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Review

Piscine insights into comparisons of anoxia tolerance, ammonia toxicity, stroke and hepatic encephalopathy☆

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Abstract

Although the number of fish species that have been studied for *both* hypoxia/anoxia tolerance and ammonia tolerance are few, there appears to be a correlation between the ability to survive these two insults. After establishing this correlation with examples from the literature, and after examining the role Peter Lutz played in catalyzing this convergent interest in two variables, this article explores potential mechanisms underpinning this correlation. We draw especially on the larger body of information for two human diseases with the same effected organ (brain), namely stroke and hepatic encephalopathy. While several dissimilarities exist between the responses of vertebrates to anoxia and hyperammonemia, one consistent observation in both conditions is an overactivation of NMDA receptors or *glutamate neurotoxicity*. We propose a glutamate excitotoxicity hypothesis to explain the correlation between ammonia and hypoxia resistance in fish. Furthermore, we suggest several experimental paths to test this hypothesis.

Keywords: Glutamate excitotoxicity; NMDA receptors; Ammonia; Glutamate neurotoxicity; Fish models; Anoxia; Hyperammonemia

Contents

☆ This paper derives from a presentation at a Memorial Symposium in honor of Dr. Peter Lutz held at Florida Atlantic University on September 23rd, 2005.

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1. Introduction

Researchers working with lower vertebrates, especially fishes, have an intuitive sense that some species are resistant to environmental perturbations or are "tough" (e.g., toadfish, carp, killifish, etc.) while others are more sensitive or are "delicate" (e.g., rainbow trout). This perception no doubt stems from comparing the natural environments in which these species live (e.g., "muddy waters" vs. flowing streams), their differential susceptibility to standard laboratory treatments (e.g., anesthesia and surgery), and their abilities to withstand, or not, multiple insults in a toxicological setting. However, in order for this perception to have a basis, there must be underlying commonality in mechanisms of resistance or sensitivity to multiple stressors. Yet, physiological and toxicological studies that directly evaluate more than one stressor in combination are still in the minority. This review attempts to compare the effects of two stressors in fish, hypoxia/anoxia and elevated ammonia (hyperammonemia, HA), first to see if in fact resistance to one reflects resistance to the other, and if so, to then explore potential common mechanisms. As background, we also focus on two important diseases in humans (and mammalian models) resulting from these particular stressors, namely, stroke and hepatic encephalopathy (HE), respectively. However, before addressing these issues, we wish to first discuss the role that Peter Lutz had on some of the authors in catalyzing this converging interest in hypoxia and hyperammonemia.

2. Peter Lutz and hypoxia: how we made the connection with hyperammonemia

In the early 1980's at about the time that one of us (PJW) joined the faculty at the Rosenstiel School of Marine and Atmospheric Science, Peter Lutz's laboratory was starting to exploit the turtle model of hypoxia resistance (for review, see Lutz and Milton, 2004), and it was not uncommon for his laboratory to have at least half a dozen to a dozen students, postdocs, and technicians, all of whom seemed to share Peter's incredible enthusiasm for science, discovery, and just plain having fun. The stark comparison to a junior faculty member with very little funding and no students was a bit distressing, since it seemed that all the "good" graduate students the division accepted wanted to work with Peter, the turtles, and his cool group (who could blame them!). However, seeing Peter's lab dynamic was a wake up call similar to one that many young Assistant Professors still go through: if you want to earn grants and tenure (and more importantly have lots of fun every day you come to work), it's time to find your own experimental system and niche, and emulate mentors like Peter. He was always expansive and generous in discussing science and life, and he always kept hypoxia simmering on the back burner of the collective mindset of the Walsh laboratory at least in part by involving me on the committees of his many students.

However, it was not until summer of 2004 when my laboratory started discussing hypoxia more actively and the confluence of events is instructive in terms of the many ways in which serendipity can work to push science forward and how even human tragedy can contribute. Just prior to the Fish Congress in Manaus, Brazil, I had received an invitation from Peter Lutz and Chris Wood to participate in a memorial symposium they were organizing to honor Bob Boutilier (another champion in hypoxia research) to take place the following summer in Barcelona; I thought it was a cruel joke. I certainly counted Bob as a close science friend and wanted to participate, but we had never directly collaborated, and there was the small matter of my lab not doing anything significant in hypoxia in 20 years. They insisted, and I started to think, well, we are doing some work on fish brains and ammonia, and the brain is a central target of hypoxia, so maybe I can bluff my way through a talk if I mention brains and fish enough. Once at the Fish Congress, things started crystallizing a little more in that Clemence Veauvy and I heard a superb talk by one of our co-authors on this current paper (LTB) on how some work that he and Mike Wilkie had done showed that down-regulation of Ca^{2+} fluxes through NMDA receptors/channels seemed to be one very important component in anoxia resistance in goldfish and turtle neurons (Buck et al., 2004; Shin et al., 2005).

By an unusual coincidence I (LTB) was attending the Brazil 2004 meeting to take part in a memorial symposium for the late Peter Hochachka when I discovered that Pat Walsh shared a common interest in ammonia toxicity, anoxia and NMDA receptors with MPW and me. About 18 years prior to this meeting I entered the Hochachka lab to work on metabolic downregulation. I adopted the freshwater turtle as a model and quickly learned that Peter Lutz was the anoxia-tolerant brain expert and I actively and nervously sought him out at a meeting in 1990 to ask for advice and discuss our common interests. It turned out I had nothing to be nervous about, Peter (L) enthusiastically discussed and shared knowledge on all aspects of anoxia-tolerance. Since then we have had a drink or two or a meal, and compared notes at many scientific meetings and I've even tried to entice his graduate students to the great white north. His work on ion channel arrest, neurotransmitters and neuromodulators in anoxia-tolerant turtles and fish had a huge impact on my experiments and thoughts on the problem of how a brain can survive without oxygen. In the fall of 2004 he served as an external examiner for my first PhD student; sadly however, he couldn't attend the thesis defense. I also knew Peter (L) was very supportive of my career during those critical moments when one is securing a research grant or an academic promotion. I am forever grateful to Peter (L) for all of those discussions and

camaraderie over the years and for laying the ground work for our present collaboration.

So, beginning in 2004, the authors started to make the connection between the hypoxia literature and the mammalian HE literature where NMDA receptors were one of two or three major targets of the hyperammonemia that caused HE. Maybe we had the germ of an idea, that perhaps there were common mechanisms underlying responses to hypoxia and hyperammonemia, and we have Peter Lutz to thank for catalyzing the theme of the current article. Sadly, of course, Peter passed before even the Boutilier Symposium took place. It seemed that in the space of less than three years we had lost three giants in hypoxia research (Hochachka, Boutilier and now Lutz); we write this article in tribute to yet another great.

3. Hypoxia and ammonia tolerance in fish: is there a connection?

To our knowledge, there are no direct comparisons in the same experiments of ammonia and hypoxia tolerance on any given species of fish, despite the high likelihood that fish experiencing hypoxia may also experience high ammonia levels (and vice versa). In fact, literature values for tolerance of these two variables are often in different "units". Methods for evaluating the effect of chemical toxic substances on aquatic life have been standardized for over 40 years (24, 96 h LC_{50} tests) but this is not the case for measurements of the impact of low dissolved oxygen on aquatic organisms. Until recently, many studies of ammonia toxicity in fish (for review, see Ip et al., 2001; Randall and Tsui, 2002) have been performed in the context of EPA water quality regulations, and are often expressed as 96-hour LC_{50} values (in either mg/L or mM); notably pH, and thus the portion of ammonia as NH_3, is a rather important corollary water condition that must be controlled and reported. Some studies of hypoxia in fish that have been concerned with establishing toxicological indices per se focused primarily on chronic tests for the most sensitive life stages of salmonids. Other studies only roughly quantified how long it takes for fish to lose equilibrium or die from hypoxia, but were more concerned with the oxygen concentration at which regulation of oxygen consumption rate begins to break down (the so-called P_{crit}, or partial pressure of oxygen where oxygen consumption begins to fall in oxy-regulators) (Doudoroff and Shumway, 1970; Davis, 1975; U.S. EPA., 1986). Despite this difference in what is an important index of tolerance for the two stressors, the relationship between susceptibility to ammonia and hypoxia in fish where the two (albeit limited) datasets are available is striking (Fig. 1). Apart from our intuitions regarding tough vs. delicate species, is this relationship a coincidence, or are there mechanisms that might explain it? Since there is less information on fish to explore this relationship, we turn to comparisons of the mechanisms of effect of low oxygen and high ammonia in two related human diseases.

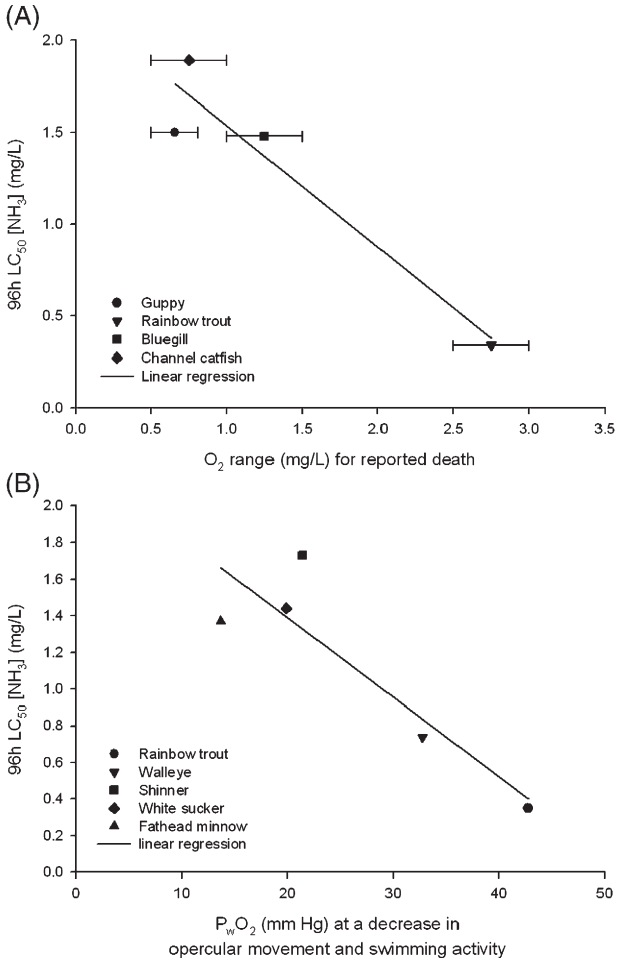

Fig. 1. Comparison of ammonia tolerance vs. hypoxia tolerance in several species of fish. (A) Median oxygen concentration (mg/L) for which death was reported. Values are taken from Weber and Kramer (1983) and Kramer and Mehegan (1981) for the guppy (*Poecilia reticulata*)(t=25 °C) and from Marvin and Heath (1968) for rainbow trout (*Oncorhyncus mykiss*) (t=12 °C), bluegill (*Lepomis macrochirus*) and channel catfish (*Ictalurus punctatus*) (t=25 °C). 96 h LC_{50} [NH_3] values are taken from the US EPA 1984 report (for rainbow trout, mean values only taken from 50–400 g fish). (B) Oxygen tension in the water (mm Hg) for which a decrease in opercular movement and in swimming activity was observed in a study by Gee et al. (1978). All fish were tested at t=16 °C at similar graded hypoxia conditions. 96 h LC_{50} [NH_3] values are taken from the US EPA 1984 report (for rainbow trout, values only including 50–400 g fish). For the shiner, "genus values" were considered since the species of shiner were different for the values of P_wO_2 (*Notropis cornutus, N. atherinoides and N. hudsonius*) and for the 96 h LC_{50} values (*Notropis crysoleucas, N. lutrensis, N. spliopterus and N. whipplei*).

4. Ischemia/stroke vs. anoxia tolerance

Compared to anoxia—ischemia is a very different insult to the brain. While no mammal survives either insult for more than a few minutes, ischemia is probably more of a stress for anoxia-tolerant species than anoxia. Ischemia not only results in a lack of oxygen to the brain but also a lack of nutrient supply (glucose) and waste removal (lactate and protons). Although we are not aware of any direct measurement of ischemia tolerance in turtle, goldfish or crucian carp brain, high brain glycogen stores, increased buffering capacities and the ability to acutely depress metabolic rate would logically extend ischemic survival time. Additionally, turtle neurons are known to survive oxygen and glucose deprivation (ischemia mimic) for periods much longer than rat neurons (P. Bickler personal communication).

Two recent excellent review volumes treating this topic from a comparative perspective are available (Lutz and Boutilier, 2004; Somero, 2004), and now other articles in the present symposium (Peréz-Pinzón, 2006-this issue; Milton and Prentice, 2006-this issue; Storey, 2006-this issue), such that we are only required to briefly summarize the mechanisms of anoxia susceptibility and tolerance. In anoxia susceptible animals (humans and most mammalian models), during either environmental oxygen limitation or restricted blood flow to the brain (ischemia), the toxic cascade at the neural cellular level includes the following events, more or less in sequence (for review, see Drew et al., 2004) (Fig. 2). 1) A switch from aerobic metabolism to anaerobic glycolysis decreases ATP yield, and given constant utilization, especially by ion motive ATPases, a decline occurs in the neuronal ATP content and subsequently in the ability of the ATPases to maintain ion gradients; this phase may also be associated with an increase in extracellular adenosine and small neuroprotective changes in intracellular calcium. 2) Neuronal loss of K^+ and gain of Na^+ and Ca^{2+} (i.e., collapse of ion gradients or depolarization) follows. 3) Which in presynaptic glutamatergic (excitatory) neurons leads to excess GLU release and stimulation of post-synaptic GLU receptors, including NMDA receptors (NMDAr) allowing additional Ca^{2+} to permeate the cell. 4) Additional release of intracellular Ca^{2+} stores from the ER and mitochondria further exacerbates the intracellular Ca^+ increase. 5) Reactive oxygen species (ROS) production and oxidative damage, which is especially pronounced on reperfusion, can exacerbate mitochondrial damage. 6) All contribute to loss of ion homeostasis, neuronal swelling, excitotoxicity, membrane blebbing and rupture, and eventual death of the neuron (Lutz et al., 2003).

By contrast, anoxia-tolerant organisms utilize several tools to combat the above events, and to short-circuit the negative cascade in the very early stages. It is now clear that ATP demands are reduced in some species by reducing ion movement across the plasma membrane (the so-called "ion channel arrest" mechanism; see below). ATP demands are further reduced by downregulating "non-essential" pathways such as protein synthesis (Fraser et al., 2001; Smith et al., 1996) and by a general decrease in tissue function, such as contraction by cardiac muscle (Stecyk et al., 2004) and reduced electrical activity in the brain (Lutz et al., 2003). Adenosine concentrations increase rapidly following the onset of anoxia and likely plays a key role in coordinating these responses, it has also been coined a "retaliatory molecule" (Nilsson and Lutz, 1992; Perez-Pinzon et al., 1993 rev. Buck, 2004). A common theme amongst animals that survive long-term anoxia is an overall reduction in metabolic rate such that ATP demand can be sustained by glycolytically-derived ATP generation alone.

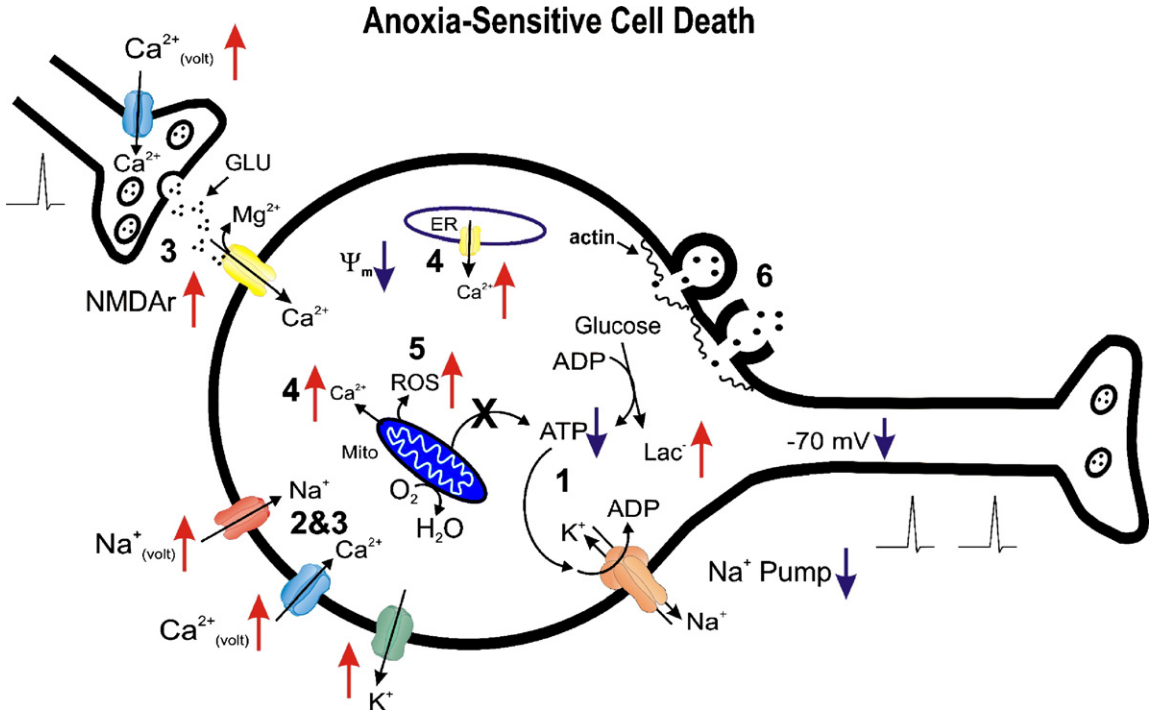

Fig. 2. Sequence of events leading to excitotoxic cell death (necrosis) in ischemic and/or anoxic neurons. Blue (dark) arrows represent decreases and red (light) arrows increases. 1) Depletion of oxygen leads to the loss of mitochondrial ATP production (X) and electrochemical gradient. Increased glycolytic ATP production alone cannot sustain ion motive pumps, such as the Na^+ pump; lactate levels increase, [ATP] and cellular pH decreases. 2) Na^+, K^+, and Ca^{2+} gradients cannot be maintained and leak down their respective concentration gradients resulting in a depolarized membrane potential (Ψm). 3) Voltage-gated Na^+ and Ca^{2+} channels open depolarizing the cell further and leading to vesicular release of neurotransmitters, such as glutamate. A depolarized Ψm also removes the Mg^{2+} blockade of the NMDA receptor making it more easily activated by increases in synaptic [glutamate] and permitting further increases in cellular [Ca^{2+}]. 4) Ca^{2+} is also released from the endoplasmic reticulum and mitochondrial matrix; although the timing is uncertain, this may represent a short term second messenger mediated protective process (Bickler et al., 2000). 5) Decreased O_2 levels, release of mitochondrial Ca^{2+} and uncoupling can promote reactive oxygen species (ROS) generation leading to lipid peroxidation, DNA and protein damage, although this is more likely during reperfusion and re-oxygenation. 6) Ultimately, cell swelling and reduced ATP levels cause membrane blebbing and rupture. Legend: Lac, lactate; GLU, glutamate; ER, endoplasmic reticulum; mito, mitochondrion, $Na^+_{(volt)}$ voltage gated and non-gated channels; Ψm, plasma membrane potential.

5. Hepatic encephalopathy

The human pathology hepatic encephalopathy can be characterized, at least superficially, by the simple phrase "when the liver fails, the brain ails" (M.D. Norenberg, University of Miami, personal communication): the human/vertebrate liver processes a wide range of toxic substances including ammonia, and when this organ fails (temporarily or permanently), at the very least plasma ammonia levels increase with ensuing pathological effect on the brain and other nervous tissue. However, the simplicity of this statement belies the incredible complexity of this disease due to several factors: 1) the manifold causes of liver dysfunction, including inborn genetic errors of the ornithine-urea cycle (O-UC), liver cirrhosis, alcoholism, viral hepatitis infection, acetominophen-induced acute liver failure, pre-hepatic "portal" hypertension (which shunts blood flow away from the liver), etc.; 2) the nature of the time course of ammonia buildup in the plasma (i.e., rapid vs. slow, acute vs. chronic); 3) the growing recognition that the sequence of pathological reactions may differ substantially depending on the particulars in 1) and 2); and 4) that parallel infection/inflammation can also alter the course of the disease and symptoms. There have been several comprehensive reviews of HE, and details on much of the following summary can be found in these reviews (e.g., Cooper and Plum, 1987; Hazell and Butterworth, 1999; Cooper, 2001; Butterworth, 2001; Brusilow, 2002; Jones et al., 2003). The condition of Hepatic Encephalopathy (HE) refers specifically to the neuropsychological deficits (e.g., sleep disorder, cognitive impairment, coma, etc.) that stem from the liver dysfunction. One feature of the disorder is that any given liver disease may or may not necessarily involve all the symptoms of HE, so the classification terminology of the stages of the disease in the literature can itself be a little complex and confusing. However, there are at least three consistent mechanistic features of the disease, and its replication in rodent models in which HA/HE can be elicited by various experimental means (e.g., bolus i.p. injection with ammonium salts, surgical shunting of blood away from liver, killing the liver cells with chemicals etc.), namely *cerebral swelling/edema, glutamate excitotoxicity*, and *reactive oxygen species (ROS) generation*.

5.1. Cerebral swelling

There are two different kinds of brain edema in HE: *astrocytic* (cellular) edema which is the actual swelling of astrocytes, the cells comprising the glia (not neurons), and *vascular* edema in which the breakdown of the blood brain barrier (BBB) and the endothelial layer contributes to a more generalized swelling of the neurons and astrocytes. Vascular edema is rather complex and its causes are still not well understood, but they appear to involve ammonia's impairment of transport mechanisms in common with water transport across other more familiar complex epithelia (e.g., kidney tubules, intestines, etc.). However, it is astrocytic edema where we really wish to focus because it is more easily understood in terms of direct ammonia effects, and ammonia probably has more direct and early effects on these cells than the BBB per se during the progression of the disease. In HE, there is a clear swelling of brain astrocytes (rather than neurons) such that the cells visually resemble Type II Alzheimer's astrocytes (where in histochemical sections, cells appear swollen with very characteristic nuclear appearance, Norenberg, 1977). This astrocytic edema appears to be a direct consequence of intra-astrocytic glutamine (GLN) accumulation. As part of the normal recycling of glutamate (GLU) neurotransmitter from the synaptic cleft, astrocytes specifically take up GLU where intra-astrocytic glutamine synthetase (GS) then converts GLU to GLN by addition of ammonia at the expense of ATP (Fig. 3). The GLN is then transported back to the presynaptic neuron where it is processed to GLU and repackaged into synaptic vesicles for synaptic release, completing the so-called GLU–GLN cycle. The exclusive localization of brain GS to astrocytes (Norenberg and Martinez-Hernandez, 1979) underscores their central role in this cycle. When circulating ammonia increases due to liver (or other) pathologies, or even if there is an ammonia surge following a meal, it easily crosses the BBB, and astrocytes then also become the primary intracranial means of ammonia detoxification (Cooper et al., 1979). Unfortunately, pathologically high ammonia concentrations disrupt the GLU–GLN cycle: most notably, astrocytic GLN levels rise (presumably because export processes cannot keep pace with ammonia supply and GS action) and simple osmotic cell swelling of astrocytes occurs. In an experimental sense, the importance of GS to this pathology has been clearly demonstrated by the ability of prior injection of GS inhibitors (e.g., methionine sulfoximine, MSO) to prevent this swelling and ensuing cognitive impairment in rodent models (Takahashi et al., 1991, 1992; Hirata et al., 1996, 1999; Sugimoto et al., 1997; Brusilow, 2002). Unfortunately, MSO has other toxic side effects precluding its clinical use in treating HE. However, the relatively simplified explanation above belies the complexity of the so-called glutamine hypothesis, and a more complete discussion can be found in Zwingmann and Butterworth (2005), including both pros and cons for this hypothesis.

5.2. Glutamate excitotoxicity

Despite the global *reductions* in brain GLU caused by the action of GS, there is also localized synaptic *excess* of GLU. In part, this excess is due to a direct inhibitory effect of ammonia on the astrocytic EAAT-1 (GLAST) and EAAT-2 (GLT-1) transporters (Norenberg et al., 1997a,b; Knecht et al., 1997; Chan et al., 2000). This local synaptic excess serves to overstimulate post-synaptic GLU receptors, most notably the NMDAr (Butterworth, 2001). It has also been proposed that ammonium ions have a more direct effect on the NMDAr, which potentiated NMDA-mediated currents possibly by removing the Mg^{2+} block from the NMDAr due to a general depolarization of neuronal membranes by ammonia (Fan and Szerb, 1993). Regardless of the mechanism, ammonia makes the NMDAr more susceptible to activation, leading to an increase in intracellular calcium and to neuronal death. Evidence for the importance of this pathway derives from NMDAr antagonists (e.g., MK-801) that provide significant

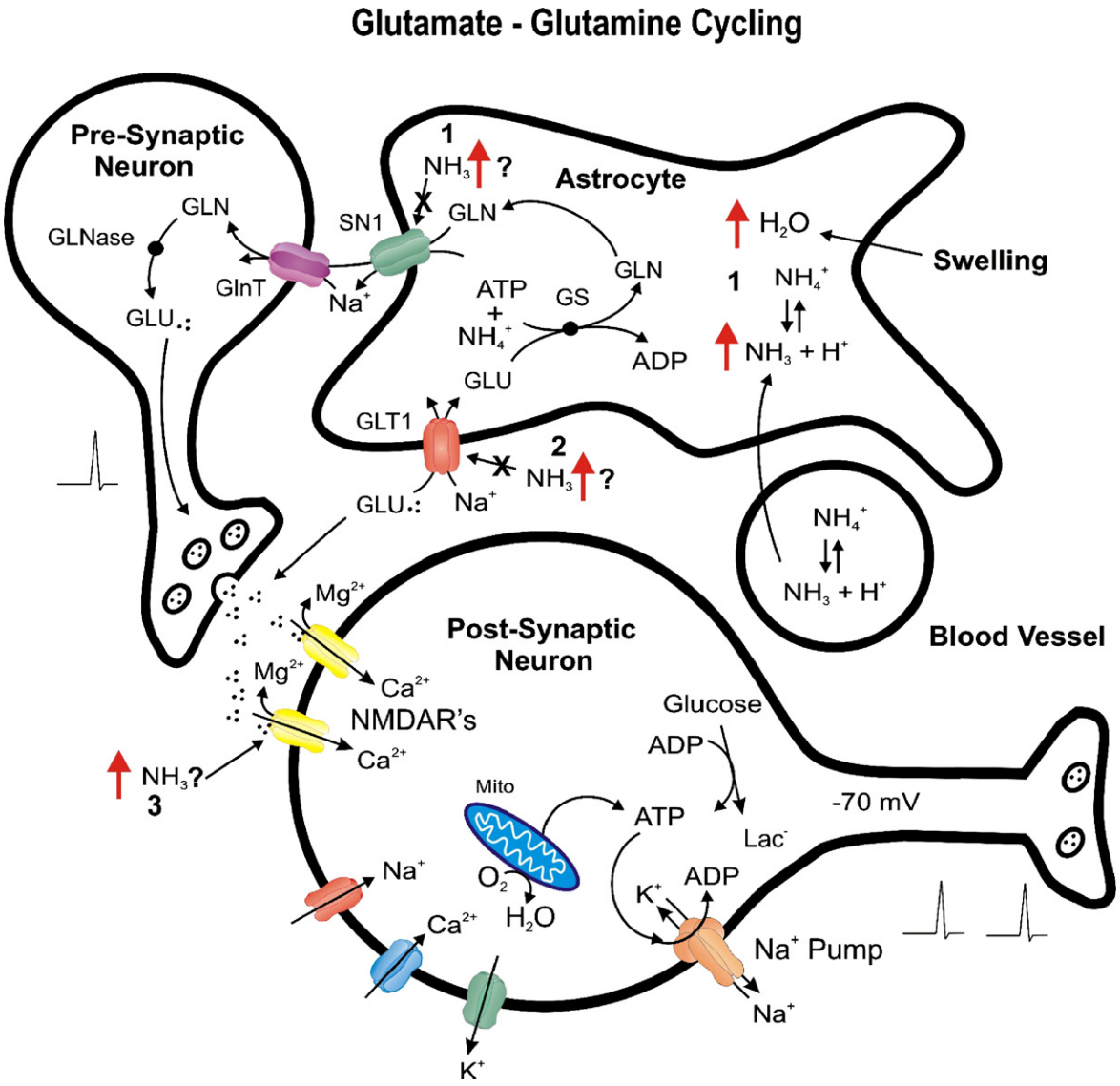

Fig. 3. Glutamine/Glutamate (GLN/GLU) cycle in neurons and astrocytes. Glutamate released into the synaptic cleft is taken up by Na^+ driven co-transport (GLT1) into astrocytes. Within the astrocyte GLU is enzymatically transformed to GLN by an ATP and NH_4^+ coupled reaction. GLN is transported by a glutamine/Na^+ co-transporter (SN1) to the extracellular space and taken up by neurons by another Na^+ coupled transporter (GlnT) and converted back to GLU by GLN synthetase and re-packaged into pre-synaptic vesicles. There is also a Na^+ independent glutamine transport pathway but it is not shown. Two major effects of ammonia toxicity are shown. 1) Astrocytic swelling from intracellular accumulation of GLN. This is the result of putative inhibition of GLN export from the cell and osmotic swelling. 2) Excess synaptic GLU is thought to be caused by NH_3 inhibition of astrocytic GLU uptake; the net effect is hyperstimulation of post-synaptic NMDAr. Acute NH_3 exposure may excite NMDAr directly. Legend: GS represents glutamine synthetase; GLNase, glutaminase; GLT1, glutamate transporter; GlnT, neuronal glutamine tranporter; SN1, astrocytic glutamine transporter.

protection from death when pre-injected into rodent models prior to what would normally be a lethal acute ammonia exposure (Hermenegildo et al., 1996).

5.3. Reactive oxygen species

ROS can be produced in several complex interactive ways in HE, but appear to mainly modulate the progression of the disease in the sense that they are either *downstream* of NMDAr effects, or are caused by processes apart from the direct effects of ammonia on astrocytes/neurons. For example, the excess intraneuronal calcium caused by glutamate excitotoxicity is now known to have a parallel in astrocytes where it has only recently been noted that NMDAr are present which can mediate similar effects as in neurons (see Schliess et al., 2002, 2003). Thus, increased calcium can likely cause increased ROS in both neurons and astrocytes by effects on superoxide metabolizing enzymes (Schliess et al., 2003). Inflammation/sepsis in organs other than the brain can also cause release to the general circulation of inflammatory cytokines that may have effects on the brain. Finally, the release of benzodiazepine-like substances has been recognized as a characteristic of HE for a number of years, although their source is unknown. These substances can interact with peripheral type benzodiazepine receptors (PTBR) in astrocytes and cause the formation of free radicals (Jayakuma et al., 2002). Free radicals and ammonia induce the mitochondrial

permeability transition pore (MPTP) in astrocytes, further exacerbating ROS production and cell swelling (Rama Rao et al., 2005). While an important modulator of toxic responses in HE, these effects generally appear to be less primary than the effects of astrocyte swelling and glutamate excitotoxicity.

6. Comparison of stroke and HE

In summary, susceptibilities to anoxia in stroke, and to ammonia in HE are compared in Table 1. Notably, the energy (ATP) deficit that is central to anoxia is not a major characteristic of HE; while there are direct effects of ammonia on energy generating enzymes, these are typically at concentrations far in excess of those known to cause HE (Cooper and Plum, 1987) and changes in brain ATP status follow, rather than precede, neurological impairment. Further, the astrocytic swelling that is a hallmark of HE is generally not seen in stroke. However, at least in hypoxia susceptible mammals, one key element of the two conditions that is shared in common is glutamate excitotoxicity. While these pathways are initiated in different ways in the two conditions (e.g. ammonia effects on astrocytic GLU transporters to increase synaptic [GLU] *vs.* hypoxia/energy-deficit induced depolarization of pre-synaptic cells to increase synaptic [GLU]), they share in common the overactivation of NMDAr. With this background in mind, we turn now to the more limited literature on hypoxia and ammonia effects in fish.

Table 1
Comparison of key characteristics of anoxia/stroke in hypoxia sensitive mammals to those of hepatic encephalopathy

	Stroke	HE
Energy (ATP) deficit	Yes	No
NMDAr activation	Yes	Yes
Reactive oxygen species	Yes	Yes
(but different sources and targets, and mainly upon reperfusion in anoxia/stroke)		
Astrocytic swelling	No	Yes
Neuronal swelling	Yes	No
Mitochondrial permeability		
Transition pore (MPTP)	Yes	Yes
(downstream of NMDAr effects)		

7. NMDAr-mediated mechanisms of anoxia toxicity and tolerance in fish

Aquatic oxygen levels can vary dramatically and oxygen limited environments are not uncommon; for example, tide pools have a wide diurnal variation in oxygen availability, ranging from hyperoxic during daylight hours to severely hypoxic at night (Dejours, 1981). Flood plains, such as the Amazon River basin, experience dramatic seasonal variations; high water and organic matter combine to greatly reduce dissolved oxygen levels. A similar phenomenon occurs in coastal marine waters and in open-ocean. Perhaps familiar to some is the severely hypoxic water created by winter ice in northern freshwater lakes. Low temperatures, reduced light penetration and impaired atmospheric gas exchange caused by ice cover kill off aquatic plant life while animal life consumes the remaining O_2, often leading to anoxic conditions that result in "winterkill" of susceptible fishes (Barica and Mathias, 1979). Neurons are generally regarded as the most anoxia-sensitive cells and are therefore the natural place to look for cellular adaptations permitting anoxic or hypoxic-survival. Indeed, hypoxia-tolerant neurons are found in every Order of vertebrates but most research has focused on a few species within these Orders (reptiles, fishes and amphibians). Neurons from turtles represent an extreme; these cells virtually switch off and the turtle brain becomes almost isoelectric throughout the anoxic winter dormancy. Anoxia-tolerant fishes such as the crucian carp and the goldfish differ with turtles in that they remain active in the water column and therefore their neurons must also remain active during anoxic periods (Nilsson, 2001). Other fishes, such as aestivating African lungfish, enter dormant, suspended animation states during dry periods, which are possibly associated with at least some degree of hypoxia (Johansen et al., 1976). It is unknown but likely that the lungfish has adapted a more turtle-like strategy.

Although there are numerous so-called hypoxia-tolerant fishes, most are unable to withstand anoxic conditions for more than a few minutes, or at most hours. Two notable exceptions are the crucian carp (*Carassius carassius*) and the goldfish (*Carassius auratus*), which along with the western painted turtle (*Chrysemys picta*), are the most anoxia-tolerant vertebrates known and capable of living without oxygen for weeks or months at the low temperatures characteristic of lakes, streams and ponds during winter months (see Lutz and Nilsson, 1997; Bickler et al., 2002 for reviews). A key to survival for each is their ability to conserve ATP through hypometabolism, which is achieved through the downregulation of energy turnover and/or the upregulation of energy efficient pathways (Hochachka and Lutz, 2001).

A common strategy of anoxia tolerance in the western painted turtle, crucian carp and goldfish is their reliance on large reservoirs of glycogen in the liver, which act as massive fuel depots for anaerobic glycolysis (fermentation). Indeed, the glycogen stores in the livers of the western painted turtle, goldfish and crucian carp are the largest reported in vertebrates (Hochachka and Somero, 2002). A potential complication of this strategy is the corresponding metabolic acidosis that accompanies anaerobic glycolysis and lactate production. In western painted turtle, acidosis is minimized by decreasing overall metabolic rate by more than 90% under anoxic conditions, rendering it virtually dormant (Jackson, 2002). The associated metabolic acid is buffered by the turtles' very high concentrations of extracellular HCO_3^- and by CO_3^{2-} found in the skeleton and shell of the animal (Jackson, 2002). In the anoxic goldfish metabolic rate is depressed by approximately 70% (Van Waversveld et al., 1989). Although minimum levels of activity are maintained in goldfish and crucian carp, their overall rates of activity are greatly reduced leading to further energy conservation (Nilsson et al., 1993). Unlike the turtle, however, self-pollution with H^+ is avoided by transferring anaerobically-derived lactate to the muscle where it is subsequently converted to pyruvate and then ethanol via alcohol dehydrogenase (Shoubridge and Hochachka, 1980). Due to its high diffusibility and water solubility ethanol is readily excreted across the gills,

preventing substantial blood ethanol accumulation (5 mmol L^{-1}) and impaired brain activity (Lutz et al., 2003).

Metabolic depression also occurs in the brain. Microcalorimetry experiments using brain slices taken from western painted turtle demonstrated that anoxia caused 40% decreases in metabolic rate (Doll et al., 1994), but it should be noted that metabolic budget calculations by Lutz's group suggested that ATP consumption likely decreased by more than 90% under anoxic conditions (Lutz et al., 1984). In anoxic carp brain slices, ATP consumption was reduced by approximately 30% (Johansson et al., 1995). One means of reducing ATP demands in the brain is to reduce "flux" through ion channels, or "channel arrest" (Hochachka, 1986), thereby decreasing the overall energy required to maintain transmembrane ion gradients. This can be achieved through "spike-arrest" in which neuronal excitability is reduced as characterized by a decreased tendency for neurons to evoke action potentials, or "ion-leak" arrest in which transmembrane ion movements in non-firing neurons is lowered. In western painted turtle, there is ample evidence of ion channel arrest. For instance, Chih et al. (1989) reported decreased K^+ leakage via membrane K^+ channels, while Peréz-Pinzón et al. (1992) reported a decrease in the density of voltage-gated Na^+ channels. Decreases in NMDA receptor activity and density have also been reported in anoxic turtles, which would not only contribute to metabolic depression but also protect against excitotoxicity (Bickler et al., 2000; Shin and Buck, 2003; Shin et al., 2005).

Ion channel arrest doesn't appear to be a major protective mechanism in anoxic crucian carp and goldfish. Lutz and Nilsson (1997) pointed out that unlike dormant turtles, these fishes remain relatively active in the anoxic water column (albeit at reduced rates) making channel arrest less likely. Indeed, changes in K^+ membrane permeability do not occur in anoxic crucian carp (Johansson and Nilsson, 1995). However, the electrical activity of the retina and optic tectum is depressed during anoxia in the crucian carp, possibly by enhanced GABA release, to the point that these animals are effectively blind (Johannson et al., 1997). We have preliminary data from goldfish suggesting that channel arrest of the NMDAr occurs in the goldfish in a manner similar to the western painted turtle. Using brain slices taken from the telencephalon, which is analogous to the mammalian hippocampus (Xu et al., 2003), we used whole-cell patch-clamping to monitor NMDAr activity over 40 min of anoxia. Exposure of the slices to this stressor caused 50–60% reductions in NMDAr receptor activity that were comparable to those observed in the western painted turtle (Wilkie and L.T. Buck, unpublished observations). These findings suggest that, unlike in mammals, western painted turtle and goldfish NMDAr's are regulated in a way that rapidly suppresses electrical activity in the brain during oxygen limitation. Perhaps such a mechanism also accounts for greater ammonia tolerance in the goldfish, and other ammonia tolerant fishes?

8. Ammonia exposure and tolerance in fish

If fish are similar to mammals in the contrasting characteristics of how they cope with anoxia and hyperammonemia, where astrocyte swelling is a major feature of the latter, then it is difficult to see how the relationship shown in Fig. 1 can be explained by common mechanisms. However, more in-depth mechanistic data on the abilities of fish to survive ammonia exposure are just beginning to emerge (for review of earlier literature, see Ip et al., 2001; Randall and Tsui, 2002; Tsui et al., 2004), and they appear to point to at least one key difference from mammals, namely in the effect of ammonia on brain swelling. Before we address brain swelling, we first wish to point out that the limited data available (two species) for effects of ammonia on cerebral energy metabolism show mixed results. A study on rainbow trout (Arillo et al., 1981) showed that brain ATP and NADH levels were disrupted when fish were exposed to ammonia concentrations near the LC_{50} value but weren't affected under conditions of sublethal exposure. However, exposure to sublethal concentrations of ammonia disrupted cerebral amino acid metabolism. A more recent toadfish study revealed that brain mitochondrial metabolism was also not severely disrupted by high concentrations of ammonia (up to 60% of the 96 h LC_{50}) (Veauvy et al., 2002). It will be interesting to determine if, as in mammals, disruption of brain energy metabolism per se is not a key explanation for toxicity of ammonia in fish.

The potential for brain swelling in fish has only been examined in one study of a species that is highly tolerant to ammonia exposure (the gulf toadfish, *Opsanus beta*) where 96 h LC_{50} values are 10 mM, (Wang and Walsh, 2000). Veauvy et al. (2005) recently demonstrated that despite substantial chronic exposure to sublethal concentrations of ammonia (1/3 of the 96 h LC_{50}) or acute exposure to lethal concentrations of ammonia (1 to 3 times the 96 h LC_{50}), toadfish showed no signs of brain swelling as assessed by water status using magnetic resonance imaging. (Notably, the methods employed were the same as those which could detect neuronal swelling during anoxia exposure in non-tolerant common carp (*Cyprinus carpio*); Van der Linden et al., 2001). Fish including toadfish certainly have high levels of brain glutamine synthetase, and ammonia exposure did lead to substantial increases in toadfish brain [GLN] (Veauvy et al., 2005). Also as expected, pre-injection of toadfish with MSO prevented this increase in brain [GLN]. However, unlike mammalian models, MSO did not change brain water status nor did it ameliorate symptoms and lethality from ammonia, but actually accelerated lethality, strongly suggesting that GS is a requirement for ammonia tolerance in the toadfish. Since MSO injection would inhibit GS in all toadfish tissues, our study could not conclude which tissue(s) was important to GS's detoxification of ammonia. We speculate that ammonia does not induce brain swelling in the toadfish model, for one of two reasons: (1) While brain GLN levels do rise, the *turnover* of GLN may be such that it can be rapidly exported from the brain to be processed or stored by other tissues. Since toadfish have high levels of a CPSase (Carbamoylphosphate synthetase) isoform that preferentially uses GLN (CPSase III, Anderson and Walsh, 1995), it is possible that they are pre-disposed to high systemic GLN turnover in general. Examination of brain GLN export transporters in (toad)fish could be expected to yield interesting results. (2) If the accumulation of GLN in toadfish brain is localized to a very small area or number of cells, global

brain swelling and its lethal effects may not occur. In this regard, one of the reasons why astrocytic swelling in mammals can be so disruptive when ammonia is elevated is that brains typically have a high proportion of astrocytes to neurons, approaching a ratio of 10:1 in some species. To date, data are scant in fish systems assessing the localization of brain GS, and the astrocyte to neuron ratio, but they suggest that GS may be localized to a thin layer of brain cells (the ependymoglial cells), and that generally astrocyte:neuron ratios in brains of lower vertebrates may not be as high as in mammals (Norenberg, 1983). Interestingly, two other studies have also demonstrated a lack of MSO effect in protecting fish from ammonia effects (Tsui, 2005; Ip et al., 2005), although pre-injection periods were less than 1 h such that full GS inhibition might not have been reached. For comparison, inhibition of toadfish GS in vivo by MSO requires 3 h for 25% inhibition and 16 h for a 80% inhibition (Veauvy et al., 2005). However, if further studies bear out the apparent lack of effect of MSO in protecting fish from ammonia insult, this would suggest a fundamental difference in GLN handling and brain swelling during ammonia toxicity in fish vs. mammals. (3) A third possibility for lack of brain swelling in fish is that the BBB may be structured differently, making fish less susceptible to the vascular edema mentioned above. While the general functional permeability of the BBB in fish is similar to mammals (i.e., it is "tight"), virtually no information is available on the structure of the BBB in fish as it relates to ammonia transport and metabolism (Cserr and Bundgaard, 1984). Clearly, all of the above are fertile areas for research in understanding the mechanisms of how fish like the toadfish can resist brain swelling, and perhaps discovering an explanation for why fish in general seem to be more tolerant of ammonia insults than mammals (Ip et al., 2001).

If as the limited dataset above suggests, ammonia-induced disruptions of cerebral energy metabolism and brain swelling are *not* key causes of toxicity in fish, does glutamate neurotoxicity play a role? Some evidence to date in fact suggests an involvement of NMDAr-mediated events. Using ammonium acetate i.p. injection at 21 mmol·kg^{-1}, Tsui (2005) discovered that this concentration killed 60% of test individuals of the oriental weather loach (*Misgurnus anguillicaudatus*). However, pre-treatment of fish with 2 mg kg^{-1} of MK-801, an NMDAr blocker, prevented all mortalities. We have found similar results with the plainfin midshipman (*Porichtys notatus*, a non-ureotelic toadfish relative) using a different end-point and exposure method. When fish were exposed to a waterborne 10 mM ammonium chloride concentration, MK-801 pre-injection (2 mg kg^{-1}) caused a delay in the time to unconsciousness (C. Veauvy and P.J. Walsh, unpublished results).

At least in the goldfish, ammonia appears to directly affect NMDAr function. Using the same goldfish brain slice model used to study anoxia tolerance (above), whole-cell patch-clamping was used to directly measure NMDAr currents in the presence of an ammonia (CH_3COONH_4) concentration of 10.0 mmol L^{-1}. Ammonia caused a reversible potentiation of the NMDAr currents (Fig. 4, M. Pamenter, M.P. Wilkie, and L.T. Buck, unpublished observations), as predicted based on mammalian models of ammonia toxicity. However, it was also notable that the

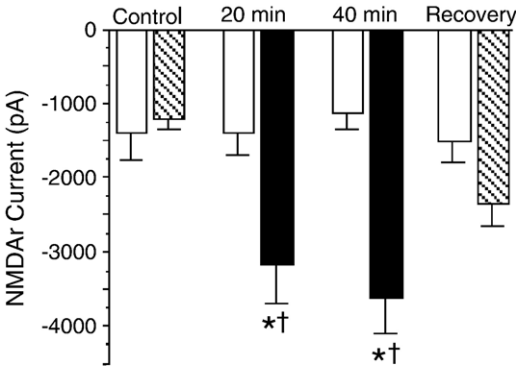

Fig. 4. A summary of NMDAr current data measured using whole cell patch-clamping of 300–400 μm brain slices, taken from the goldfish telencephalon, during a normoxic (99.5% O_2/0.5% CO_2, open bars) 40 min exposure to 10 mmol L^{-1} ammonium acetate (CH_3COONH_4; shaded bars) or sodium acetate controls (CH_3COONa, open bars). Hatched bars represent currents measured in experimental slices prior to and/or following exposure to high ammonia. Methods were similar to those described by Shin and Buck (2003) apart from the composition of the bath saline, which was (in mmol L^{-1}): NaCl 125, KCl 2.0, NaH_2PO_4 2.0, $NaHCO_3$ 20, glucose 20, imidazole 5.0, $MgCl_2$ 1.0, $CaCl_2$ 2.5, pH 7.6. Data are the mean and SEM during the pre-exposure (N=11), ammonia exposure (N=10–11) or post-exposure (n=10) periods. Asterisks indicate experimental (high ammonia) values that are significantly different from control data, while daggers represent values that are significantly different from pre-exposure measurements.

resting membrane potential (E_{rest}) was unaltered by ammonia, at approximately −70 mV for the duration of the 40 min exposures. This finding differs from observations by Fan and Szerb (1993) who reported that ammonia depolarized E_{rest} by approximately 15 mV in rat brain CA1 pyramidal neurons in hippocampal slices using intracellular recording techniques, which they argued removed the intracellular Mg^{2+} block of the NMDAr. It is well established that the underlying mechanism of NMDAr activation involves the electrophoretic displacement of Mg^{2+} from the receptor's channel by AMPA mediated excitatory post-synaptic potentials and depolarization. (see Wenthold et al., 2003 for review). The absence of altered E_{rest} in goldfish could therefore suggest that the vertebrate NMDAr is directly stimulated by NH_4^+, not indirectly as proposed by Fan and Szerb (1993). Alternatively, perhaps goldfish neurons are resistant to the possible depolarizing effects of NH_4^+, which could be an adaptation contributing to the unusually high tolerance of the goldfish to ammonia. Indeed, we recently reported that the 96 h LC_{50} for NH_3 was approximately 290 μmol L^{-1}, one of the highest values ever reported for an exclusively water breathing, non-ureogenic teleost (Etches et al., 2005). This tolerance is underscored by the comparable survival times of the goldfish and the extremely ammonia tolerant mudskipppers following IP injections of 20 μmol g^{-1} ammonium acetate (Ip et al., 2005). These studies also implicated the NMDAr in the toxic response of the goldfish to ammonia, by demonstrating increased survival times at these ammonia concentrations following the blockage of the NMDAr antagonist MK801 (Ip et al., 2005).

However, it is clear that all fish may not be affected by ammonia exposure through involvement of NMDAr-mediated pathways. Ip et al. (2005) have also recently shown that pre-injection of two species of mudskippers with MK-801 had no

effect on ammonia tolerance. It is not clear to us how these species might be incorporated into comparisons like the one we have made in Fig. 1, since these are both air-breathing fish. Nonetheless, the results of Ip et al. (2005) and a related review article (Ip et al., 2004) point out that there are many biochemical strategies by which fish species can process ammonia and adapt to its toxicity. It is possible that NDMAr-mediated responses to ammonia are common in only some fish species and mammals.

9. Conclusions and prospects for future research

This review has shown several similarities and dissimilarities between the stressors of low oxygen and high ammonia in vertebrates. While it is clear that the mammalian diseases of stroke and hepatic encephalopathy are rather complex, there is at least one pathway that is shared between them, namely glutamate excitotoxicity and excess stimulation of NMDA receptors. While the commonality of mechanisms of the two diseases is not extensive, it is large enough that scientists in these two areas of research would be well served by continuing to examine the findings in these complementary fields.

What is also clear from our review is that the mechanisms of ammonia toxicity in fish are probably less complicated than in mammals. It appears that while brain GLN production is an important facet of survival, brain swelling may not take place to the same extent as in mammals; GS inhibitors like MSO when applied to fish do not appear to extend survival during ammonia toxicity as in mammals, and in some cases appear to exacerbate ammonia's effects. However, it also appears as if NMDAr-mediated effects of ammonia are common in many (but perhaps not all) fish species, and that in these simpler systems where brain swelling might not be an issue in ammonia toxicity, NMDAr effects might be at the core of the toxic response to ammonia. Since NMDAr-mediated effects are so central to toxic anoxia responses in all animals (once the energy supply has been disrupted), we propose that the common aspects of resistance to high ammonia and low oxygen as seen in Fig. 1 relate to differential susceptibility of NMDAr's in these different species. This "glutamate excitotoxicity" hypothesis might manifest itself (and be subject to experimental testing) in several ways such that we predict that more ammonia/anoxia-tolerant species may have: (1) lower densities of GLU containing synaptic vesicles, or lower quantities of GLU released per action potential; (2) lower densities of NMDAr on post-synaptic membranes; (3) higher thresholds for activation of NMDAr; (4) shorter channel open times (and therefore lower Ca ion fluxes).

The $26{,}000^{+}$ described species of fish present us with a diversity of habitats relative to low oxygen and high ammonia tolerance. We encourage researchers to examine these dual tolerances to determine if the early trends shown in Fig. 1 are valid, and to discover additional model species to understand human diseases like stroke and HE.

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