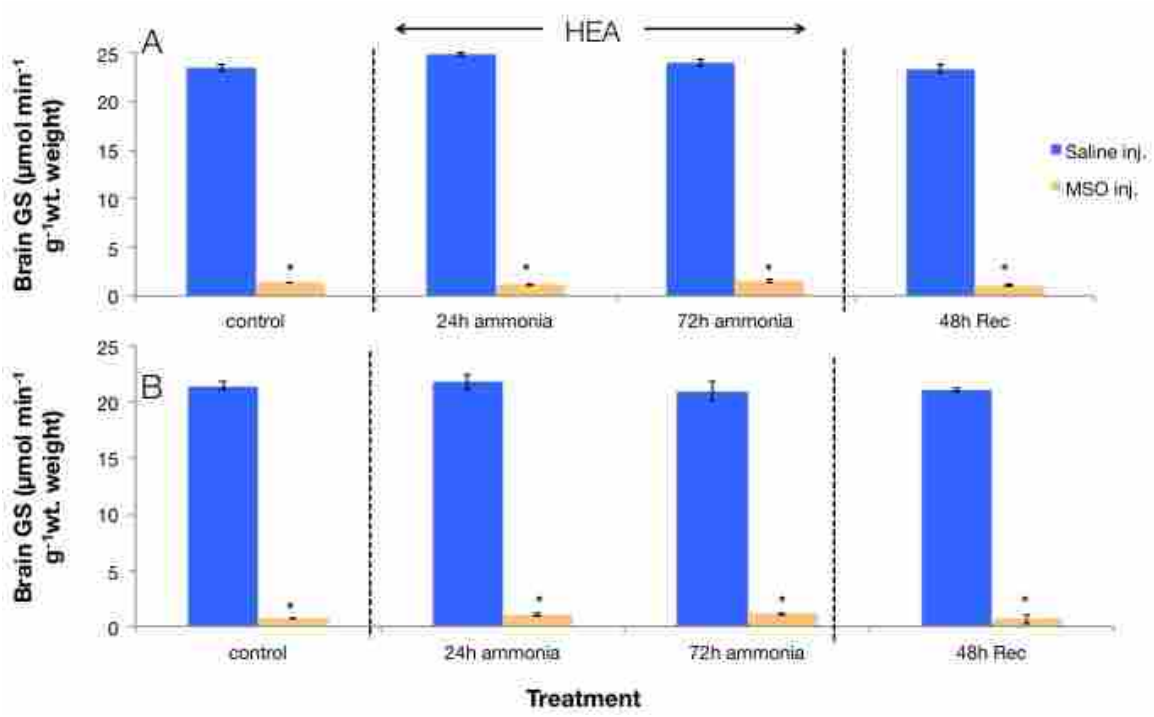
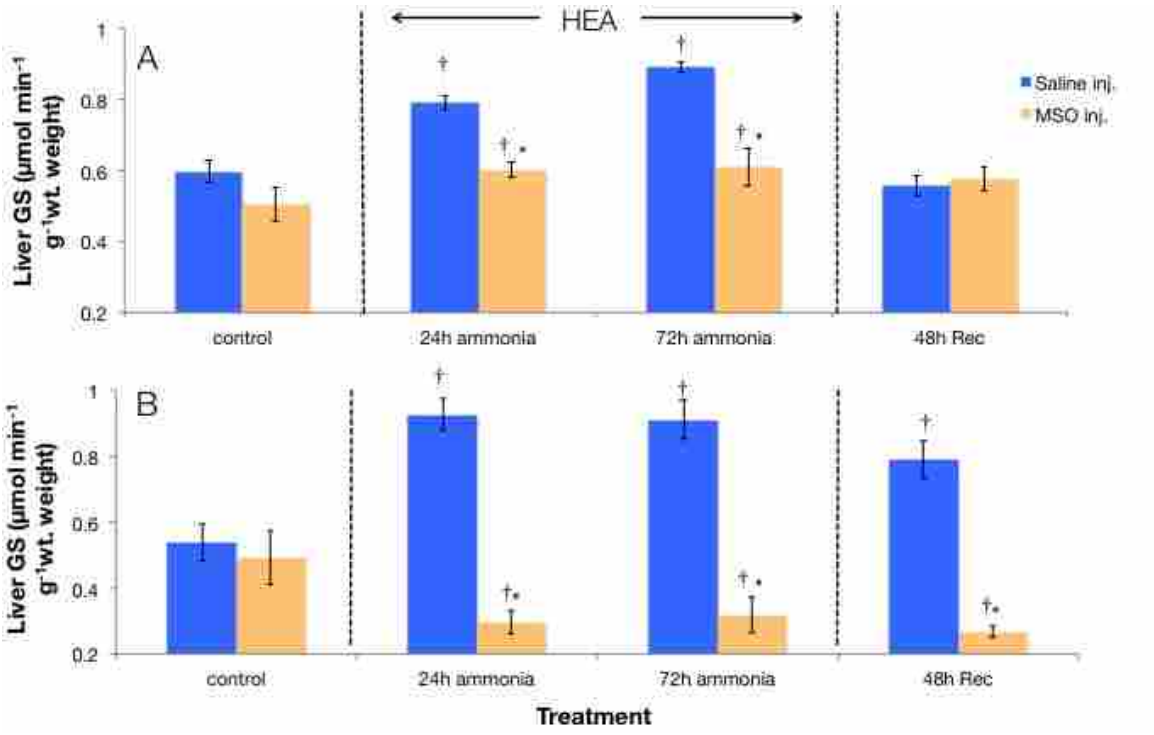

A
HEA
Brain GS (µmol min-1 g-1wt. weight)
25
20
15
10
5
0
control
24h ammonia
72h ammonia
48h Rec
Saline inj.
MSO inj.
B
Brain GS (µmol min-1 g-1wt. weight)
25
20
15
10
5
0
control
24h ammonia
72h ammonia
48h Rec
Treatment

Figure 2.7: Effects of MSO on Liver Glutamine Synthetase (GS) Activity in Goldfish and Rainbow trout exposed to High External Ammonia (HEA).

Liver GS activity in saline injected or MSO injected (10mg kg^{-1}), (A) goldfish (B) rainbow trout. Dagger denotes significant differences ($p<0.05$) from the respective pre-ammonia exposure values in the saline injected or MSO injected fish. Asterisks denote significant ($p<0.05$) between the saline injected and MSO injected fish at each treatment interval. All values are presented as mean ± SEM. N=5-8 for goldfish and rainbow trout for each treatment.

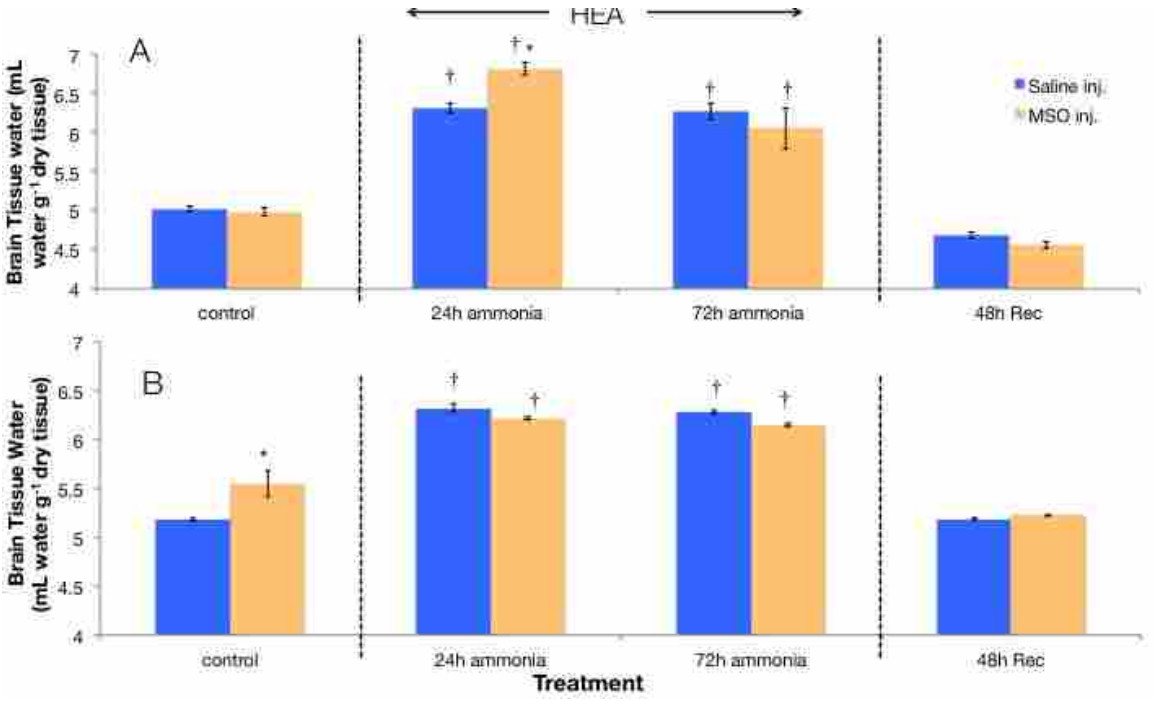

HEA
A
B
Liver GS (µmol min⁻¹ g⁻¹wt. weight)
1
0.8
0.6
0.4
0.2
Saline inj.
MSO inj.
control
24h ammonia
72h ammonia
48h Rec
Treatment

for 24h or 72h, and activity remained elevated after 48 h of recovery in ammonia-free water (Fig 2.7B). In contrast, there was a 65% decrease in liver GS activity in MSO-treated rainbow trout, but this did not appear to significantly lower the liver glutamine concentrations in these fish (Fig 2.5B).

Despite the inhibition of brain glutamine accumulation during HEA in the MSO-injected fish (Fig. 2.4), brain water content still increased by 26% when the MSO-injected goldfish were exposed to high external ammonia, and the increase was not significantly different from that observed for the saline injected fish at HEA (Fig. 2.8A). Similarly, brain water content increased about 21% when trout were exposed to high external ammonia following either MSO or saline injection. After 48h recovery in ammonia-free water, water content was restored to control levels in both the saline-treated and MSO-injected goldfish and rainbow trout that had been exposed to ammonia (Fig. 2.8).

Figure 2.8: Effect of MSO on Brain Swelling in Goldfish and Rainbow trout Exposed to High External Ammonia (HEA).

Fish were either saline injected or MSO injected (10mg kg^{-1}), (A) goldfish (B) rainbow trout. Dagger denotes significant differences ($p<0.05$) from the respective pre-ammonia exposure values in the saline injected or MSO injected fish. Asterisks denote significance ($p<0.05$) between the saline injected and MSO injected fish at each treatment interval. All values are presented as mean ± SEM. N=5-8 for goldfish and rainbow trout for each treatment.


HEA
A
Brain Tissue water (mL water g-1 dry tissue)
7
6.5
6
5.5
5
4.5
4
Saline inj.
MSO inj.
control
24h ammonia
72h ammonia
48h Rec
B
Brain Tissue Water (mL water g-1 dry tissue)
7
6.5
6
5.5
5
4.5
4
control
24h ammonia
72h ammonia
48h Rec
Treatment

DISCUSSION

Effects of HEA on Brain Water Content

Research on brain swelling in mammals (Norenberg *et al*. 2005; Albrecht and Norenberg 2006; Norenberg *et al*. 2009) suggests that swelling could be related to an increase in brain glutamine levels, as glutamine acts as an osmolyte. However, this hypothesis has not been directly tested. In the present study, the predictable increase in glutamine levels when fish were exposed to HEA provided an opportunity to directly determine if glutamine contributes to brain swelling.

A simple technique was developed to quantify changes in brain water content, as opposed to Magnetic Resonance Imaging (MRI; Van der Linden *et al.* 2001; Veauvy *et al.* 2005), or markers of intracellular fluid volume such as 3-O-methyl[^{3}H]-D-glucose (Bender *et al*. 1998; Issacks *et al*.1998; Cheol *et al*. 2004; Jayakumar *et al*. 2006; Panickar *et al*. 2009) and ^{14}C-inulin (Cohen *et al*. 1968; Milligan and Wood 1986; Zielinska *et al*. 2004). Here the overall change in water content was measured directly within brain tissue carefully dissected from the cranium, and then dried to constant weight. A limitation, however, was that it was not possible to determine whether the astrocytes themselves were swelling in the brain (Norenberg 1977; Swain *et al.* 1991; Cordoba and Blei 1995). Given that the astrocytes comprise 50-90% of cellular makeup of the mammalian brain (Pope 1978; Norenberg 1995; Norenberg 1998, see Lewitus *et al*. 2012 for a recent critique), however, it can be deduced that any water taken-up by the brain was likely via glial cells. Further support for this hypothesis was generated by our *in vitro* experiments which allowed us to look at cell lines primarily composed of glial cells, and which indicated that rainbow trout and

goldfish brain cell lines exhibited extensive vacuolization when exposed to toxic concentration of ammonia (Chapter 3). It was notable that the increased brain water content associated with HEA could be reversed with no visible pathology in either goldfish or trout. This may be because, unlike mammals, the cranial cavity of fishes is relatively large and able to accommodate increases in brain volume (Van der Linden *et al*. 2001). In mammals, the brain is much more sensitive to ammonia-induced encephalopathy (Felipo and Butterworth 2002). Thus, a larger cranial cavity could be an additional factor that contributes to the generally greater tolerance of fish to increased brain water content compared to other vertebrates, particularly mammals (Walsh *et al.* 2007; Eddy, 2005). At first glance, the associated increases in brain glutamine, which takes place mainly in astrocytes (Kosenko *et al*. 2003; Walsh *et al.* 2007), supported the hypothesis that glutamine contributed to increased water accumulation by increasing the intracellular osmolarity (Brusilow 2002). However, subsequent experiments with MSO in which glutamine accumulation in the brain was prevented suggested otherwise. In agreement with earlier work (Sanderson *et al*. 2010), both ammonia-tolerant goldfish and ammonia-sensitive rainbow trout were able to withstand exposure to HEA, despite the inhibition of brain glutamine synthetase by MSO. It was predicted that glutamine accumulation by the astrocytes of the brain would be reduced following MSO injection, and this would prevent the increases in ICF osmolality thought to cause water uptake by astrocytes (Brusilow 2002; Albrecht and Norenberg 2006). Because glutamine accumulation was greatly reduced with MSO treatment but swelling persisted during HEA, it seems unlikely that the accumulation of glutamine in the brain causes brain swelling which often

culminates in the increased intra-cranial pressure and brain herniation that causes death in vertebrates experiencing hyperammonemia (Felipo and Butterworth 2002; Walsh *et al*. 2007). Thus, swelling of astrocytes does not appear to be the direct result of an increase in intracellular glutamine, as previously suggested for mammals by several groups (Zielinska *et al*. 2003; Norenberg *et al*. 2005; Zwingmann and Butterworth 2005; Jayakumar *et al*. 2006a). Rather, other factors must be causing swelling.

If glutamine does not contribute to water uptake by the brain, another possibility is that water uptake was related to the generation of ROS and reactive nitrogen species (NO; Kosenko *et al.* 1994; Albrecht and Norenberg 2006). The ammonia-induced over-activation of NMDA receptors reduces the activity of antioxidant enzymes such as catalase, superoxide dismutase (SOD) and glutathione peroxidase, leading to the accumulation of ROS (Kosenko *et al*. 2003).

The key to the high hypoxia/anoxia tolerance of the goldfish (Bickler and Buck 2007; Walsh *et al.* 2007) is its high constituent levels of antioxidant enzymes such as brain glucose-6-phosphate dehydrogenase and glutathione peroxidase, which allow them to combat increased ROS production (Luschak *et al*. 2001) following periods of oxygen starvation. This may explain why the threshold required to cause brain swelling was higher in the goldfish compared to the more hypoxia- and ammonia-sensitive rainbow trout, which has lower constituent levels of antioxidant enzymes (Luschak *et al.* 2001).

Brain swelling could also be caused by ammonia-induced changes in ion concentration within the cerebrospinal fluid (CSF) caused by impaired ATP production (Kosenko *et al.* 1994). By impairing ATP production, ion pumps such as the Na^+/K^+-ATPase would starve of their energy supply and lead to a breakdown of transmembrane ion gradients in not only astrocytes but neurons. Similar disturbances to CNS ion balance have been reported during periods of oxygen starvation in vertebrates. Cserr *et al.* (1988) reported that prolonged anoxia caused changes in brain and CSF ion composition in freshwater turtles (*Chrysemys picta bellii*). Following an anoxic period, elevated K^+, Ca^{2+}, Mg^{2+} and lactate were noted. Meyer *et al*. (1971) noted that anoxia caused CSF sodium to decrease and potassium to increase.

Increases in extracellular K^+ concentration have also been noted in mammals experiencing ammonia toxicity (Hawkins *et al.* 1973; Benjamin and Quastel 1975). Ammonia also causes depolarization in cultured astrocytes, which causes extracellular K^+ to increase and a decrease in uptake of K^+ in the astrocytic cultures (Bender and Norenberg 1998). It would be very useful in future studies to examine the ion concentrations in the brain and CSF because it would help us to gain insight on whether ion homeostasis is responsible for brain swelling.

Another manner by which the swelling of astrocytes could be occurring is by over-expression of aquaporin-4. Aquaporins are proteins that are embedded in the cell membrane which regulate water flow and they are mostly found in the astrocytes (Jung *et al*. 2004). Margulies *et al.* (1999) reported that aquaporin-4 gene and expression increased in response to hyperammonemic conditions. It was also reported

that astrocytes cultured under hyperammonemic conditions (5mmolL^{-1} NH_4Cl) led to increased protein expression of aquaporin-4 (Rao *et al.* 2003). It has also been reported that oxidative stress upregulates gene expression of aquaporin-4 (Arima *et al*. 2003; Norenberg *et al.* 2005; Cauli 2010).

Effects of HEA on Blood and Tissue Ammonia, and Glutamine Concentration

Based on plasma ammonia measurements at 1 mmolL^{-1} T_{Amm} , it appears that goldfish are no more adept than trout at restricting ammonia entry and/or excreting ammonia against an inwardly directed water to ammonia gradient. It was notable that in both species, plasma ammonia concentrations were always less than in the surrounding water, suggesting that the fish were either restricting ammonia entry and/or excreting ammonia against the inwardly directed gradient. It is therefore possible that the gills of goldfish and trout were undergoing gill remodelling to prevent ammonia uptake. Gill remodelling in both goldfish and the closely-related crucian carp (*Carassius carassius*) takes place in response to reduced water temperature and changes in oxygenation (Sollid *et al.* 2005, Sollid and Nilsson 2006; Perry *et al.* 2010). At lower temperatures and/or when O_2 is abundant, there are reductions in the functional surface area of the gill through the formation of an interlamellar cell mass (ILCM) covering the lamellae, which are the functional sites of gas exchange on the fish gill (Sollid *et al.* 2005). During hypoxia, fishes are also able to increase the functional respiratory area of the gills by increasing their blood pressure to open lamellar vasculature (Nilsson 2007). Thus, it is possible that gill remodelling was facilitating ammonia excretion by the goldfish and/or trout. Indeed, Perry *et al.* (2010) demonstrated that Rh glycoproteins were present in the ILCM, which would have the

advantage of promoting ammonia excretion and increasing the inwardly directed diffusion gradient favouring ammonia uptake.

The goldfish does not appear to be more efficient at converting ammonia to less toxic end products such as urea or glutamine than the rainbow trout (Arillo *et al.* 1981; Schenone *et al*. 1982). Since goldfish do not have a urea cycle (Felskie *et al*. 1998), it is unlikely that their greater ammonia tolerance compared to trout is due to the conversion of ammonia to less toxic urea. Wilkie *et al*. (2011) reported that muscle urea decreases during ammonia exposure in goldfish, and is accompanied by only small elevations of urea excretion. It seems unlikely that the glutamine synthesis capacity was any greater in goldfish compared to trout. The increase in brain glutamine as a consequence of HEA exposure (Fig. 2.4) was consistent with previous research on goldfish and other fishes (Levi *et al*.1974; Arillo 1981; Ip *et al*. 2004a; Sanderson *et al*. 2010). Indeed, glutamine concentrations (Fig. 2.4 and 2.5) and GS activity increased in goldfish liver but not the brain (Compare Figures 2.4 and 2.5), which could have lowered circulating ammonia levels. However, GS activities were similar and also increased in the trout, suggesting that differences in glutamine synthesis capacity were not the reason for the greater tolerance of the goldfish.

Another factor for the higher ammonia tolerance of goldfish compared to the trout could be due to their ability to tolerate increased amounts of ammonia in the brain (Arillo 1981; Ip *et al*. 2005). The brain ammonia concentrations in the goldfish exposed to HEA were two-fold higher than that of rainbow trout (Fig. 2.3). The results were comparable with Wilkie *et al*. (2011) who reported 6000 $\mu molkg^{-1}$ wet weight brain ammonia for goldfish, but substantially less than reported by Ip *et al.*

(2005) who observed ammonia concentrations of 18 $\mu molkg^{-1}$ brain of mudskipper exposed to 100 $mmolL^{-1}$ ammonia.

The absence of any change in brain ammonia concentration following MSO treatment in trout exposed to ammonia was comparable to observations made by Sanderson *et al*. (2010), who argued that glutamine synthesis was not necessary to restrict an increase in ammonia during HEA. The similar findings here support this conclusion. Like Sanderson *et al.* (2010), large decreases in glutamine production were observed without any additional ammonia accumulation during HEA exposure in trout.

Sanderson *et al*. (2010) argued that other metabolic pathways may have provided alternate routes to prevent further increases in brain ammonia when glutamine synthesis was inhibited (by MSO) in trout during exposure to HEA. In the absence of glutamine synthesis following MSO treatment, they demonstrated that increased ammonia would also result in less glutamate consumption and the transamination of glutamate to alanine, aspartate and gamma amino butyric acid (GABA) as a means to prevent ammonia toxicity. Perhaps this "reserve capacity" to transaminate glutamate to these alternate amino acids is greater in the goldfish compared to the trout, which could explain why brain ammonia concentrations decreased when the fish were treated with MSO during HEA exposure. To test this hypothesis, it would be useful to measure these amino acids, along with the transaminase enzymes and glutamate dehydrogenase in goldfish and trout during HEA exposure following MSO administration. However, such work is beyond the scope of this thesis.

CONCLUSIONS

The present study supports the premise that formation of glutamine from toxic ammonia in the brain is a defense against hyperammonemia. However, the fact that the water uptake by the brain persists even in the absence of appreciable glutamine formation indicates that the glutamine does not likely cause brain swelling during hyperammonemia in goldfish or trout. Rather, it suggests that ammonia-induced brain swelling is the result of other factors such as ROS production, but this possibility requires more research. The fact that water accumulation in the brain of the goldfish is reversible suggests that in fish there is a large cranial capacity to allow brain swelling to take place without damaging the brain, unlike which occurs in mammals. This would be of considerable advantage for fish, which may be prone to increased ammonia following feeding (Wicks and Randall 2002) and may be exposed to environmental stressors such as HEA or hypoxia in unpredictable aquatic ecosystems.

Chapter 3

Development of an *In Vitro* Model to Study the Neurotoxic Effects of Ammonia in the Anoxia-Tolerant Goldfish (*Carassius auratus*) and Ammonia-Sensitive Rainbow Trout (*Oncorhynchus mykiss*)

ABSTRACT

Whole animal models are conventionally used to assess the effects of toxic substances such as ammonia on different organisms, but to deduce the mechanism(s) of toxicity, multiple levels of organization need to be examined which includes the use of cell culture preparations. In this study, two fish brain cell lines, goldfish (*Carassius auratus*) cerebellum cell line (GFB3C) and rainbow trout (*Oncorhynchus mykiss*) brain cell line (RTB), were used to perform fluorometric assays to determine how ammonia affected cell viability, morphology and metabolism. The cells displayed immunoreactivity to polyclonal antibodies of glial fibrillary acidic protein (GFAP), but not to neuronal filament (NF) antibody, indicating that the cell's phenotype was that of glial cells and not neurons. Consistent with this conclusion was the presence of significant glutamine synthetase (GS) activity in both sets of cells, a characteristic of glial cells. After 24h and 48h exposures to NH_4Cl greater decreases in cell viability were measured in RTB using Alamar Blue than in the GFB cell lines. The IC_{50} of NH_4Cl for GFB3C was 187mmolL^{-1} (48h), and 67mmolL^{-1} (48h) for RTB, indicative of the greater tolerance of goldfish cells to ammonia. Ammonia toxicity was characterized by vacuolization of cells, which was much more pronounced in RTB cells than in goldfish cells. In both GFB3C and and RTB exposure to ammonia resulted in increased glutamine synthetase activity. To determine if glutamine accumulation within cells affected cell viability, cells were pre-treated with methionine sulfoximine (MSO), which inhibits the glutamine synthetase (GS) mediated conversion of ammonia to glutamine. MSO did not reduce GS activity in GFB3C and RTB. Taurine, which acts as an osmoprotectant, did not increase cell

viability of GFB3C but increased cell viability of RTB by 20%. In agreement with the findings of previous *in vivo* studies, the response of the GFB3C cell lines to ammonia supports the conclusion that the goldfish nervous system is more ammonia tolerant than that of trout.

INTRODUCTION

Ammonia can arise in aquatic environments as a result of the degradation of organic matter or from anthropogenic sources such as sewage effluents, landfill leachate and agricultural effluents (Ip *et al*. 2001; Randall and Tsui 2002; Eddy 2005). Ammonia is highly toxic and inhibits ammonia excretion by fishes by decreasing blood – water NH_3 diffusion gradients, leading to increases in internal ammonia (Wilkie 2002). Marked increases in internal ammonia are also seen following feeding in fishes (Wicks and Randall 2002), and after vigorous exercise (Driedzic and Hochachka 1976; Wang *et al*. 2004). Ammonia exists as either un-ionized NH_3 or ionized as NH_4^+, but at physiological pH (pH 7.8; Wilkie and Wood 1991) most ammonia exists as NH_4^+ because of its high apparent dissociation constant (pK) of ~ 9.5 (at 15°C) (Cameron and Heisler 1983; Wright *et al*. 1995). However, it is likely that ammonia crosses the blood brain barrier as NH_3, which is more diffusible than ionized NH_4^+.

When brain and blood ammonia concentrations become toxic, nervous system function is altered in at least one of three ways: 1) Astrocyte swelling, 2) N-methyl-D-aspartate (NMDA) receptor over-activation, and 3) Increases in reactive oxygen species (ROS) (Albrecht and Norenberg 2006; Brusilow 2002; Felipo and Butterworth, 2002; Walsh *et al*. 2007; Jayakumar *et al.* 2006b; Wilkie *et al*. 2011). Astrocytic swelling may be caused by ammonia detoxification by the glutamine synthetase pathway, which leads to increases in brain glutamine concentration (Kosenko *et al.* 2003; Walsh *et al*. 2007). It has been proposed that glutamine accumulation leads to increased intracellular osmolarity in the astrocytes, which leads to increased water uptake and astrocyte swelling. Over-activation of the NMDA

receptor may cause stimulation of secondary messenger pathways and an increase in intracellular calcium leading to cell death. ROS are generated as a consequence of the over-activation of NMDA receptors because an increase in calcium leads to an increase in astrocyte as well as neuronal ROS (Schliess *et al*. 2002).

Standardized methods of evaluating toxicity use whole animals to elucidate the effects of toxicants. Although this technique allows for a comparative model to evaluate the effects of toxicants on human beings, better models are required to look at the mechanisms by which these toxicants affect specific organs within the organism. The main advantage of cell cultures is that processes can be studied within an isolated physiological environment (Freshney 2005). *In vitro* models can provide insight into the physiological mechanism involved in the way the particular toxicant influences different organisms at the cellular or tissue level.

The studies conducted to deduce the mechanism of ammonia toxicity have mainly been restricted to *in vitro* mammalian models. Researchers have employed whole animal organ slices such as brain slices (Zielinska *et al*. 2003, 2004), as well as primary cultures (Isaacks *et al*. 1998; Murthy *et al.* 2001; Panickar *et al.* 2009), but no one has to date used piscine cell lines.

The goal of this study was to evaluate whether brain cell lines from goldfish and rainbow trout that were suitable for studying the mechanisms of ammonia toxicity and tolerance in fishes. Once the cells were characterized for cellular identity, the goal was to then use the brain cell lines to test the hypothesis that goldfish cell lines are ammonia tolerant when compared to those of ammonia-sensitive rainbow trout. To learn more about the underlying causes of cell death and cell swelling under

hyperammonemic conditions, the hypothesis that glutamine synthetase plays an essential role in the detoxification of brain ammonia was also tested.

To test these hypotheses, cell viability was measured using Alamar Blue in the presence and absence of ammonia for both cell lines (GFB3C-goldfish cerebellum, and RTB-rainbow trout brain) to deduce whether goldfish brain cells are more tolerant to ammonia. Subsequent experiments using the glutamine antagonist methionine sulfoximine (MSO) were performed to determine if MSO improved cell viability in the presence of ammonia. The osmoprotectant amino acid taurine (Albrecht 1998) was then used to determine if increases in external osmotic pressure improved cell viability by decreasing osmotic gradients between the extracellular fluid and the intracellular fluid of RTB and GFB3C cells lines.

MATERIAL AND METHODS

Cell Lines

Cell lines were acquired from the lab of Dr. L.E.J. Lee at Wilfrid Laurier University. GFB3C was derived from the cerebellum of male goldfish (*Carassius auratus)* brain on Dec 8th, 2004 (Bufalino BSc. Thesis 2008) and RTB was derived from the whole brain of rainbow trout (*Oncorhynchus mykiss*) on Sept 30th 1989 (Unpublished).

Both cell lines were maintained with a growth medium containing L15 Leibovitz Media (11415, Invitrogen), supplemented with 10% fetal bovine serum (FBS) (F1051, Sigma-Aldrich), 10 % Hank's Buffered Saline Solution (HBSS) (14170, Invitrogen), 1% penicillin and streptomycin in glutamine (10378, Gibco) and 0.1% Tylosin (T3397, Sigma-Aldrich). GFB3C was maintained at 26^{o}C and RTB at 15^{o}C. From this point, 10%FBS/L15 will be used to refer to growth media. Cells were maintained in 75 cm^{3} culture flasks (353135, BD Falcon).

When a flask reached confluence, the cells were split into multiple flasks (passaged). Passaging of cells involved using TrypLE Select (12563, Gibco) to dissociate cells from the bottom of the flasks after the medium was removed. After three minutes of gentle agitation of the flask, the cell suspended solution was removed, placed in a 15mL sterile conical tube (352097, BD Falcon) and centrifuged at 1000 rpm for three minutes. The supernatant was removed and the pellet was re-suspended in 10% FBS/L15 media, distributed between two flasks, and the cells allowed to grow until they were once again confluent (as described in Dayeh *et al*. 2005).

Experimental Protocols

Series 1: Cell Type Characterization

Prior to conducting experiments using ammonia, it was important to deduce the types of neural cells present. Immunohistochemistry techniques were therefore employed to distinguish if the types of cells present in the GFB3C and RTB cell lines were more like neurons, or if they more closely resembled astrocytes, a type of glial cell. Accordingly, the cells were dissociated using TrypLE from flasks and placed in conical tubes. These were then centrifuged for three minutes at 1000 rpm. The supernatant was removed and the pellet was re-suspended in 10mL 10%FBS/L15 media. These suspended cells were then plated on microscope glass slides specific for fluorescent antibody detection (22-267-104, Fisher Scientific), and incubated at 26^{o}C for GFB3C and 15^{o}C for RTB for 24 hours. The medium was then removed from each slide and the slides washed with phosphate buffered saline (1X; Scheppler *et al.* 2000). Ice-cold methanol was placed on each slide for three minutes. The cells were once again washed with PBS before 10% goat serum solution prepared in PBS was applied to each slide for one hour to block any non-specific binding. Anti-glial fibrillary acidic protein (GFAP) derived from human and raised in rabbit (G9269, Sigma-Aldrich) and monoclonal anti-neurofilament 200 (NF) derived from pig and raised in mouse (N0142, Sigma-Aldrich) were diluted 1:80 and 1:400 times, respectively, in 1% PBS. The slides were exposed to GFAP and NF antibody, and incubated at 26^{o}C for 2.5 hours. Primary antibody incubation was followed by incubation with fluorescein isothiocyanate (FITC) conjugated anti-rabbit IgG developed in goat (F0382, Sigma-Aldrich) secondary antibody diluted 1:320 in 1% PBS for GFAP, and with FITC conjugated anti-mouse IgG developed in goat (F8521,

Sigma-Aldrich) diluted 1:80 in 1 % PBS (wt/vol.). After incubation the slides were washed with PBS and stained with 4', 6-diamidino-2-phenylindole (DAPI) (D1306, Invitrogen) which is a fluorescent stain that binds to A-T rich regions in the DNA, thus staining the nucleus. DAPI was diluted to 1µg ml^{-1} in PBS for three minutes. Slides were rinsed with PBS twice and coverslips were mounted using Gel Mount Aqueous Mounting Medium (G0918, Sigma-Aldrich). The slides were viewed using a phase contrast microscope (Nikon E600) and fluorescence was detected using a mercury lamp and FITC filter. Additionally, images were collected using a confocal laser-scanning microscope (Olympus FluoView FV1000, Hamburg, Germany).

This procedure was also used for dissociated goldfish brain cells, which were acquired by gently scraping fresh goldfish brain on glass slides, and goldfish brain slices which acted as positive controls. For the goldfish brain slices, prior to the immunohistochemistry protocol being performed, a subsection of the brain was embedded in Tissue Tek* optimal cutting temperature compound (OCT; 25608-930, VWR International) by placing the brain in OCT and then slowly freezing the sample using liquid nitrogen. The 10-micron slices were generated using a cryostat (Leica CM3050) at -16^{o}C and placed on glass slides, and prepared for confocal microscopy as described above.

Series 2: Standard Curve Generation for Viability and Cell Numbers

Cell density experiments were performed to measure the relationship between Alamar Blue (DAL1100, Invitrogen) uptake and cell density. This experiment was also conducted to deduce the number of cells required to provide a consistent reading of

relative fluorescent units (RFU), to ensure that the same number of cells were used for each ammonia exposure experiment. Two 96-well plates (353072, BD Falcon) were used for this assay, one for GFB3C and one for RTB cell lines. Following cell suspension, the cells were counted using a hemocytometer and trypan-blue exclusion dye. This 100% cell solution was used to prepare a series dilution (50%, 25%, 12.5%, 5% 2.5%, 1%) of cell suspensions. Approximately 100µL of each solution was added to each well of the plate and incubated for 24 hours. After 24 hours, the cells were subjected to Alamar Blue (AB) and 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) treatment to calculate the number of viable cells that were present in each diluted solution Dayeh *et al*. 2005; see below).

Series 3: Determination of the Viability of Goldfish Brain Cell Lines and Rainbow Trout Cell Lines in the Presence of Ammonia.

Using the standard curve generated in the Series 2 experiments, cells were plated in 96-well plates at 2 x 10^4 cells/well in 10% FBS/L15 for 24 hours to allow for cell adhesion. Following this 24-hour incubation (19^{o}C and 23^{o}C for RTB and GFB respectively), cells formed a confluent layer in each well, and the medium was removed by carefully inverting the plate onto paper towels. Each well was then rinsed using 200 µL of exposure medium L15/ex (Dayeh *et al*. 2003) prior to treatment with ammonia. The plate was once again inverted onto paper towels to remove the rinsing solution. Exactly 100 µL of ammonia solutions (of various concentrations) made in L15/ex was added to each well. Subsequent to the 48 h exposure, the medium was carefully removed from each well using a pipette and placed into individual bullet

tubes. These tubes were frozen (-20^{o}C) for later determination of osmolality and ammonia analysis. The plates were once again rinsed using L15/ex rinsing solution, and inverted onto paper towels to remove the rinsing solution. AB and CFDA-AM assays were then conducted as described in Dayeh *et al*. (2005).

Series 4: Ammonia and Methionine Sulfoximine (MSO) exposures

Cells were plated in 96-well plates at 2 x 10^4 cells/well in 10% FBS/L15 for 24 hours to allow for cell adhesion. After 24-hour incubation (19^{o}C and 23^{o}C for RTB and GFB respectively), cells formed a confluent layer in each well. The media was removed by carefully inverting the plate onto paper towels. Each well was then rinsed using 200 µL of L15/ex prior to treatment with 100 µL MSO (M5379, Sigma-Aldrich) at 0.01, 0.05, 0.1, 0.5, 1, 5 mmolL^{-1} made in L15/ex. After 24 hours, the exposure medium were taken out of the wells using a syringe and replaced with the same MSO solutions, which included ammonia (100 mmolL^{-1} for GFB3C and 25 mmolL^{-1} for RTB). The cells were left at room temperature for 48 hours. After 48h, the solution was carefully removed using a pipette from each well and placed into individual bullet tubes, which were frozen (-20^{o}C) for later osmolality and ammonia analysis. After 48 hours, AB assays were conducted to measure cell viability (see below for analytical details; Dayeh *et al*. 2005).

Series 5: Ammonia and Taurine exposures

Cells were plated and wells were rinsed as described for the Series 4 experiments (see above). 100 µL of Taurine (1, 2.5, 5, 10 $mmolL^{-1}$) with ammonia (100 $mmolL^{-1}$ for GFB3C and 25 $mmolL^{-1}$ for RTB) made in L15/ex was added to each well for 48 hours, at which time the exposure media was carefully removed using a pipette and placed in individual bullet tubes. These solutions were frozen (-20^{o}C) for later osmolality and ammonia analysis. AB assay was conducted on the cells (as described in Dayeh *et al*. 2005).

Analytical Techniques and Preparation of Solutions

Exposure Sample Preparation

All ammonia samples were prepared by dissolving ammonium chloride (Sigma-Aldrich Chemical Co., St. Louis) in L15/ex. L15/ex is a basic exposure medium composed of inorganic salts and carbohydrate source (See Appendix A). To prepare culture solutions containing MSO or taurine, respective solutions of MSO (Sigma-Aldrich Chemical Company, St. Louis, MI) or taurine (Acres Organics; Fair Lawn, NJ) were dissolved in L15/ex to prepare a concentrated 100 $mmolL^{-1}$ solution. The corresponding concentrated solution was then filter-sterilized using a 50ml syringe and 0.2µm syringe filter (VWR, 28145-501). The sterile solution was diluted using serial dilutions in sterile L15/ex to make the various concentrations of exposure media.

Sufficient NH_4Cl was added to the appropriate MSO and taurine solutions to yield ammonia concentrations of 100 $mmolL^{-1}$ and 25 $mmolL^{-1}$ to match the

concentrations of ammonia to which the GFB3 cells and RTB cells were exposed, respectively.

Before using the various media for exposures, osmolality (291 to 330 mOsm) and pH (~7.4) were measured using a vapour pressure osmometer (Vapro5520, Wescor, Logan UT) and pH meter (Radiometer, Copenhagen, Denmark) with electrode (GK 2401C, Radiometer) to ensure that cell viability was not influenced by osmotic or pH changes.

Methodology Used to Measure Cell Viability Using Alamar Blue and CFDA

Cell viability was initially assessed by using the fluorescent indicator dyes: AB and CFDA-AM (Schirmer *et al*. 1997). Fluorescence was determined (expressed in relative fluorescence units; RFUs) in cells treated with these dyes at different time points and concentrations of NH_4Cl added to the media. In each case, fluorescence was quantified using a Spectra Max® microplate reader (Molecular Probes Gemini XS, Molecular Devices, Sunnyvale, CA).

Alamar Blue and CFDA assays are widely accepted in *in vitro* toxicology assays (Back *et al.* 1999; Dayeh *et al*. 2005). Alamar Blue measures the metabolic activity endpoint and CFDA-AM measures the membrane integrity endpoint. Alamar Blue is a blue non-fluorescent dye resazurin, which is converted to resorufin (pink) by mitochondrial dehydrogenases and cytoplasmic esterases. The conversion of resazurin to resorufin correlates to high metabolic activity. Resorufin fluoresces at an excitation wavelength of 530nm, which is detected at an emission wavelength of 590nm. (Back *et al*. 1999; Dayeh *et al*. 2005; Schreer *et al*. 2005).

To determine how ammonia influenced cell membrane integrity, cells were treated with CFDA-AM, a fluorogenic chemical that is converted by non-specific plasma membrane esterases in living cells to carboxyfluorescein dye (Bopp and Lettieri, 2008). The carboxyfluorescein is retained inside the cells from which it emits a fluorescent signal detected at a wavelength of 530nm when it is excited at 485nm. A decrease in the retention, and hence RFU, of this dye indicates a decrease in membrane integrity or esterase activity (Dayeh *et al*. 2005).

Quantification of Glutamine Synthetase Activity in Cells Following MSO Treatment

Cells were plated in 12-well plates (353043, BD Falcon) at 6 x 10^5 cells/well for GFB cell line and 3 x 10^5 for RTB cell line in 10% FBS/L15 for 24 hours to allow for cell adhesion. After 24 hour incubation (19^{o}C and 23^{o}C for RTB and GFB respectively), cells formed a confluent layer in each well. MSO exposures were conducted as described above. After the completion of the 48 hours exposure, cells were trypsinized and centrifuged in 0.6ml bullet tubes for two minutes at 10,000 rpm. The supernatant consisting of trypsin was replaced with 500 µl of 1x PBS. The cells in PBS were once again centrifuged for two minutes at 10,000 rpm. These bullet tubes containing cell pellet and PBS were then immediately snap-frozen in liquid nitrogen and stored at -80^{o}C, until determination of glutamine synthetase. The glutamine synthetase assay was performed as outlined by Shankar and Anderson (1985). Glutamine synthetase is an enzyme, which metabolizes nitrogen by catalyzing glutamate and ammonia to form glutamine. This assay was based on the formation of γ-glutamylhydroxymate in the presence of glutamate and hydroxylamine, which

results in the generation of pyruvate. The generated pyruvate reacts with hydroxylamine and ferric chloride reagent, which was measured colorimetrically at 540 nm using the plate spectrophotometer. Cell homogenate was used to determine glutamine synthetase activity.

Quantification of Ammonia in Cell Media

Ammonia concentrations were determined enzymatically (glutamate dehydrogenase; GDH) using a commercial assay kit (AA0100, Sigma-Aldrich) in which quantities of total ammonia in the cell homogenate were based on differences in the absorbance at 340nm caused by the oxidation of NADPH to NAD^+ following the addition of GDH to the assay mixture. To ensure accuracy of the standard curve, the ammonia concentration of commercial ammonia standard (588 μM; Sigma-Aldrich) was also determined for each series (Refer to Appendix C).

Statistical Analysis

GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA) was used to calculate the IC_{50} as well as the cell viability using AB and CFDA-AM. Data were analyzed using one-way analysis of variance (ANOVA), followed by a Tukey-Kramer post-hoc test when significance was noted. In instances where significant variation amongst the standard deviations for a given data set was noted, a non-parametric ANOVA, followed by Dunn's post-hoc test was used to test for statistically significant differences. All significant differences were determined at the $P<0.05$ level. All statistical analysis was conducted using GraphPad InStat, Version 3.02 (GraphPad Software, Inc., San Diego, CA, USA).

RESULTS

RTB and GFB3C cell morphology and immunohistochemistry

Cell appearance was fibroblastic under a light microscope for both GFB3C (Fig. 3.1 A, D) and RTB cells (Fig. 3.2 A, D). Both GFB3C and RTB cell lines were immunostained with anti-GFAP and anti-NF. Green fluorescence indicated positive GFAP (Fig. 3.1 C and 3.2 C) or NF (Fig. 3.1 F and 3.2 F). The primary antibody alone and secondary antibody alone showed no fluorescence when observed using a FITC filter, which acted as negative control (images not shown). NF positive staining was not observed for either GFB3C (Fig. 3.1 F) or RTB (Fig. 3.2 F), indicating there were no neurons present in either of the cell lines. DAPI stained well for the nuclei in GFB3C (Fig. 3.1 B, E) and RTB (Fig. 3.2 B, E). Positive controls using goldfish brain slices subjected to the immunostaining protocol confirmed that both anti-GFAP and anti-NF specifically bound to glial cells and neurons, respectively (Fig. 3.3 C, F).

Changes in the cell morphology of cultured GFB3C and RTB cells in response to Ammonia

No obvious changes were observed in the morphology of GFB3C cells at total ammonia concentrations of 5 $mmolL^{-1}$ (Fig. 3.4 A, B), but moderate vacuolization was seen in the RTB cells which were exposed to ammonia at the same concentration (Fig. 3.4 D, E). At 100 $mmolL^{-1}$ of ammonia, some vacuolization of the GFB3C cells was noted, along with more extensive vacuolization in the RTB cells (Fig. 3.4 C, F).

Figure 3.1: Goldfish brain cells (GFB3C) probed with anti-Glial fibrillary acidic protein (anti-GFAP) and anti-Neurofilament (anti-NF) primary antibodies. (A, D) Visualization of GFB3C cell lines using phase-contrast microscopy to illustrate key ultrastructural features including the presence of fibrous extensions characteristic of astrocyte-type glial cells (arrow) and other features including star-like stuctures with projections. (B,E) The same cells stained with DAPI, and visualized under ultraviolet (UV) light to localize the cell nuclei. (C) GFB3C visualized under FITC filter to detect green fluorescence emitted by FITC conjugated-IgG used to identify GFAP positive cells. (F) GFB3C was also probed for anti-NF immunoreactivity, but none was detected. Scale: 1 cm = 50 μm.

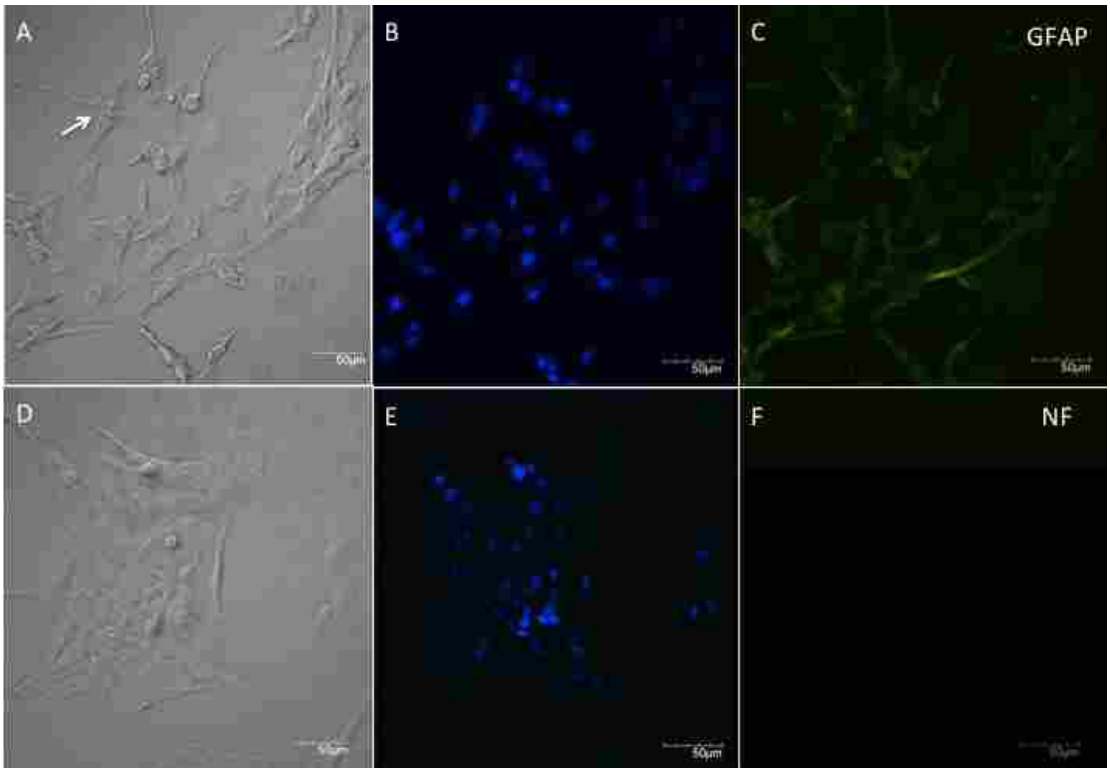

A
B
C
GFAP
D
E
F
NF

Figure 3.2: Rainbow trout brain cells (RTB) probed with anti-Glial fibrillary acidic protein (GFAP) and anti-Neurofilament (NF) primary antibodies. (A,D) Visualization of RTB cell lines using phase-contrast microscopy to illustrate key ultrastructural features including the presence of fibrous extensions characteristic of astrocyte-type glial cells (arrows), and other features including star-like structures. (B,E) The same cells stained with DAPI, and visualized under ultraviolet (UV) light to localize the cell nuclei. (C) RTB visualized under FITC filter to detect green fluorescence emitted by FITC conjugated-IgG used to identify GFAP positive cells. (F) RTB was also probed for anti-NF immunoreactivity, but none was detected. Scale: 1 cm = 50 μm.

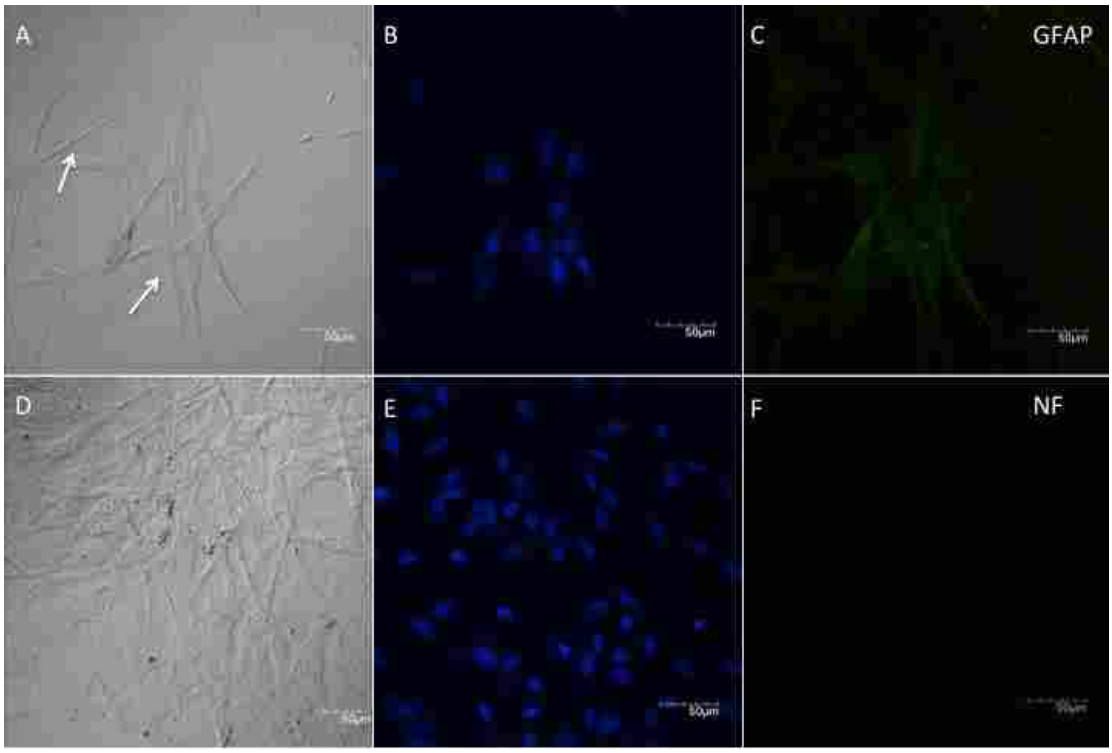

A
B
C
GFAP
D
E
F
NF

Figure 3.3: Goldfish brain slices (positive control) probed with anti-Glial fibrillary acidic protein (GFAP) and anti-Neurofilament (NF) antibodies. (A,D) Visualization of goldfish brain slice using phase-contrast microscopy to illustrate key ultrastructural features of astrocytes (fibrous). (B,E) The same cells stained with DAPI, but visualized under ultraviolet (UV) light to localize the cell nuclei. (C) Brain slice visualized under FITC filter to detect green fluorescence emitted by FITC conjugated-IgG used to identify GFAP positive cells, or (F) NF positive cells. Scale: 1 cm = 50 μm.

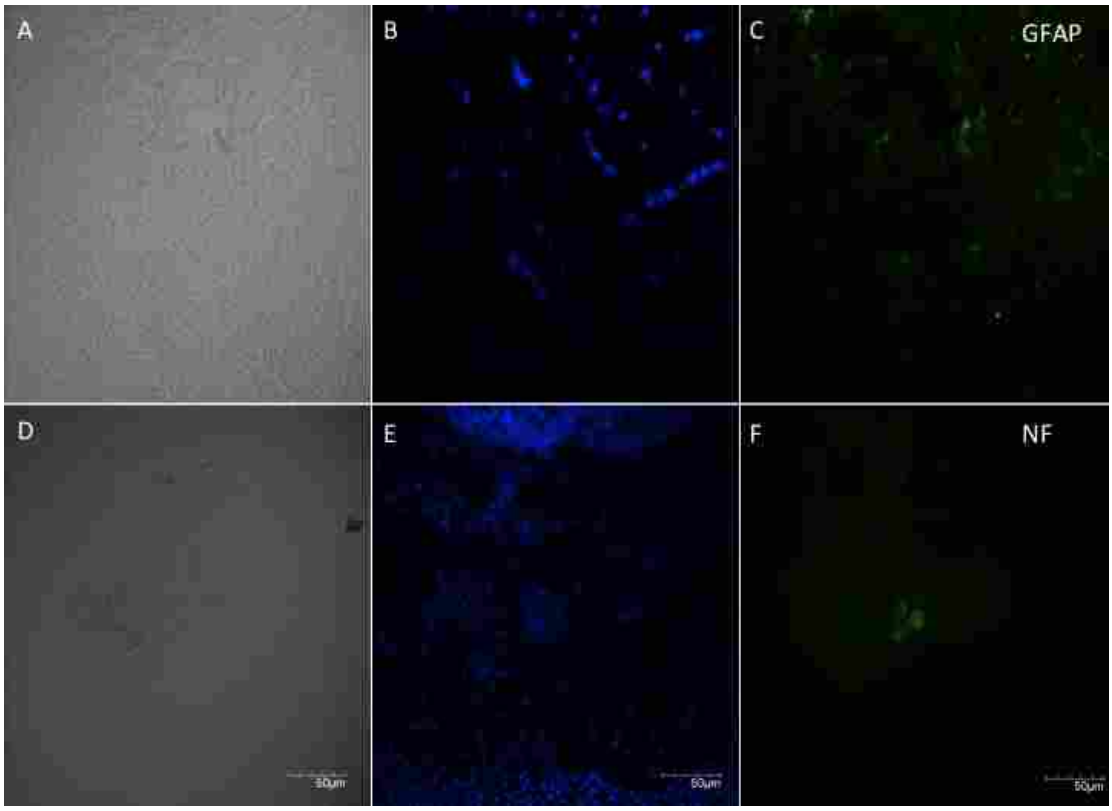
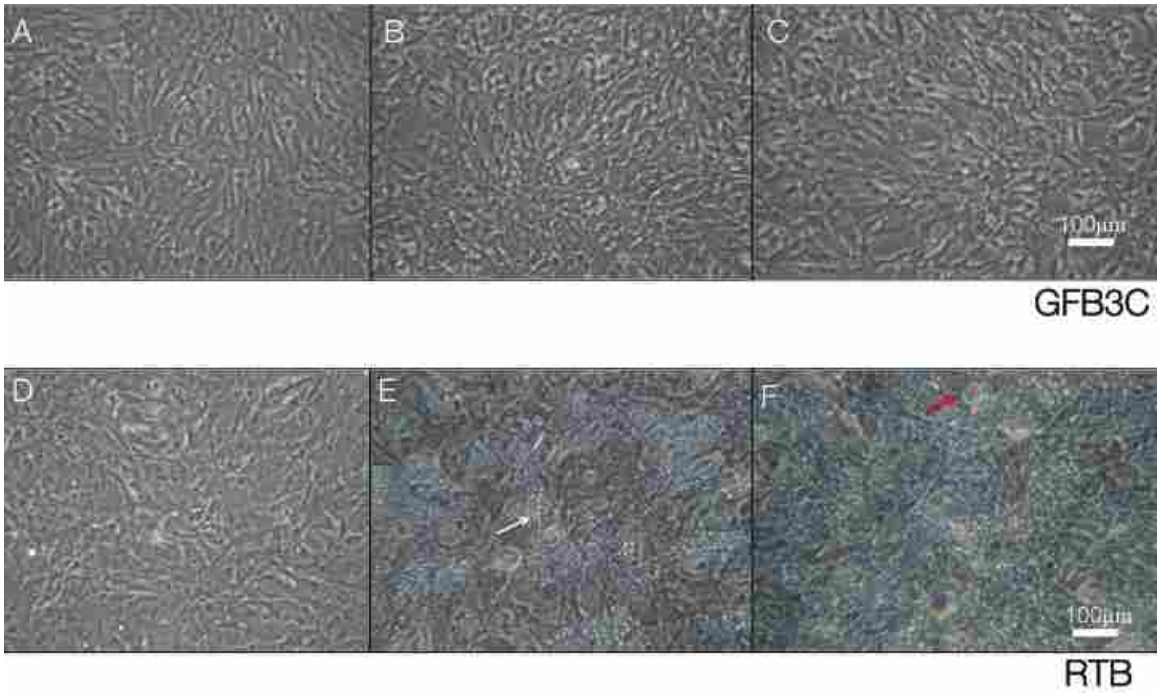

A
B
C
GFAP
D
E
F
NF
50μm
50μm
50μm

Figure 3.4: Phase-contrast Micrographs of GFB3C and RTB cells after being treated with Ammonium Chloride. GFB3C and RTB were exposed to 5mM and 100mM ammonia for 24 h. Both sets of cells exhibited fibrous and radial morphology consistent with glial cells. However, when exposed to NH_4Cl, the GFB3C morphology looked more dendritic, while RTB cells exhibited dose-dependent vacuolization (indicated by arrows) Scale: 1cm = 50 μm.


A
B
C
100µm
GFB3C
D
E
F
100µm
RTB

Quantification of Cell Viability and Number Using Alamar Blue and CFDA

The fluorescence of cells caused by AB decreases in cells with an impaired metabolism; it is entirely absent if the cells are dead (Back *et al*. 1999). On the other hand, CFDA-AM is an indicator of cell membrane integrity and is useful for evaluating cell viability (Dayeh *et al.* 2005). Prior to performing cell viability assessments, standard curves of cell viability against cell number were generated using either Alamar Blue or CFDA to determine which was most suitable for ammonia toxicity testing (Fig. 3.5). For each a significant linear correlation was found between fluorescence intensity [expressed as RFUs)] and cell number for both the GFB3C and RTB cells. The relationship between viable cell number (C) and RFU was, RFU =1374.8LogC -3820.7 (r^2=0.95) for GFB3C, and RFU = 1486.1LogC - 3708.2 (r^2=0.97) for RTB using the AB assay, and RFU=268.02LogC-790.1 (r^2=0.89) for GFB3C and RFU=529.49LogC-1486.2 (r^2=0.86) using CFDA. Based on the steeper slope of the cell viability vs. cell number relationship, AB proved to be the most sensitive indicator of cell viability. Thus, for all subsequent experiments a total of $2.5x10^4$ cells/well GFB3C cells and $2.0x10^4$ cells/well RTB cells were plated into each well, and AB was used to determine how exposure to ammonia affected cell viability.

In GFB3C cells, cell viability was maintained up to ammonia concentrations of 100 $mmolL^{-1}$ before significantly decreasing by approximately 20 %. In contrast, significant reductions in cell viability were observed at 25 $mmolL^{-1}$ NH_4Cl (Fig. 3.6 A). Notably, CFDA treatment of the GFB3C cells revealed that there was no change

Figure 3.5: Fluorescence response of GFB3C and RTB to Alamar Blue and CFDA-AM. Standard curve was generated for plating cells by comparing increased cell numbers to relative fluorescent units. Cells were plated at different cell concentrations in 96-well plate and incubated at room temperature for 24h. Six replicates were used for each cell concentration. They were exposed to Alamar Blue and CFDA-AM. Each data point represents the mean of 6 well replicates.

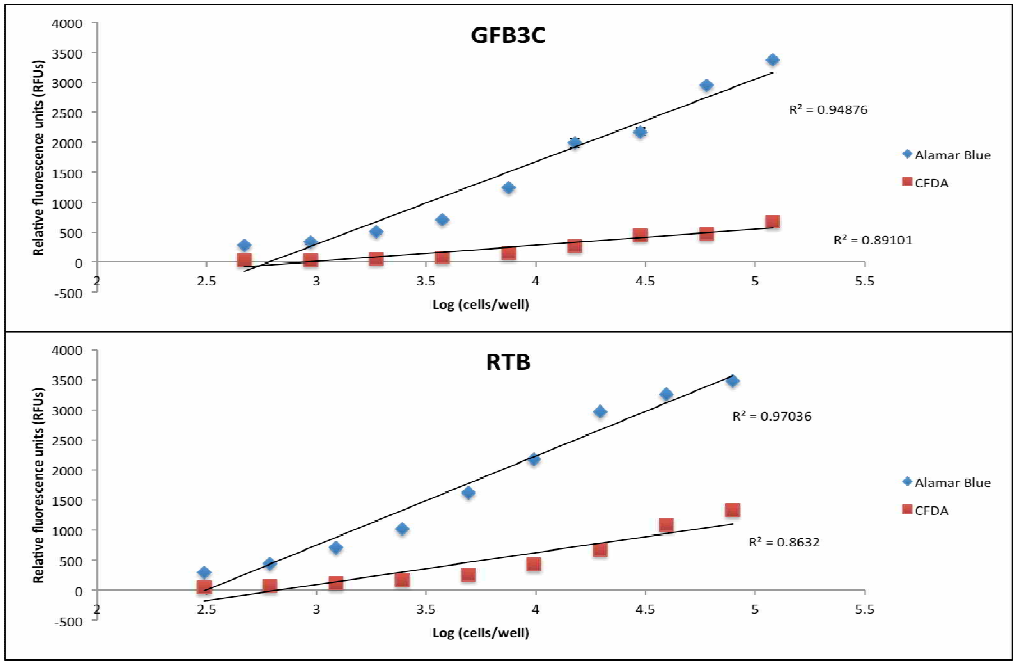
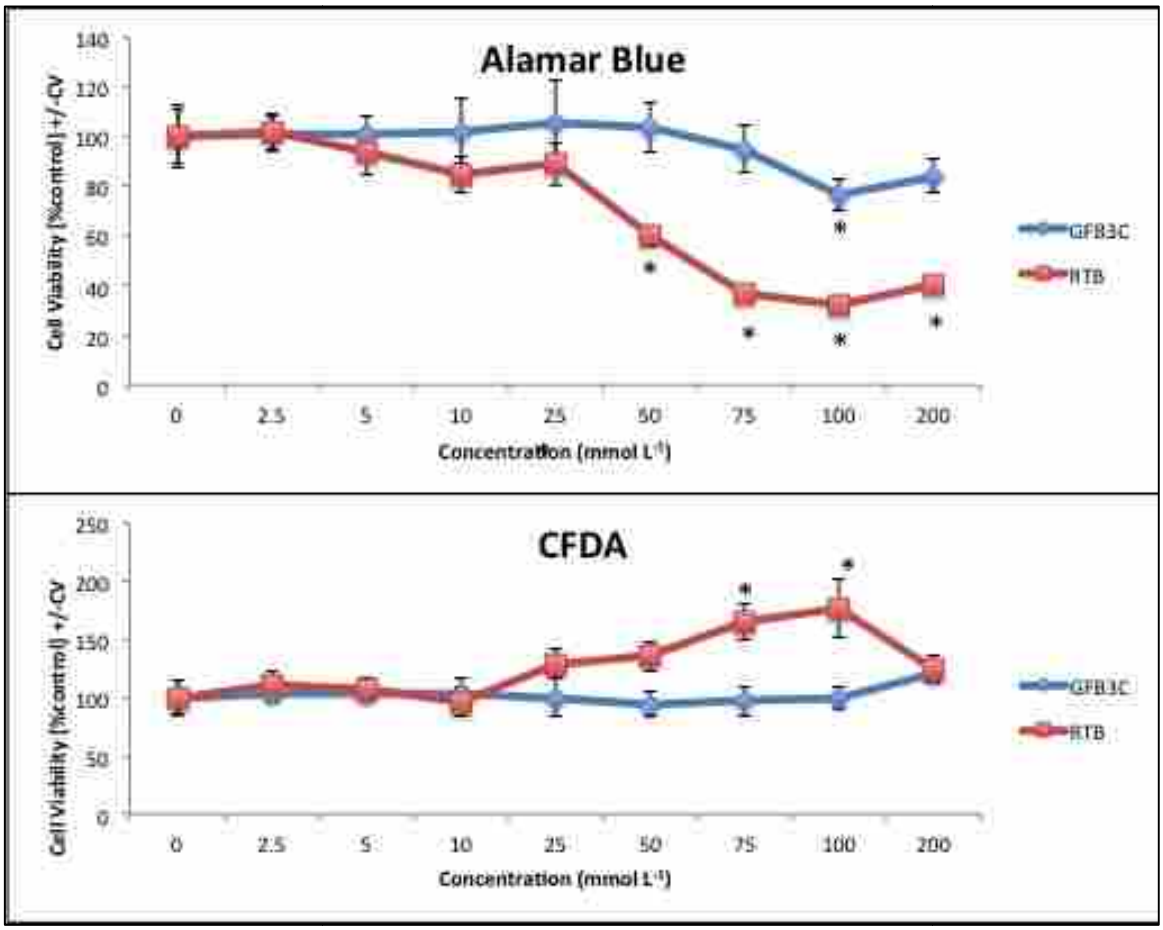

GFB3C
Relative fluorescence units (RFUs)
Log (cells/well)
R² = 0.94876
R² = 0.89101
Alamar Blue
CFDA
RTB
Relative fluorescence units (RFUs)
Log (cells/well)
R² = 0.97036
R² = 0.8632
Alamar Blue
CFDA

Figure 3.6: Cell viability of GFB3C and RTB cell lines during exposure to increasing concentrations of Ammonia (NH_4Cl) using (A) Alamar Blue, and (B) CFDA. Graphs were generated by comparing increased cell numbers to relative fluorescent units. Cells were plated in 96-well plate and incubated at room temperature for 24h and then exposed to NH_4Cl for 48h. Six replicates were used for each concentration of NH_4Cl. Alamar Blue and CFDA-AM assays were conducted. Each data point represents the mean of 6 well replicates. Data presented as the mean % relative fluorescence units [RFU (± CV)] in which control cells, not exposed to ammonia, have an RFU value of 100%.


Alamar Blue
Cell Viability (%control) +/-CV
140
120
100
80
60
40
20
0
0
2.5
5
10
25
50
75
100
200
Concentration (mmol L-1)
GFB3C
RTB
*
CFDA
Cell Viability (%control) +/-CV
250
200
150
100
50
0
Concentration (mmol L-1)
GFB3C
RTB

in the integrity of the plasma membrane over the entire range of ammonia concentrations (2.5-200 $mmolL^{-1}$) examined. In RTB cells, however, cell membrane integrity appeared to increase at concentrations of ammonia greater than 25 $mmolL^{-1}$ (Fig. 3.6 B).

Calculating the IC_{50} using Alamar Blue

Exposure of the GFB3C and RTB cell lines to L15/ex containing NH_4Cl concentrations ranging from 1$mmolL^{-1}$ to 1000$mmolL^{-1}$ for 24 h and 48 h resulted in a dose-dependent decrease in the viability of both GFB3C cells and RTB cells. In each case the RTB cells were more sensitive, with respective IC_{50} values of 118 and 67 $mmolL^{-1}$ at 24 h and 48 h, compared to values of 1165 $mmolL^{-1}$ and 187 $mmolL^{-1}$ for the GFB3C cells (Fig. 3.7).

Effects of Methionine Sulfoximine on Ammonia toxicity and Glutamine Synthetase Activity

GFB3C and RTB cells were pre-treated with methionine sulfoximine (0.01, 0.05, 0.1, 0.5, 1, 5 $mmolL^{-1}$) for 24 hours and then treated with pre-determined concentrations of ammonia, which proved to be effective for causing a change in cell viability in experiments described above. GFB3C were exposed to 100 $mmolL^{-1}$ of ammonia and RTB were exposed to 25 $mmolL^{-1}$ of ammonia. Alamar Blue assays were performed after 48 hours of ammonia exposure. It was found that although no change in GFB3C cells, after 48 h exposure RTB cell viability increased by 40% when pre-treated with

Figure 3.7: IC_{50} of GFB3C and RTB cell lines to Ammonia for 24h and 48h. GFB3C and RTB were exposed to NH_4Cl concentrations between 10 and 1000 mM. Each data point represents cell viability at different concentration of NH_4Cl (± 1 standard deviation). The IC_{50} was determined by calculating the concentration at which 50% cell viability occurred from the logarithmic curve.

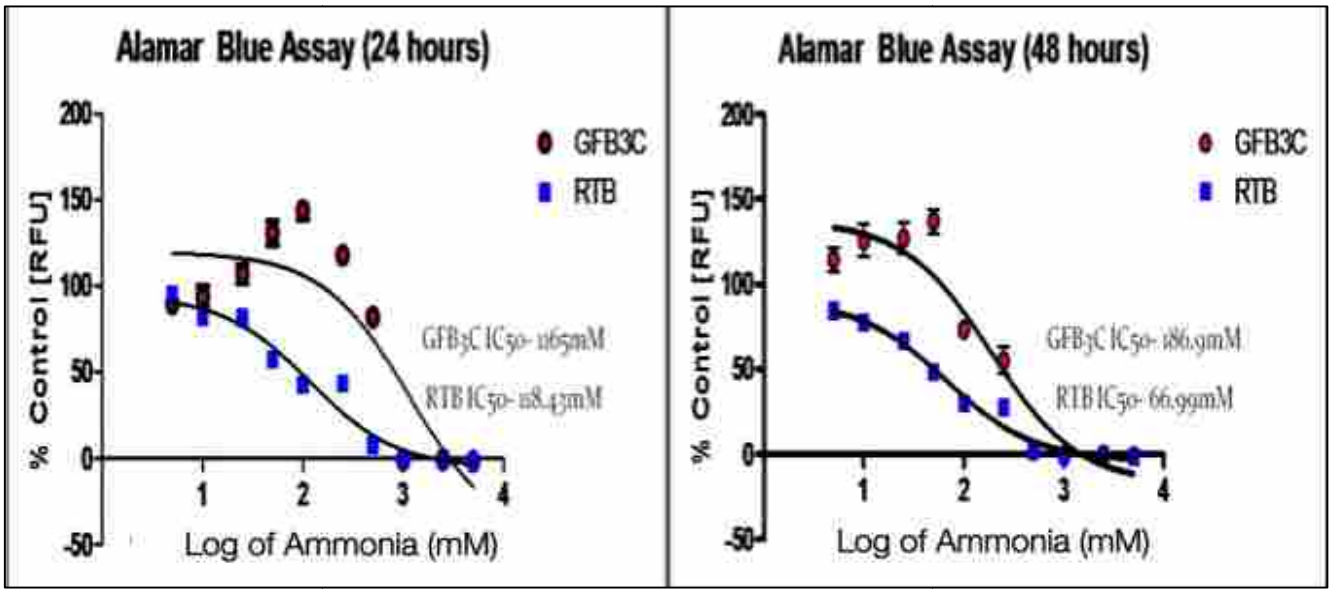

Alamar Blue Assay (24 hours)
% Control [RFU]
Log of Ammonia (mM)
GFB3C
RTB
GFB3C IC50- 1165mM
RTB IC50- 118.43mM
Alamar Blue Assay (48 hours)
% Control [RFU]
Log of Ammonia (mM)
GFB3C
RTB
GFB3C IC50- 186.9mM
RTB IC50- 66.99mM

Figure 3.8: Effects of MSO on cell viability of GFB3C and RTB cell lines in the presence of Ammonia measured with Alamar Blue. Cells were plated in 96 well plates and pre-exposed to MSO for 24h prior to being exposed to ammonia and cell viability was measured using Alamar blue at 48h post ammonia exposure. Each data point represents the mean viability of 6 well replicates. Cell viability of ammonia-treated cells increased significantly for RTB when co-exposed to 0.5, 1, and 5 mmol L^{-1} MSO. GFB3C cell viability for ammonia treated cells also increased significantly when co-exposed to lower concentrations of MSO. Dagger denotes significant differences ($p<0.05$) between control and ammonia exposed cells and asterisks denotes significant differences ($p<0.05$) between the control and MSO treated cells. Data presented as the mean % relative fluorescence units [RFU (± CV)] in which control cells, not exposed to ammonia, have an RFU value of 100%.

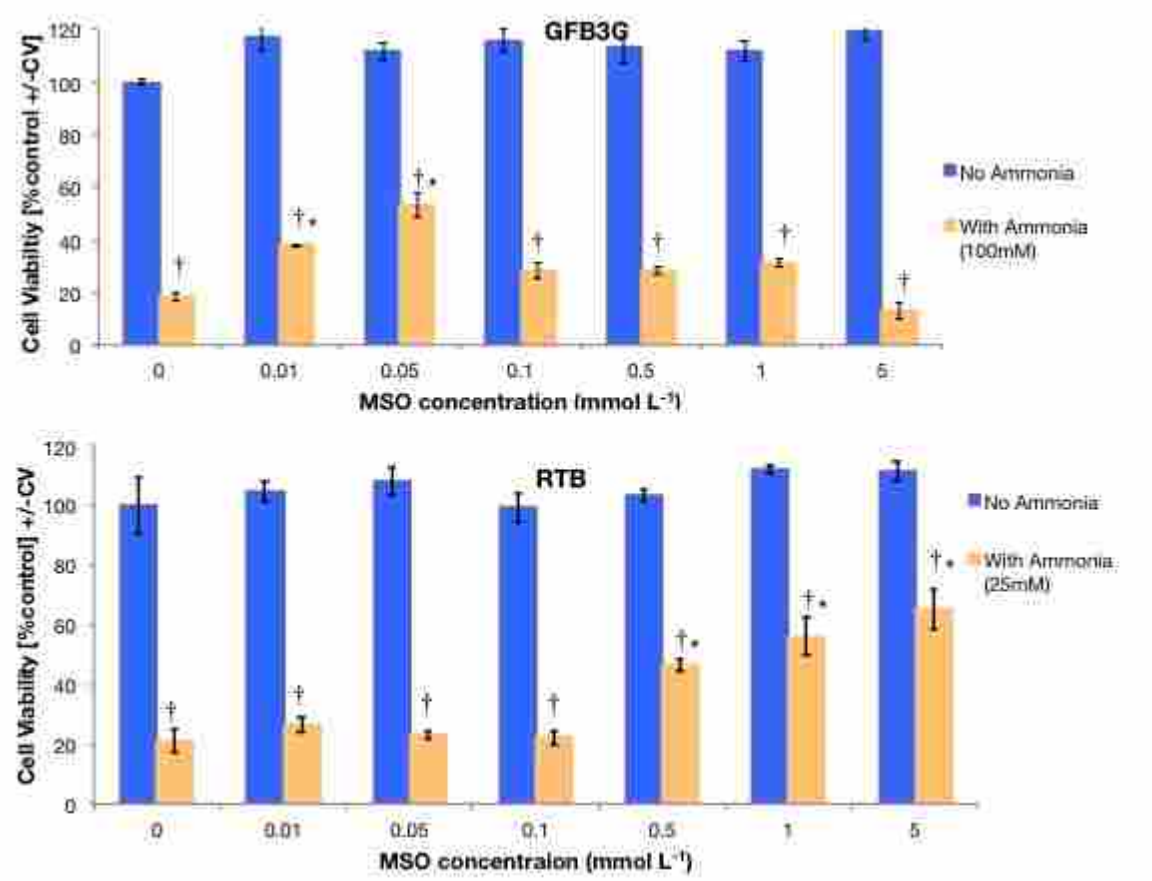
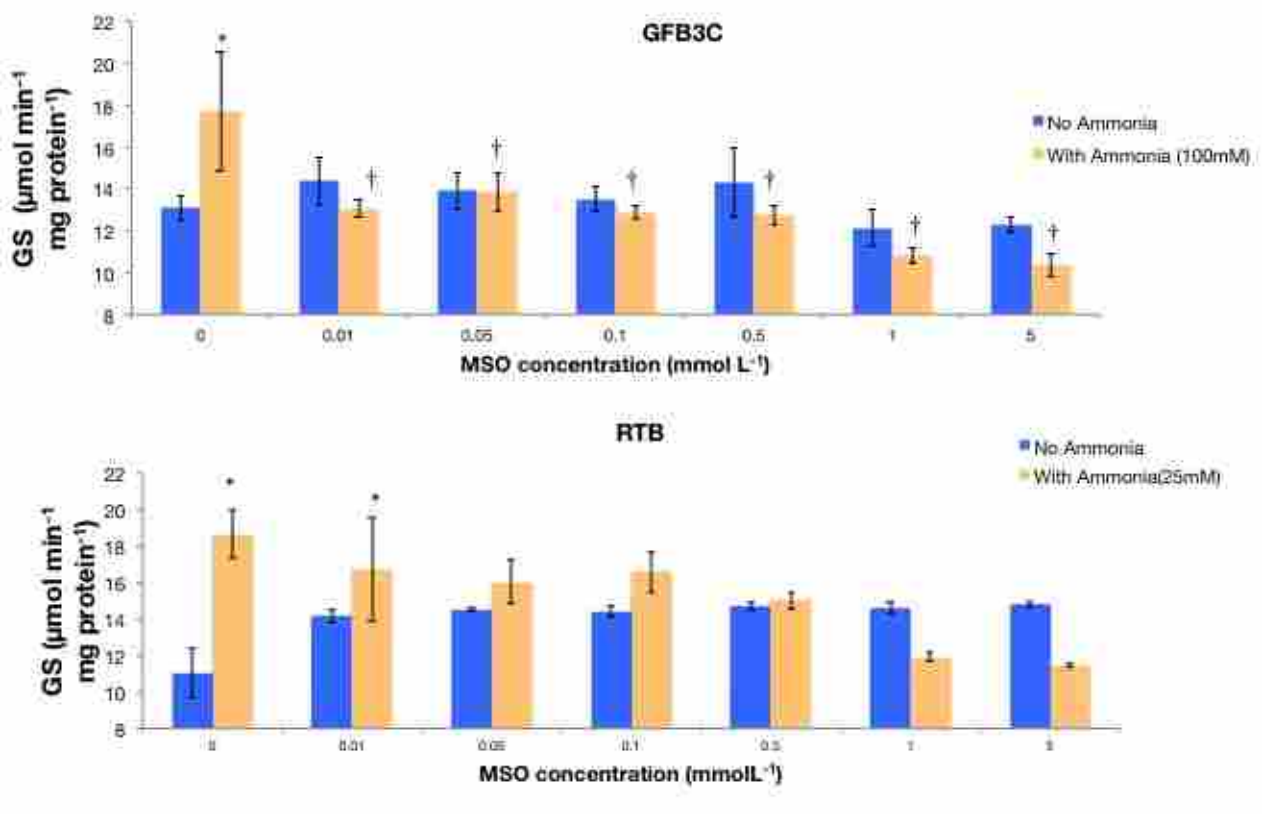

GFB3G
Cell Viability [%control +/-CV]
120
100
80
60
40
20
0
0
0.01
0.05
0.1
0.5
1
5
MSO concentration (mmol L-1)
No Ammonia
With Ammonia (100mM)
RTB
Cell Viability [%control] +/-CV
MSO concentralon (mmol L-1)
No Ammonia
With Ammonia (25mM)

Figure 3.9: GS activity in the presence of MSO. GS activity increased in cells exposed to ammonia but did not decrease significantly as a result of being exposed to MSO. Dagger denotes significant differences ($p<0.05$) between control and ammonia exposed cells and asterisks denotes significant differences ($p<0.05$) between cells with or without ammonia at each treatment interval. All values are presented as mean ± SEM. N=6 for goldfish and rainbow trout cell lines.


GFB3C
GS (µmol min-1 mg protein-1)
No Ammonia
With Ammonia (100mM)
MSO concentration (mmol L-1)
RTB
GS (µmol min-1 mg protein-1)
No Ammonia
With Ammonia(25mM)
MSO concentration (mmolL-1)

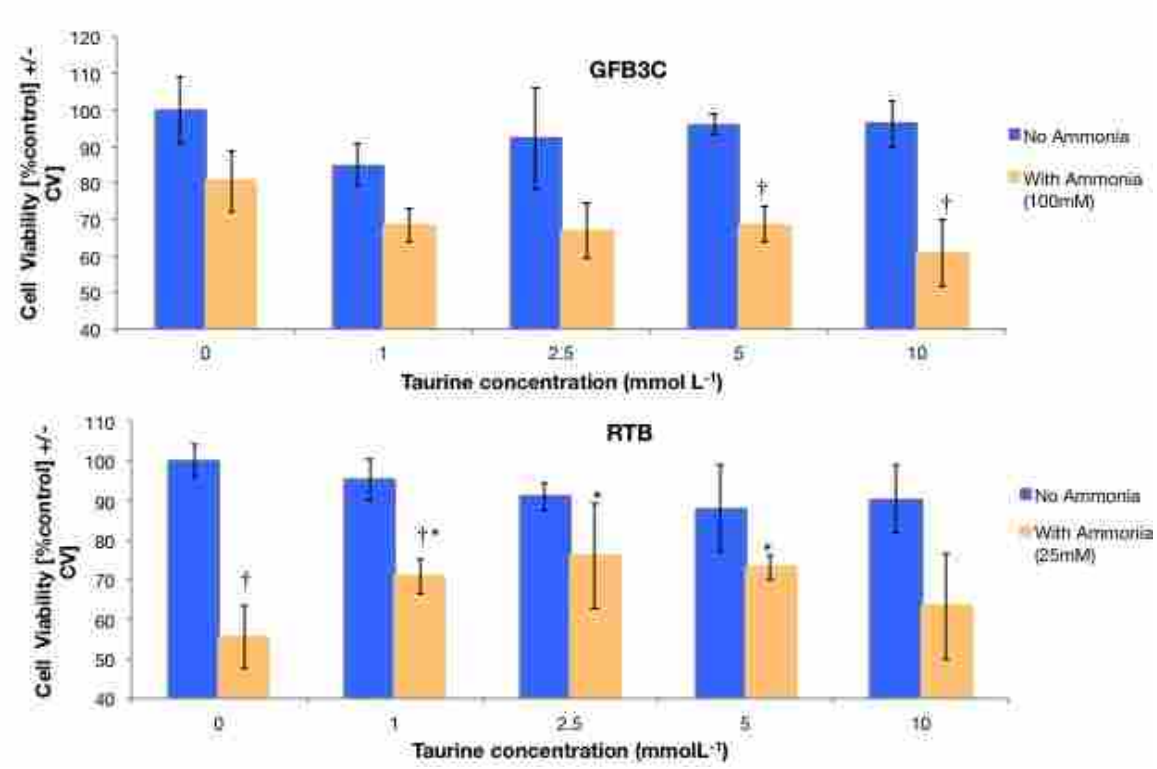
5 mmolL^{-1} of MSO (Fig. 3.8).

Glutamine synthetase activity did increase, 31% in GFB3C and 64% in RTB (Fig. 3.9), when cells were exposed to ammonia but glutamine synthetase was not inhibited significantly by methionine sulfoximine as predicted earlier. There were no significant differences in cell viability between cells treated with only MSO or MSO in the presence of ammonia (Fig. 3.8).

Taurine and Ammonia Toxicity

Taurine treatment appeared to have no effect on GFB3C cells when they were exposed to ammonia (100 mM). In contrast, taurine treatment appeared to increase cell viability during ammonia exposure in RTB cells, which was significantly increased by 20% at a taurine concentration of 5mM (Figure 3.10 B). Thus, RTB cells are more sensitive to the swelling than GFB3C.

Figure 3.10: Effects of Taurine on cell viability of GFB3C and RTB in the presence of Ammonia. Cells were plated in 96 well plates and exposed to solutions consisting of taurine plus or minus NH_4Cl (25 mM). Cell viability was measured using Alamar Blue at 48h post exposure. Each data point represents the mean of 6 well replicates. RTB cell viability increased significantly after 48 hours. Dagger denotes significant differences ($p<0.05$) between control and ammonia exposed cells and asterisks denotes significant differences ($p<0.05$) between the control and taurine treated cells. Data presented as the mean % relative fluorescence units [RFU (± CV)] in which control cells, not exposed to ammonia, have an RFU value of 100%.


GFB3C
Cell Viability [%control] +/- CV]
120
110
100
90
80
70
60
50
40
0
1
2.5
5
10
Taurine concentration (mmol L-1)
No Ammonia
With Ammonia (100mM)
†
†
RTB
Cell Viability [%control] +/- CV]
110
100
90
80
70
60
50
40
0
1
2.5
5
10
Taurine concentration (mmolL-1)
No Ammonia
With Ammonia (25mM)
†
†*
*
*

DISCUSSION

Cell Characterization

Ammonia is primarily detoxified in the astrocytes (Norenberg and Martinez-Hernandez 1979) via the conversion of ammonia and glutamate to glutamine in a reaction catalyzed by GS (Mommsen and Walsh 1991; Felipo and Butterworth 2002; Walsh *et al*. 2007). The GFB3C and RTB cell lines employed to conduct this study are therefore ideal for studying the role of astrocytes in ammonia toxicity and tolerance due to their morphological and physiological characteristics similar to those of astrocytes. Phase-contrast microscopy revealed that both cell lines had a fibroblastic morphology, a feature usually associated with astrocytic cells (Gregorios *et al.* 1985a) (GFB3C, Figure 3.1 A, D; RTB, Figure 3.2 A, D). Each tested positive for glial fibrillary acidic protein (GFAP), and showed fibrillary astrocytic like structures (Figure 3.1 A and 3.2 A) characteristic of astrocytes (Eng 1985; Back *et al.* 2011). GFAP is a protein, which makes up glial intermediate filaments. They are known to be prominent in astrocytes, and play an important role in maintaining the shape of the astrocyte (Eng, 1985).

Astrocytes are believed to be the most abundant cell type in the brain and these have been readily cultured from vertebrates including goldfish (Sivron *et al.* 1993) and trout (Frojdo *et al.* 2002). Physiologically, both GFB3C and RTB exhibited GS activity, which provided further evidence that these cells were astrocyte-like (Walsh *et al*. 2007). The astrocytes play an important role in the glutamate-glutamine cycle, in which the excitatory neurotransmitter glutamate is taken up from the synaptic cleft by glutamate: Na^+ co-transporters on the astrocytes; glutamate is then combined with

the ammonia to form glutamine (Felipo and Butterworth 2002; Walsh *et al*. 2007). Further, GS activity increased as a result of exposing cells to ammonia (Fig. 3.9), perhaps as a means of ammonia detoxification or just simply in response to increased demand for the enzyme (see below).

The absence of NF immunoreactivity strongly suggests that the cells exhibited few, if any characteristics of neurons. Neurofilament-200 (NF) is a common marker of intermediate filaments of neurons in primary culture preparations. In addition, other morphological characteristics of neurons, including cell bodies accompanied by polar extensions were absent.

Using Alamar Blue and CFDA as indicators of cell viability

The response of the GFB3C and RTB cell lines was quantified using various fluorometric indicator dyes and by evaluating changes in the structure of cells using micrographs. Two of the most widely used assays of cell viability are AB and CFDA (Back *et al.* 1999). Preliminary experiments indicated that AB provided more stable and reproducible results than CFDA. As noted in previous *in vitro* literature (Bopp and Lettieri 2008; Schreer *et al.* 2005; Dayeh *et al*. 2009), AB measures the metabolic activity of cells, which is a more direct measure of cell viability than CFDA-AM which is an indicator of membrane integrity. AB also was more sensitive to changes in cell number than CFDA-AM, as demonstrated in preliminary assays of cell viability vs. cell number. More importantly, AB was a more sensitive indicator of physiological changes brought about by ammonia exposure, than was CFDA-AM. CFDA-AM indicated the affected plasma membrane of cells, by measuring esterase activity (Dayeh *et al*. 2005). However, CFDA-AM may not be an appropriate marker

for ammonia toxicity, because ammonia causes extensive vacuolization in cells, which results in the release of intracellular esterases which could inadvertently increase CFDA activity even though the cells are no longer intact (Dayeh *et al*. 2005). Since the CFDA measures the esterase activity both inside and outside the cells, it makes it a less reliable indicator of cell viability (Ganassin *et al.* 2000; Dayeh *et al*. 2003).

Ammonia Toxicity

The IC_{50} values of ammonia after 48h determined using cell lines were 100 and 200 times higher for trout and goldfish, respectively, than the 96 h LC_{50} values reported during *in-vivo* studies (Wilkie *et al.* 2011). These results are consistent with what has been reported in other *in vitro* toxicology studies. Bols *et al*. (1985) reported a good correlation between the IC_{50} and LC_{50} values but there was also an order of magnitude of difference between the two values (as reported by Segner and Lenz 1993). Segner *et al.* (1987) reported that the route of uptake is important when taking into consideration the toxicity of metals. For instance, many metals accumulate in the gill tissue and cause toxicity by interfering with gill-mediated ion exchange and with ion uptake by fishes, leading to ion losses and osmotic disturbances that cause death (Niyogi and Wood). In such instances, death cannot be correlated to what is observed *in vitro* because the factors leading to toxicity are not a direct consequence of the metals acting on internal organs.

Ammonia is different from metals in that it acts directly on the nervous system,

causing astrocytic swelling (Buttertworth 2010). Cell vacuolization can be considered as a measure of toxicity (Dayeh *et al.* 2009). It was found that vacuolization in RTB cells occurred at low concentrations of ammonia near 5 $mmolL^{-1}$, while GFB3C cells did not show any increase in vacuolization until exposed to 100 $mmolL^{-1}$ of ammonia, which exceeds the amount of ammonia found in the environment.

These *in vitro* findings are consistent with the *in vivo* results presented in Chapter 2, which indicated that the concentration of ammonia required to cause brain swelling in goldfish was five times more than what was required to generate the same brain swelling in rainbow trout. Dayeh *et al*. (2009) similarly showed that effluents from paper-mills, which contained ammonia caused vacuolization in RT gill cell lines. These authors suggested that vacuolization was due to the uptake of un-ionized ammonia, which diffused into acidic organelles such as lysosomes and endosomes, causing an ionic imbalance, which in turn drew water into these organelles, leading to vacuolization. The vacuoles seen in the cells exposed to ammonia were similar to the ones seen by Gregorios *et al*. (1985a) when they exposed primary rat astrocyte cultures to 10 $mmolL^{-1}$ ammonia. Consistent with Gregorios *et al.* (1985a) an increase in extracellular debris was also noted. It is notable, that similar vacuolization and debris from cell death were seen in the RTB, but not the GFB3C cell lines, at similar concentrations of ammonia (Fig. 3.4).

Effects of MSO on ammonia exposed cell lines

MSO inhibits glutamine synthetase which enzymatically converts ammonia and glutamate to glutamine. A decline in glutamine synthetase activity as a result of MSO was not observed in the present *in vitro* work, in contrast to the *in vivo* experiments. Cells exposed to ammonia in the presence of MSO appeared to be vacuolized (micrographs not shown) which could indicate that glutamine accumulation does not cause swelling.

Norenberg and Bender (1994), Willard-Mack *et al*. (1996), and Zwingmann *et al*. (2000) all showed that MSO reduced astrocyte swelling *in vitro* which in turn means that it should also increase cell viability. We found that cell viability, as measured by Alamar Blue assay, did increase in RTB cells pre-treated with MSO. Viability of RTB cell line increased by 20-40% when exposed to $1mmolL^{-1}$ or $5mmolL^{-1}$ MSO. The GFB3C cell line did not show a significant increase in viability, but this could be due to the fact that the concentrations of MSO used were not high enough for an effect to be noted. Similar to higher concentrations of ammonia were used. Jayakumar *et al*. (2006) did not find a correlation between astrocyte swelling and intra-cellular glutamine concentrations in primary cultures of cortical astrocytes from rat brain, despite the fact that MSO resulted in reduced brain swelling. In addition to this, MSO is a known convulsant, and is thought to cause an increase in extracellular glutamine (Albrecht and Norenberg 1990), which could impact cell viability at high concentrations.

In addition to the measurement of glutamine synthetase activity, glutamine concentrations could have also been measured to prove that glutamine was infact

being decreased as a result of MSO exposures.

Effects of taurine on ammonia exposed cell lines

Zelinska *et al*. (2003) showed a decrease in swelling of brain slices after they were exposed to taurine, suggesting that swelling is due to an increase in intracellular osmolarity. Taurine is an inhibitory neurotransmitter, and is also considered to be an osmoprotectant (Albrecht 1998). It is believed that taurine is released by astrocytes to counteract the excitation of neurons when the brain is exposed to ammonia (Albrecht 1998). The most significant increase in viability when cells were exposed to taurine was seen in RTB cells after 48h. Although, taurine did increase the cell viability of RTB, it did not affect GFB3C. This suggests that RTB cells were more sensitive to volume changes induced by ammonia than the GFB3C cells.

Taurine is known to act in different ways in protecting the brain during ammonia exposure. As an agonist of GABA receptors, it may help counteract the over-activation of NMDA receptors by (i) attenuating Ca^{2+}-driven cascades (Saransaari and Oja 2000), (ii) it is known to counter free radical production and, (iii) its neuroprotectant properties attenuate brain swelling (Zelinska *et al*. 2003; Albrecht and Wegrzynowicz 2005). Studies show that extracellular taurine increases when brain slices are exposed to ammonia (Zielinska *et al.* 1999). Similar results were seen in rat cortical astrocytes when they were exposed to ammonia (Albrecht *et al.* 1994) and in cultured rabbit Müller cells (Faff-Michalak *et al.* 1994; Faff *et al.* 1997). Quantification of taurine in the cerebrospinal fluid of goldfish and rainbow trout

exposed to ammonia, or in cell culture media of ammonia-treated GFB3C or RTB cell lines should be considered for future studies.

SUMMARY and CONCLUSIONS

The cell lines GFB3C and RTB had morphological and physiological properties that strongly resembled those of astrocytes, a site of action of ammonia in the nervous system of vertebrates. The GFB3C cells were able to survive under higher concentrations of ammonia than the RTB cells, which was consistent with the greater tolerance previously observed *in vivo* (Wilkie *et al.* 2011). The use of Alamar Blue as an indicator for cell viability rendered more consistent results than CFDA-AM. Consistent with the *in vivo* findings, prior treatment of the cell lines with MSO did not attenuate the effects of ammonia in GFB3C but did improve RTB viability, despite an absence of inhibited GS activity. These findings further support the hypothesis that the goldfish possesses distinct mechanisms that allow it to withstand higher external concentrations of ammonia than do the more ammonia-sensitive trout. Increases in the osmoprotectant amino acid taurine also improved RTB cell viability but not that of GFB3C, suggesting that the goldfish brain cells are not as sensitive to ammonia-induced osmotic disturbances as those of the rainbow trout. In conclusion, the findings of the present study indicate that both GFB3C and RTB cell lines could be an ideal model for studying the effects of ammonia and other toxicants on astrocytes in the nervous system of fishes, and the underlying mechanisms of brain swelling in not only fishes but also vertebrates in general.

Chapter 4

Mechanisms of Ammonia Neurotoxicity

An Integrative Approach to Study the Mechanisms of Ammonia Toxicity

This thesis used *in vivo* and *in vitro* based methods to provide an integrative understanding of the mechanisms involved in ammonia toxicity in vertebrates. Two fish species, the ammonia-tolerant goldfish and the ammonia-sensitive rainbow trout were studied using whole animal approaches and brain cell lines. Experiments were conducted to determine the mechanism of brain swelling during exposure to high external ammonia. Two hypotheses were tested: 1) Goldfish survival at high external ammonia is related to their ability to recover from ammonia-induced brain swelling more quickly than rainbow trout and 2) increased intracellular glutamine is the cause of brain swelling in both goldfish and trout.

Rainbow trout appeared to be more susceptible to ammonia-induced water uptake by the brain than goldfish; with an ammonia concentration threshold for brain swelling that was 50% lower than in goldfish. While survival appeared to be related to greater resistance to brain swelling in the goldfish, the goldfish did not appear to have a greater capacity to recover from brain swelling following ammonia exposures, as originally hypothesized.

The findings of the present study did not support the hypothesis that astrocytic swelling was a direct consequence of intra-astrocytic glutamine (GLN) accumulation. The importance of glutamine synthetase (GS) was demonstrated by using the GS inhibitor methionine sulfoximine (MSO), which did not prevent swelling in trout or goldfish during HEA. Thus, the brain can likely detoxify ammonia via other metabolic pathways not examined in this thesis. These findings support the work of Sanderson *et al*. (2010), who also reported that MSO treatment had no effect on brain

ammonia levels in rainbow trout exposed to high external ammonia. Thus, the long-standing hypothesis that GS may be critical for ammonia detoxification should be reconsidered. To follow-up these findings, similar *in-vitro* experiments should be carried out with a higher dosage of MSO.

Alternate Hypotheses to Explain Water Accumulation by the Brain During HEA.

***Does Glutamate Excitotoxicity Contributes to Cell Swelling During HEA*?** Exposure to ammonia directly activates the N-methyl-D-aspartate (NMDA; Fan and Szerb 1993; Hermenegildo *et al*. 2000; Wilkie *et al.* 2011) receptor, and this is compounded by the later accumulation of excess glutamate (Butterworth 2001). It has been proposed that ammonium ions are able to remove the intrinsic magnesium block from NMDA receptors, resulting in the general depolarization of neuronal membranes by ammonia, which affects the NMDA receptors directly (Fan and Szerb 1993). Ammonia therefore makes the NMDA receptor more susceptible to activation, which leads to an increase in intracellular calcium, which in turn can lead to neuronal death by causing cell swelling. The underlying causes of cell swelling may be related to corresponding reductions in ATP synthesis and ionic disturbances in the astrocytes (Kosenko *et al.* 2004; Norenberg *et al.* 2009). Increased rates of glutamine synthesis (Norenberg *et al.* 2009) could deplete intracellular glutamate stores and therefore impair the citric acid (TCA), which provides reducing equivalents needed to drive ATP synthesis via oxidative phosphorylation in the mitochondria (Sanderson *et al.* 2010).

Hermenegildo *et al*. (1996) demonstrated the importance of NMDA receptors in rodents subjected to excessive ammonia conditions through the administration of NMDA receptor antagonists such as MK801. Many of these antagonists, particularly MK801, were found to protect the rodents from death. Based on the findings of my thesis, I propose that MK801 likely contributes to survival by preventing cell swelling due to ROS production arising from over-activation of the NMDA receptor. To test this hypothesis, NMDA receptor antagonists such as dizocilpine (MK-801) and ketamine could be administered to trout and goldfish prior to HEA exposure. Additionally, cultured brain slices could be used to directly measure water uptake and changes in the intracellular fluid volume astrocytes in response to ammonia in the presence or absence of NMDA receptor antagonists. Such a preparation could also be used to directly measure ROS production arising from excitoxicity, or other sources, during exposure to HEA.

Is Ammonia-Induced Brain Swelling Caused by Free Radicals? NMDA receptor over-activation could also lead to neuronal death by causing cell swelling due to the generation of nitric oxide and reactive oxygen species (Kosenko *et al.* 2003). Over-activation of the NMDA receptor leads to an influx of calcium which binds to calmodulin activating NOS which leads to generation of NO. Calcium can also enter the post-neuronal mitochondria leading to the collapse of the mitochondrial membrane potential altering mitochondrial respiration chain. Thus, causing an increase in ROS generation (Kosenko *et al.* 2003). Indeed, there is evidence of lipid peroxidation in the plasma membrane of cultured astrocytes after ammonia treatment (Murphy *et al*. 1992). The generation of free radicals can be blocked by the

simultaneous addition of catalase and superoxide dismutase to the culture preparation. The administration of antioxidants also has a beneficial effect in experimental hepatic encephalopathy (HE)/hyperammonemia using rat models (Willard-Mack *et al*. 1996).

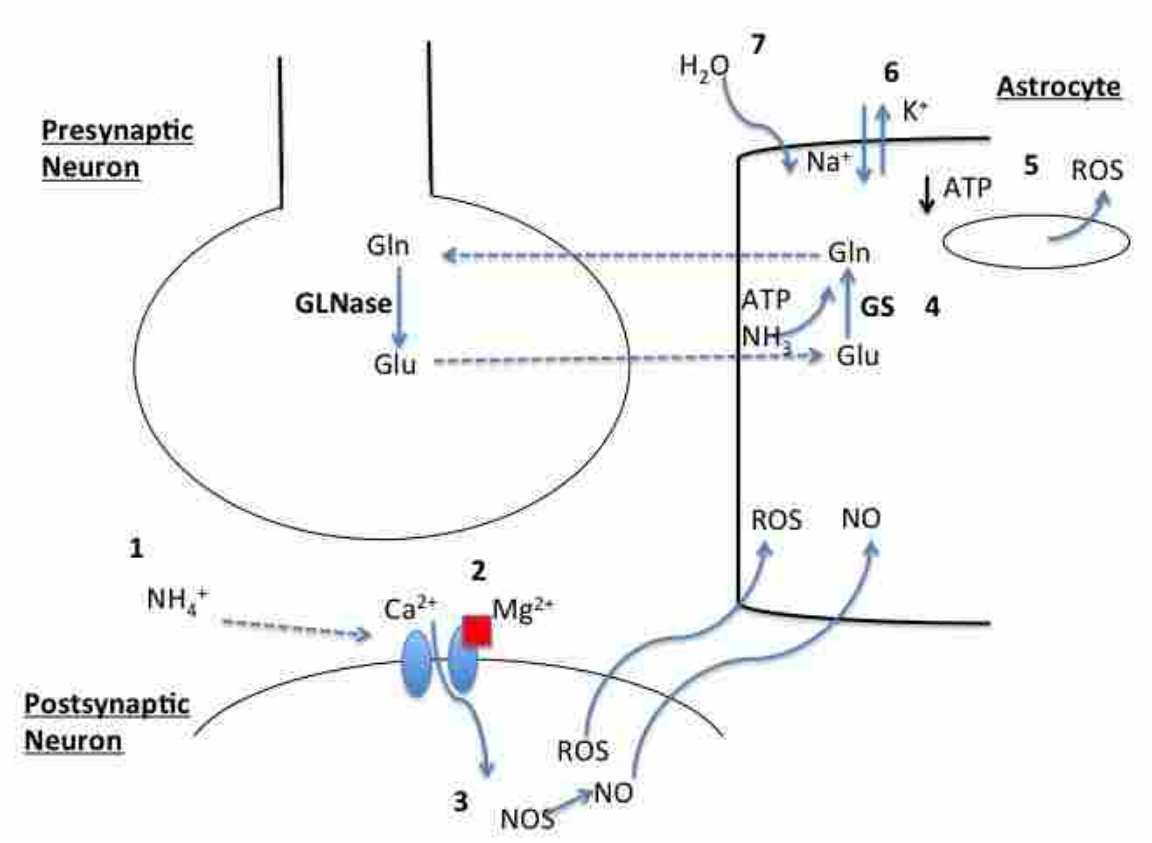
The free radical nitric oxide (NO) is also generated during hyperammonemia (Albrecht 1998). Nitric oxide synthase (NOS) activity has been shown to be elevated in experimental models of HE, and increased brain NO production was shown in portacaval shunted rats given ammonia infusions (Butterworth 2001). It would be very useful to use the *in vitro* and *in vivo* models of fish ammonia toxicity developed during my thesis to determine the relationship between ROS production and brain swelling in fishes. Whole fish ROS production can be measured indirectly by quantifying thiobarbituric acid reactive substances (TBARs) and conjugated dienes (Storey 1996; Lushchak *et al.* 2001), along with antioxidant enzymes such as such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (Kosenko *et al*. 1998; Lushchak *et al*. 2001).

Proposed Model for Ammonia Toxicity in Vertebrates

Based on the data generated in my thesis, and a survey of the literature on ammonia toxicity in vertebrates, I propose that free radicals likely contribute to astrocytic swelling during HEA exposure in fishes, and that ROS are generated due to the direct effects of ammonia on the NMDA receptors of neurons. According to this model, increased ammonia causes over-activation of NMDA receptors by initially leading to NH_4^+ permeation of the neuronal membranes, which causes depolarization of the cell. The depolarization leads to removal of the Mg^{2+} block from the NMDA receptor

calcium channel, which normally prevents premature activation of the receptor (Fan and Szerb, 1993). Glutamate that is present in the synaptic cleft then activates post-synaptic NMDA receptors, resulting in an excessive influx of calcium (Fan and Szerb 1993). Recent work on goldfish also suggests that ammonia may directly activate the NMDA receptor (Wilkie *et al*. 2011). The net effect would be over-activation of the NMDA receptor, and excess calcium entry and/or release into the intracellular space of the post-synaptic neuron, and the activation of intracellular enzymes including nitric oxide synthase (NOS), which produces the second messenger nitric oxide ($NO^{\cdot}$) and ROS (Kosenko 1994; 2003). NO and ROS then diffuse into the astrocytes causing more damage (Fig. 4.1). ROS are detrimental to DNA, proteins, lipids as well as plasma membrane. This can compromise cell integrity leading to cell swelling.

Figure 4.1: Schematic diagram of proposed mechanism of ammonia toxicity. 1) Increased ammonia causes over-activation of the NMDA receptor and depolarizes neurons. 2) Depolarization causes removal of Mg^{2+} block leading to an influx of calcium. 3) Calcium stimulates nitric oxide synthetase (NOS) which produces NO. ROS are also generated due to increase calcium. Both NO and ROS diffuse into astrocytes. 4) Within the astrocyte, glutamine synthetase (GS) converts glutamate (Glu) into glutamine (Gln), a reaction that requires ATP. This causes a decrease in astrocytic ATP. 5) An increase in intracellular ROS impairs mitochondrial ATP generation, leading to 6) ionic imbalances causing 7) astrocytic swelling.


Presynaptic Neuron
Astrocyte
H_2O
7
6
K^+
Na^+
5
ROS
ATP
Gln
Gln
GLNase
ATP
GS
4
NH_3
Glu
Glu
ROS
NO
1
2
NH_4^+
Ca^{2+}
Mg^{2+}
Postsynaptic Neuron
ROS
3
NOS
NO

The accumulation of ROS and NO within the astrocytes cause cell swelling. In conjunction with this, ATP within the astrocyte also gets depleted because the glutamine synthetase reaction is ATP dependent. This compounded decrease in ATP causes ionic imbalances, which leads to an increase in intracellular Na^+ and extracellular K^+. The resultant osmotic imbalance causes the astrocyte to swell further (Fig 4.1; Kosenko *et al*. 1994; Monfort *et al.* 2002).

Fishes as a Model to Study Brain Swelling

Fish could provide researchers with a simple model to study brain swelling because the fish brain is less complex. Traditionally, it was believed that only a single layer of meninges surrounds the fish brain but it has been reported that they have three layers: outer, intermediate and inner (Momose *et al*. 1988; Wang *et al.* 1995). The fish meninges are not as complex as the mammalian meninges, which consist of dura mater, archnaoid mater and pia mater layer (Nabeshima *et al*. 2004) that are comprised of multiple layers of connective tissue, which surround the brain and spinal cord (Moyes and Schulte, 2006).

The high density of water acts as a natural "shock-absorber" and provides buoyancy to support and cushion the brain within the cranium of the fish. As a result, the fish is naturally protected against "jarring" of the brain against the walls of the cranium, which can lead to brain trauma (Kardong 2001). The meninges that surround the brain of mammals provide additional padding that protects against "jarring" on land where air is less dense compared to water. Unlike the fishes,

however, this additional protection limits the capacity of the brain to swell (Nilsson 2001). A further complication is the relatively smaller size of the cranial cavity of terrestrial vertebrates versus fishes (Nilsson 2001), which leaves less room for any swelling to take place in the event of exposure to toxicants or trauma.

To conclude, ammonia exposure leads to reversible brain swelling in goldfish and trout. The underlying mechanisms are yet to be determined, but do not appear to be related to glutamine accumulation. Due to its greater capacity to swell compared to mammals, the fish could brain therefore provides an excellent model to study the physiological mechanisms of brain swelling in vertebrates. The goldfish model could provide researches with a system on which to study mechanisms involved in potentially fatal brain swelling caused by stroke, trauma or ammonia toxicity. Moreover, the reversible swelling of the brain observed in my thesis may be an important factor that allows these fishes to withstand a wide range of environmental stressors including exposure to xenobiotics, oxygen starvation and variations in environmental ammonia.

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APPENDICES

Appendix A – Formulation for the preparation of 1L of the L-15/ex (exposure medium)

Inorganic Salts	Supplier	Mass (g)
NaCl	Sigma S-5886	8.00
KCl	Sigma P-5405	0.400
$MgSO_4.7H_20$	Caledon 4860-1	0.200
$MgCl_2.6H_20$	BDH ACS 474	0.200
$CaCl_2.2H20$	BDH ACS 186	0.140
Na_2HPO_4 (anhydrous)	Sigma S-5136	0.190
KH_2PO_4 (anhydrous)	BDH ACS 657	0.060
Carbohydrate source		
D-Galactose	Sigma G5388	0.900
Sodium Pyruvate	Alfa Aesar 113-24-6	0.550

Appendix B – Water Ammonia Assay

Ammonia stock prepared (25mM) and fresh standards prepared

Std [NH4] (µM)	Vol. 25mM stock (µl)	Vol. Well water (µL)
0	0	10000
2.5	1	9999
5	2	9998
10	4	9996
25	10	9990
50	20	9980
75	30	9970
100	40	9960

Preparation of reagents used:

- Sodium Salicylate: Make up immediately before use. Caution: avoid inhaling or contact with skin. Sodium salicylate is stored in the dark at room temperature.

  Preparation: 10g salicylic acid/25ml e-pure
- Sodium Nitroprusside: Pre-prepared and stored in a light-tight bottle in the refrigerator. Caution: Poisonous

  Preparation: 0.2g sodium nitroprusside/500ml e-pure
- Alkaline Hypochlorite: This reagent is made of two components:

  (a) Alkaline sodium citrate which is prepared by dissolving 175g of sodium citrate in 500 ml of 1N NaOH

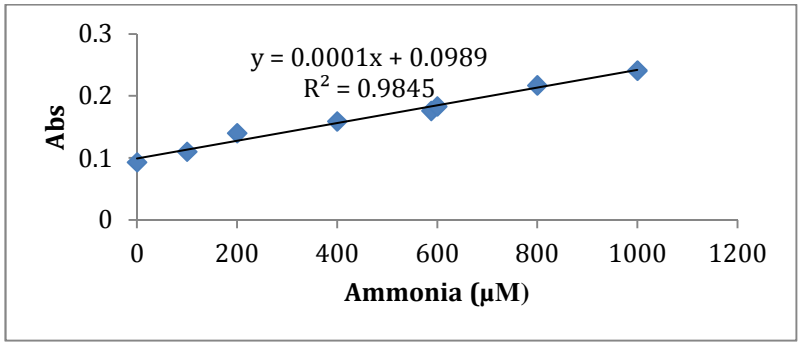
  Preparation: 1N NaOH – 20NaOH pellets in 500ml e-pure

  (b) Immediately before use, dilute a portion of alkaline sodium citrate solution with chlorine bleach in a ration of 1 part sodium citrate: 1 part bleach

Protocol:

1. In a cuvette put 100µL water sample
2. Add to this 25µL sodium salicylate then add 25µL sodium nitroprusside followed by 25µL alkaline hypochlorite
3. Incubate at room temp in dark for 90 min
4. Vortex and read at 650nm

Sample Standard Curve:



Appendix C – Plasma, Brain and Cell Ammonia Assay

Ammonia stock prepared (10mM) and fresh standards prepared

Std [NH4] (µM)	Vol. 10mM stock (µl)	Vol. Well water (µL)
0	0	10
50	5	10
100	10	10
200	20	10
400	40	10
600	60	10
800	80	10
1000	100	10

Reagents used:

Ammonia Reagent (20mL of e-pure in one vial)

Glutamine dehydrogenase (GLDH)

588uM Analytical Standard (AA0100, Sigma-Aldrich)

Protocol for Brains:

Homogenization Protocol

Prepare only those tissues you can assay in a single day, as once they are defrosted and homogenized, enzyme activity starts to fall off. Weigh frozen tissue/tube and add a volume in mls of ice-cold homogenization buffer equal to 4 times the weight of the tissue in grams. Since all the assays add up to about 310µl of homogenate, it is necessary to grind up minimum of about 100mg tissue.

Homogenization Buffer

Make up 500mls total. Since half of this is glycerol, you need to make up the components in a smaller volume of water first. In approximately 200mls distilled water make up these concentrations for a final total volume of 500mls.

	In 1 L	In 500mL
20mM K_2HPO_4	3.484g	1.74g
10mM HEPES	2.383g	1.1915g
0.5mM EDTA	0.1861g	0.9305g
1mM DTT*	0.15g	0.077g

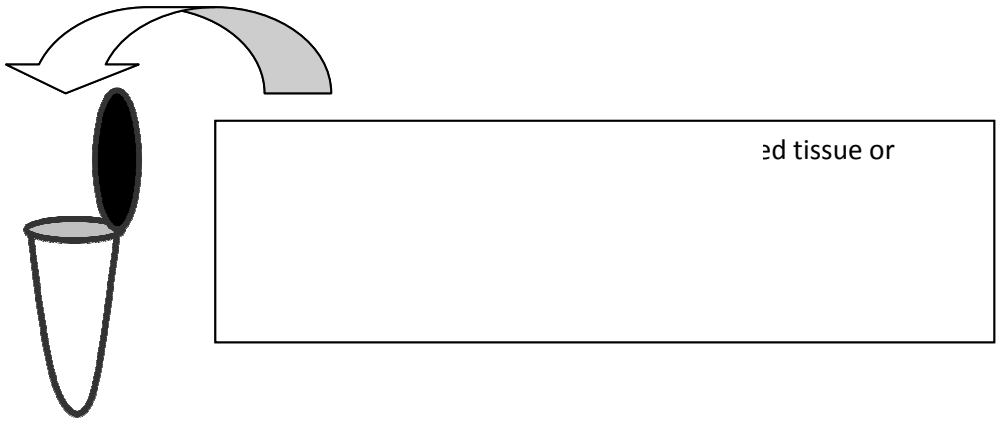
pH the above to 7.5 at room temperature
*DTT (dithiothreitol or "Cleland's reagent") ADD THIS FRESH DAILY

Bring volume to 250mls glycerol to get 50% glycerol and appropriate final concentrations of other reagents.

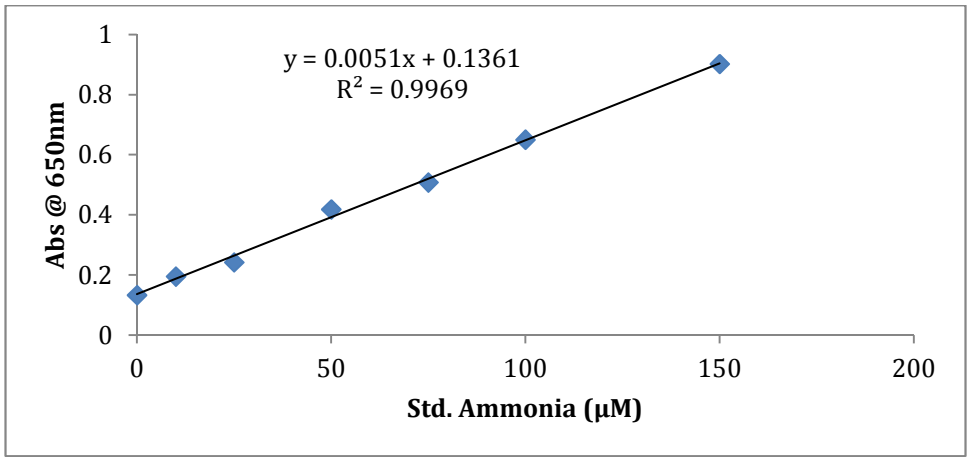
Grind away in the polytron or the sonicator keeping the sample on ice.

Centrifuge (13,000 rpm for 1 min) the small tubes in the microcentrifuge in the cold room. Centrifuge the larger tubes in the larger centrifuge for 20min at 4500rpms. Use the supernatant as indicated in the brain ammonia assay.

Protocol for Plasma:
Thaw plasma on ice and use in the assay



Sample Standard Curve:



Appendix D – Glutamine Synthetase Assay (GSase) for Tissue and Cells (Based on Shankar and Anderson 1985)

Glutamine Synthetase is an enzyme, which metabolizes nitrogen by catalyzing glutamate and ammonia to form glutamine

$$\text{Glutamate} + \text{ATP} + NH_3 \xrightarrow[\text{GS}]{} \text{Glutamine} + \text{ADP} + \text{Phosphate}$$

Tissue:

Before conducting the assay, the tissue of interest should be homogenized:

Homogenization Protocol
Prepare only those tissues you can assay in a single day, as once they are defrosted and homogenized, enzyme activity starts to fall off. Weigh frozen tissue/tube and add a volume in mls of ice-cold homogenization buffer equal to 4 times the weight of the tissue in grams. Since all the assays add up to about 310µl of homogenate, it is necessary to grind up minimum of about 100mg tissue.

Homogenization Buffer
Make up 500mls total. Since half of this is glycerol, you need to make up the components in a smaller volume of water first. In approximately 200mls distilled water make up these concentrations for a final total volume of 500mls.

	In 1 L	In 500mL
20mM K_2HPO_4	3.484g	1.74g
10mM HEPES	2.383g	1.1915g
0.5mM EDTA	0.1861g	0.9305g
1mM DTT*	0.15g	0.077g

pH the above to 7.5 at room temperature

*DTT (dithiothreitol or “Cleland’s reagent”) ADD THIS FRESH DAILY

Bring volume to 250mls glycerol to get 50% glycerol and appropriate final concentrations of other reagents.

Grind away in the polytron or the sonicator keeping the sample on ice.

Centrifuge (13,000 rpm for 1 min) the small tubes in the microcentrifuge in the cold room. Centrifuge the larger tubes in the larger centrifuge for 20min at 4500rpms. Use the supernatant as indicated in the glutamine synthetase assay.

Cells:

1. Remove exposure media (L15 ex) with plastic bulb pipette.
2. Add 100µl TypLE (Trypsin) to each well and wait for 5 minutes.
3. Observe cells under phase contrast microscope to look at cells, they should be lifting off from surface.
4. Use a pipette and pipette up and down to help cells detach from the wells. Add 200µl of L15 ex centrifuge at 1000 rpm for 5 minutes.
5. Carefully remove supernatant by using a pipette.
6. Re-suspend cells in 100µl of fresh L15 ex. Centrifuge and re-suspend again.
7. Transfer 100µl cell solution from each well to individual bullet tubes and add 200µl PCA to each bullet tube.
8. Vortex each bullet tube and freeze in liquid nitrogen
9. Thaw sample on ice when ready to conduct glutamine synthetase assay. Centrifuge sample at 1000 rpm for 5 minutes. Carefully take out 50 µl of the supernatant.
10. Vortex the rest of the sample and use for glutamine synthetase.

Glutamine Synthetase Assay:
Glutamine Synthetase Cocktail

	In 1 L	In 20mL
60mM glutamine	8.766g	0.17532g
15mM hydroxylamine	0.49545g	0.009909g
0.4mM ADP	0.17088g	0.0034176g
20mM KH_2AsO_4	3.6g	0.072g
50mM HEPES	11.915g	0.2383g
3mM $MnCl_2$	0.60993g	0.0121986g

Adjust pH to 6.7

Add 3 mM $MnCl_2$ 5min before assay. To yield 3mM $MnCl_2$ in cocktail, weigh it out in an eppendorf tube and pre-dissolve in a little bit of water. Initially adding this to the cocktail will form a cloud precipitation, which will more or less go away in about 5min. It does not need to be clear to use

Ferric Chloride reagent
Mix 1:1:1 ratio:

50% HCl: 24% Trichloroacetic acid: 10% FeCl3 in 0.2 N HCl (Both the mix and the stocks are stable at room temperature for months)

PROCEDURE:

Put 50µL of supernatant into each of two microcentrifuge tubes. One will be in a control (i.e., t=0), one will be incubation (i.e., t=10). To control add 300µL of Ferric Chloride reagent.

At the time zero, add 1ml of reaction cocktail to both controls and experimental. Cap and invert tube to mix. Incubate at room temperature for 60

minutes. At 10 min, add 300µL Ferric Chloride Reagent to experimental tubes. If you did this right, controls will be yellow, incubations will be brown.

Centrifuge tubes at 13,000 rpms in microcentrifuge for 1 min. Transfer liquid to plastic cuvettes. Record abs at 540 nm. (Abs should be measured at 10 min, 20min, 30min, and 60min to prevent inconsistencies).

Calculation

$$\frac{(\text{Abs-blank})}{0.3932^{*}/\text{L}} \quad \frac{(5)}{(0.05)\,(60)} = (\text{A-blank})\,(25.432) = \mu\text{mols/min/g}$$

*this is the slope for a standard curve of gamma glutamyl monohydroxamate in reagent soup. One does not need to repeatedly do this every day, but it is wise to check periodically.

Note: Volume can be scaled down to 0.2mL cocktail, 10µl supernatant and 60µl Ferric Chloride reagent if using microtiter plate reader to read samples. Proceed as above, but after centrifugation, remove 200µl to microtiter plate. The calculation changes to Delta A (47.096) to account or shorter path length in plates (0.54cm if using 200µL).

Schematic Diagram

Typical Absorbance for Brain Tissue:

Exposure Period	Control Abs	Experimental Abs	Delta
Control	0.065	0.98	0.915
Control	0.062	0.91	0.848
Control	0.061	0.9	0.839
24h NH4	0.068	0.97	0.902
24h NH4	0.07	0.99	0.92
24h NH4	0.071	1.03	0.959
76h NH4	0.066	1	0.934
76h NH4	0.062	0.98	0.918

Typical Absorbance for Cells:

Exposure	Control Abs	Experimental Abs	Delta
Control	0.03	0.05	0.02
	0.037	0.09	0.053
	0.044	0.06	0.016
	0.045	0.088	0.043
	0.042	0.082	0.04
	0.043	0.08	0.037
Ammonia	0.044	0.09	0.046
	0.049	0.088	0.039
	0.045	0.1	0.055
	0.042	0.12	0.078
	0.042	0.087	0.045
	0.047	0.09	0.043
MSO	0.043	0.096	0.053
	0.042	0.09	0.048
	0.046	0.091	0.045
	0.042	0.09	0.048
	0.047	0.08	0.033
	0.042	0.094	0.052

Appendix E – Glutamine Assay

For this assay use neutralized PCA extracts of the tissue

Glutamine Cocktail
Make up 20mL of cocktail
100mM Hydroxylamine NH_2OH
1.5mM ADP
20.0mM Arsenic Acid Na_2HAsO_3
Reconstitute with 20mL Hepes (50mM)
Adjust pH to 7.6

After making the glutamine cocktail, immediately before the assay add 5 units of glutamine synthetase. Then add $MnCl_2$ solution

$MnCl_2$ solution
Weight 12 mg $MnCl_2$ in a bullet tube first and then add few drops of cocktail and then

transfer to cocktail.

Ferric Chloride reagent
Mix 1:1:1 ratio:

50% HCl: 24% Trichloroacetic acid: 10% FeCl3 in 0.2 N HCl (Both the mix and the

stocks are stable at room temperature for months)

Glutamine Standards

Use 10mM glutamine stock (0.146g in 100mL 50mM Hepes)

Glutamine concentration	Volume 10mM	Volume Hepes
0	0	1
50	5	0.995
100	10	0.990
200	20	0.980
400	40	0.960
800	80	0.920
1600	160	0.840

Protocol

1. In a bullet tube add 50µl sample or standard
2. Then add 300µl cocktail (with glutamine synthetase and $MnCl_2$ added)
3. Incubate for 60 minutes
4. 100µl ferric chloride reagent added
5. Spin at 13000 rpm for 1 minutes
6. Pipette 200µl to each well of a 96 well plate
7. Read absorption at 540 nm

Sample Standard Curve: