The Journal of Experimental Biology 214, 4107-4120

doi:10.1242/jeb.057513

RESEARCH ARTICLE

The relationship between NMDA receptor function and the high ammonia tolerance of anoxia-tolerant goldfish

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Accepted 30 August 2011

SUMMARY

Acute ammonia toxicity in vertebrates is thought to be characterized by a cascade of deleterious events resembling those associated with anoxic/ischemic injury in the central nervous system. A key event is the over-stimulation of neuronal *N*-methyl-D-aspartate (NMDA) receptors, which leads to excitotoxic cell death. The similarity between the responses to acute ammonia toxicity and anoxia suggests that anoxia-tolerant animals such as the goldfish (*Carassius auratus* Linnaeus) may also be ammonia tolerant. To test this hypothesis, the responses of goldfish were compared with those of the anoxia-sensitive rainbow trout (*Oncorhynchus mykiss* Walbaum) during exposure to high external ammonia (HEA). Acute toxicity tests revealed that goldfish are ammonia tolerant, with 96 h median lethal concentration (LC_{50}) values of 199 μmol l^{-1} and 4132 μmol l^{-1} for NH_3 and total ammonia ($[T_{Amm}]=[NH_3]+[NH_4^+]$), respectively. These values were ~5–6 times greater than corresponding NH_3 and T_{Amm} LC_{50} values measured in rainbow trout. Further, the goldfish readily coped with chronic exposure to NH_4Cl (3–5 mmol l^{-1}) for 5 days, despite 6-fold increases in plasma [T_{Amm}] to ~1300 μmol l^{-1} and 3-fold increases in brain [T_{Amm}] to 6700 μmol l^{-1}. Muscle [T_{Amm}] increased by almost 8-fold from ~900 μmol kg^{-1} wet mass (WM) to greater than 7000 μmol kg^{-1} WM by 48 h, and stabilized. Although urea excretion rates (J_{Urea}) increased by 2–3-fold during HEA, the increases were insufficient to offset the inhibition of ammonia excretion that occurred, and increases in urea were not observed in the brain or muscle. There was a marked increase in brain glutamine concentration at HEA, from ~3000 μmol kg^{-1} WM to 15,000 μmol kg^{-1} WM after 48 h, which is consistent with the hypothesis that glutamine production is associated with ammonia detoxification. Injection of the NMDA receptor antagonists MK801 (0.5–8 mg kg^{-1}) or ethanol (1–8 mg kg^{-1}) increased trout survival time by 1.5–2.0-fold during exposure to 2 mmol l^{-1} ammonia, suggesting that excitotoxic cell death contributes to ammonia toxicity in this species. In contrast, similar doses of MK801 or ethanol had no effect on ammonia-challenged (8–9.5 mmol l^{-1} T_{Amm}) goldfish survival times, suggesting that greater resistance to excitotoxic cell death contributes to the high ammonia-tolerance of the goldfish. Whole-cell recordings measured in isolated brain slices of goldfish telencephalon during *in vitro* exposure to 5 mmol l^{-1} or 10 mmol l^{-1} T_{Amm} reversibly potentiated NMDA receptor currents. This observation suggested that goldfish neurons may not be completely resistant to ammonia-induced excitotoxicity. Subsequent western blot and densitometric analyses revealed that NMDA receptor NR1 subunit abundance was 40–60% lower in goldfish exposed to 3–5 mmol l^{-1} T_{Amm} for 5 days, which was followed by a restoration of NR1 subunit abundance after 3 days recovery in ammonia-free water. We conclude that the goldfish brain may be protected from excitotoxicity by downregulating the abundance of functional NMDA receptors during periods when it experiences increased internal ammonia.

Key words: ammonia toxicity, excitotoxicity, glutamate NR1 receptor, glutamine, methionine sulfoximine, MK801, urea.

INTRODUCTION

Stress and routine activities such as feeding or vigorous swimming can lead to increased blood and tissue ammonia concentrations in fish (Ortega et al., 2005; Wicks and Randall, 2002; Wang et al., 1994b), in addition to exposure to high ambient concentrations of ammonia arising from anthropogenic or natural sources (Randall and Tsui, 2002). Typical ammonia concentrations are usually less than 10 μmol l^{-1} in natural freshwaters (Environment Canada and Health Canada, 2001), but concentrations may approach 0.5 mmol l^{-1} or more in highly eutrophic waters or waters receiving ammonia-contaminated municipal, industrial or agricultural effluents (Environment Canada and Health Canada, 2001; Eddy, 2005). Increased internal ammonia, or hyperammonemia, can also result from the degradation of food or crowding in aquaculture facilities. Although fish are more tolerant to ammonia than mammals, their susceptibility to ammonia's neurotoxic effects varies widely among different species (Ip et al., 2001; Randall and Tsui, 2002; Eddy, 2005). In mammals, toxic increases in ammonia, often caused by acute liver failure or in-born errors of urea metabolism, lead to seizures, coma and eventually death (Felipo and Butterworth, 2002). Fishes exhibit similar symptoms to acutely toxic levels of ammonia including hyperventilation and hyper-excitability, followed by convulsions, coma and death (Eddy, 2005).

The injurious cascade of neurophysiological responses that characterize ammonia toxicity are similar in many respects to those associated with anoxic/ischemic injury. It is well known that anoxia

results in profound energy (ATP) deficits in the brain (Lutz et al., 2003; Bickler and Buck, 2007), but similar ATP deficits have been reported in mammals (Kosenko et al., 1994) and fishes experiencing hyperammonemia (Arillo et al., 1981; Schenone et al., 1982). During anoxia, the lack of ATP results in a run-down of transmembrane ion gradients due to insufficient ATP delivery to ion pumps such as the Na^+/K^+-ATPase. This run-down results in the excess release of the excitatory neurotransmitter, glutamate, leading to the over-activation of glutamate receptors (excitotoxicity), particularly *N*-methyl-D-aspartate (NMDA) receptors, in the brain (Bickler et al., 2000; Lutz et al., 2003). The subsequent accumulation of Ca^{2+} in post-synaptic neurons is then thought to cause the activation of lipases and proteases that compromise cell membrane integrity and ionic homeostasis, which leads to neuronal swelling and necrosis (Bickler et al., 2000). The generation of reactive oxygen species (ROS) following anoxic/ischemic episodes (Traystman et al., 1991; Luschak et al., 2001) exacerbates this loss of central nervous system (CNS) integrity by damaging membrane proteins and DNA (Storey, 1996).

Ammonia intoxication also results in the over-activation of NMDA receptors (Marcaida et al., 1992; Fan and Szerb, 1993), and the generation of ROS (Kosenko et al., 2003a). Unlike anoxia, however, ammonia-induced NMDA receptor over-activation does not appear (at least initially) to involve excess glutamate accumulation in the CNS (Hermenegildo et al., 2000). Nor does ammonia lead to neuronal swelling, but instead it causes astrocyte swelling (Brusilow, 2002; Albrecht and Norenberg, 2006). The proximate cause of astrocyte swelling is unresolved but some researchers have suggested that the glutamine synthetase (GS)-catalyzed conversion of ammonia plus glutamate to glutamine within astrocytes causes swelling by increasing the cytosolic osmolarity of the cells (Brusilow, 2002). However, glutamine synthesis is normally thought to protect against ammonia toxicity by converting ammonia to less toxic glutamine within astrocytes (e.g. Kosenko et al., 2003b; Veauvy et al., 2005).

While there are differences, the similarities between the mechanisms of acute ammonia toxicity and those associated with anoxia/ischemia suggest that anoxia-tolerant animals might also be ammonia tolerant (Walsh et al., 2007). The goldfish (*Carassius auratus* Linnaeus) and the closely related crucian carp (*Carassius carassius*) are two of the most anoxia-tolerant vertebrates known (for reviews, see Nilsson, 2001; Bickler and Buck, 2007). Their adaptations to anoxia include the ability to: conserve ATP by suppressing metabolic demand (Nilsson et al., 1993; Johansson et al., 1995); store massive amounts of glycogen to generate ATP *via* anaerobic glycolysis (Nilsson, 1990; Vornanen and Paajanen, 2006); convert lactate arising from glycolysis into ethanol to prevent metabolic acidosis (Shoubridge and Hochochka, 1980; Johnston and Bernard, 1983); and an ability to resist brain swelling (Van der Linden et al., 2001). The goldfish also has high constituent levels of antioxidant enzymes, which offset post-anoxia ROS production [reperfusion injury (Luschak et al., 2001)]. More recently, goldfish were shown to reduce NMDA receptor currents in response to anoxia, which may increase their resistance to excitotoxicity (Wilkie et al., 2008).

The present study tested the hypothesis that goldfish are ammonia tolerant using *in vivo*, pharmacological, neurophysiological and immunoblotting approaches to characterize the integrated responses of this anoxia-tolerant fish to ammonia. Experiments included acute ammonia toxicity tests to compare the ammonia tolerance of the goldfish with ammonia-sensitive rainbow trout [*Oncorhynchus mykiss* Walbaum (USEPA, 1999)]. Goldfish were also exposed to sub-lethal ammonia for 5 days to characterize how internal ammonia was handled, stored and detoxified. Pharmacological interventions known to inhibit NMDA receptor and GS activity were used to determine the relative importance that excitotoxicity and glutamine played in the responses of goldfish and trout to ammonia. The hypothesis that a protective strategy against ammonia toxicity was to decrease neuronal excitability through a downregulation of NMDA receptor activity was tested using whole-cell patch clamp recordings of NMDA receptor activity in isolated brain slices (cf. Wilkie et al., 2008). Western blot studies were then undertaken to determine if decreases in the abundance of functional NMDA receptors provided protection against excitotoxicity during exposure to high external ammonia.

MATERIALS AND METHODS

Experimental animals and holding

Juvenile goldfish (2–60 g) and rainbow trout (5–10 g each) were purchased from commercial suppliers (goldfish: Aleong International, Mississauga, ON, Canada; rainbow trout: Rainbow Springs Trout Hatchery, Thamesford, ON, Canada). Fish were held in flowing (~1–2 l min^{-1}), aerated (dissolved oxygen >90% saturation) well water [composition (in mmol l^{-1}) Na^+ ~0.8; Cl^- ~0.5; Ca^{2+} ~3; pH 8.0; temperature 12±1°C] in 110 liter tanks at Wilfrid Laurier University, ON, Canada. A separate set of goldfish (common comets; 50–100 g), purchased from the same supplier, were held in flowing dechlorinated tap water at the University of Toronto, ON, Canada (temperature 15–20°C). Fish were fed three times weekly with appropriately sized commercial pellets, but starved one week before experiments to minimize the effects that nitrogenous waste metabolism could have on their response to elevated environmental ammonia. Holding conditions and all experiments were approved by the Animal Care Committees of Wilfrid Laurier University and the University of Toronto, and followed Canadian Council of Animal Care guidelines.

Experimental protocols

Series 1 – acute toxicity of ammonia to goldfish and rainbow trout

To determine the acute toxicity of external ammonia to goldfish and rainbow trout, fish (5–10 g) were placed in darkened 10 liter buckets in groups of 5 (goldfish; N=10 buckets) or 7 (trout; N=14 buckets) containing aerated well water the day preceding acute toxicity determinations. The acute toxicity of total ammonia (T_{Amm}; sum of NH_3+NH_4^+) and un-ionized ammonia (NH_3) to rainbow trout was then determined by exposing the fish to nominal total ammonia concentrations of 0 (control), 0.5, 1, 2, 4, 8 and 16 mmol l^{-1}. Preliminary experiments suggested that goldfish were ammonia tolerant, with a 96 h half-maximal lethal concentration (LC_{50}) severalfold higher than rainbow trout. Accordingly, the 96 h LC_{50} for total ammonia and NH_3 was determined over a relatively narrow concentration range of 1.5, 3.0, 4.5 and 7 mmol l^{-1}. All exposures were done in duplicate (N=10–14 fish per nominal concentration tested), and for all experiments, control fish not exposed to ammonia were held under identical conditions. For each determination, survival of the trout or goldfish was monitored at 1, 2, 4, 6, 12, 24, 36, 48, 60, 72, 84 and 96 h. The survival data was then used to calculate the 96 h LC_{50} for T_{Amm} and NH_3 for each species using probit analysis (Probit Program, version 1.5, United States Environmental Protection Agency, Cincinnatti, OH, USA) and the measured ammonia concentration in each bucket. Because goldfish and trout inhabit freshwater, sensitivity to ammonia was expressed using NH_3, rather than NH_4^+ because ammonia mainly enters the animal as NH_3, where it is trapped as NH_4^+ inside the animal (e.g. USEPA, 1999; Wilkie, 2002).

Series 2 – effects of elevated external ammonia on blood and tissue nitrogen stores and nitrogenous waste excretion in the goldfish

Goldfish were exposed to a nominal external total ammonia concentration of 5 mmol l^{-1}, and changes in internal ammonia concentrations (plasma, brain, muscle) were measured in tissue samples collected terminally after 4 h or 1 day, 3 days or 5 days of exposure. To test the hypothesis that the fish were able to cope with high external ammonia (HEA) by switching to urea production, simultaneous measurements of urea excretion rate (J_{Urea}) were made on water samples collected during 4 h flux periods conducted under control conditions and at regular intervals during HEA (4, 24, 48, 72, 96, 120 h). Ammonia flux rates (J_{Amm}) were also determined to track rates of ammonia excretion and uptake during the HEA using the same water samples.

Ammonia exposures were conducted in a pH-controlled 70 liter re-circulating system. It was necessary to control pH to ensure that the speciation of NH_4^+ and NH_3 remained constant. The system comprised a TTT80 Autotitrator (Radiometer, Copenhagen, Denmark) connected to a PHM82 pH/mV meter (Radiometer). The pH was continuously monitored in a 40 liter head tank using a GK2401C pH electrode (Radiometer) connected to the pH meter. When pH exceeded a set point of pH 8, the autotitrator opened a solenoid valve allowing 1 mol l^{-1} HCl to enter the head tank in a drop-wise fashion. The water drained from the head tank into a wet table containing fish in individual rectangular, holding containers, ranging in volume from 0.5 liters to 2.5 liters, depending upon the size of the fish. Water was returned to the head tank using a submersible pump, and replenished daily to replace water lost to evaporation, overflow and water sampling.

Fish were transferred to their container and allowed to acclimate for a minimum of 12 h. Ammonia exposures were initiated by cutting-off water flow to each container, and by adding sufficient 1 mol l^{-1} NH_4Cl to each holding container at the appropriate pH. The pH (pH 8.0) was maintained *via* the drop-wise addition of 1 mol l^{-1} HCl to the water using a polyethylene, disposable transfer pipette (VWR CanLab, Mississauga, ON, Canada). Water samples (10 ml) were collected at 0, 2 and 4 h and frozen for later determination of water ammonia and urea. In the meantime, ammonia concentrations in the recirculation system were increased by adding sufficient amounts of 1 mol l^{-1} NH_4Cl to the lower reservoir. After 4 h, flow from the re-circulating system was re-established to the holding containers. Simultaneous control fish were held in an identical experimental set-up for 120 h, but were not exposed to ammonia.

Goldfish were sampled under control conditions (nominally ammonia-free) or after 4 h, 1 day, 2 days or 5 days of exposure to nominal [T_{Amm}] of 5 mmol l^{-1}. Immediately prior to sampling, water flow was cut-off to each chamber and a lethal dose (1.0 g l^{-1}) of the anaesthetic tricaine methane sulfonate (MS222; Syndel Labs, Qualicum Beach, BC, Canada) buffered with 2 parts $NaHCO_3$ was added to each. After 1–2 min, blood samples (50–250 µl) were collected by caudal puncture using a 26 G needle and heparinized syringe (55 U Na heparin ml^{-1}; Sigma Chemical Co., St Louis, MO, USA). A filet of tissue was then collected from the lateral musculature of the animal, which was then snap-frozen using liquid nitrogen-cooled aluminium tongs. The brain was collected by rapidly peeling back the cranium, removing the whole brain and snap-freezing it in liquid nitrogen. Blood samples were centrifuged at 10,000 ***g***, and the plasma was drawn off and transferred to 500 µl centrifuge tubes and frozen in liquid nitrogen. All tissue and plasma were stored at –80°C until analyzed for ammonia, glutamine and urea. Water samples were stored at –20°C until analyzed for water ammonia and urea.

Series 3 – effects of NMDA receptor antagonists and a GS inhibitor on ammonia tolerance in the goldfish and rainbow trout

Antagonists of the NMDA receptor, notably MK801, are known to protect mammals from ammonia toxicity, suggesting that this receptor plays a role in the neuropathological response to ammonia (e.g. Marcaida et al., 1992; Hermengildo et al., 1996). To determine if inhibition of NMDA receptor function protected trout or goldfish from ammonia toxicity, they were injected with MK801, and survival times determined during exposure to HEA. In these experiments fish (1–10 g mass) were added in groups of 4–6 to 20 liter buckets containing 10 liters of aerated well water at 14–16°C, and left overnight. They were then anaesthetized with HCO_3^- buffered MS222 (0.1 g l^{-1} for rainbow trout; 0.25 g l^{-1} for goldfish), followed by intraperitoneal (IP) injections of MK801 at doses of 0 (Cortland's saline vehicle only), 0.5, 1, 2, 4 or 8 mg kg^{-1}, and their survival times during HEA were determined (nominal [NH_4Cl]=9.5 mmol l^{-1} for goldfish and 2.0 mmol l^{-1} for rainbow trout).

Ethanol is also known to be neuroprotective against ammonia (Hermenegildo et al., 1996), and these effects are also thought to be due to ethanol's suppression of NMDA receptor activity (e.g. Lovinger et al., 1989; Weight et al., 1991). Accordingly, ethanol was administered in doses of 0 (saline vehicle), 1, 2, 4, 8 or 16 mmol kg^{-1} body mass prior to an identical ammonia challenge to that described for MK801-injected fish.

A third experiment was done on fish injected with the GS inhibitor, methionine sulfoximine (MSO), to determine if glutamine production during HEA protected against or exacerbated toxicity. As for the MK801 and ethanol experiments, trout or goldfish were transferred to 20 liter buckets containing 10 liters of aerated well water at 14–16°C the night before experiments. The next day fish were anaesthetized, and injected (IP) with MSO doses of 0 (saline vehicle only), 5, 10, 25, 50 or 100 mg kg^{-1} prior to the same ammonia challenge procedure described above, and the mean survival time at each dose of MSO determined. No measurements were made to ensure that GS activity was inhibited (but see Discussion for further details).

Series 4 – whole-cell patch-clamp recording of goldfish brain slices exposed to elevated ammonia

Whole brain from large goldfish (>50 g) was removed from the cranium following decapitation of the fish and immediately placed in a solution of oxygenated and chilled (4°C) artificial cerebrospinal fluid [aCSF; composition (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, osmolality 300–310 mOsm; pH 7.6]. Each telencephalon was then dissected from the brain while in chilled aCSF solution, and temporarily stored in 15 ml of oxygenated aCSF on ice. Within 15 min, the telencephalon was fastened to a sectioning block using cyanoacrylate glue, and submerged in ice-cold aCSF contained within the reservoir of a Vibtratome 1000 tissue sectioning instrument (Vibratome, St Louis, MO, USA). Slices were cut in the parasaggital plane (300 µm thick; 3–4 for each lobe), and gently lifted out of the reservoir using a fine paintbrush and transferred to a vial of aCSF. Previous experiments indicated that slices were viable, and capable of generating action potentials and NMDA receptor currents for up to 48 h (Wilkie et al., 2008).

Individual telencephalon slices were placed on a coverslip contained in a flow-through perfusion chamber (RC-26, Warner Instruments, Hamden, CT, USA) and whole-cell patch-clamp recordings were made as previously described (Wilkie et al., 2008). Briefly, each slice was placed in the perfusion chamber and held in

place by Lycra threads stretched between the opposing arms of a horseshoe-shaped stainless steel slice anchor (Warner Instruments). The chamber was gravity perfused with unmodified aCSF or aCSF plus ammonium acetate ($CH_3CO_2NH_4$) at a concentration of 5 mmol l^{-1} or 10 mmol l^{-1} from a 1.0 liter glass bottle fitted with an intravenous dripper. Other than ammonium acetate, the chemical composition and pH were identical to the unmodified aCSF. These concentrations of ammonia were chosen because they approximated the brain ammonia concentrations reported in earlier studies on other ammonia-tolerant fishes, and those measured in the brain of hyperammonemic goldfish in the present study. Ammonium acetate was chosen rather than ammonium chloride to achieve hyperammonemia *in vitro* but without changing the ionic composition of the aCSF.

Whole-cell patch-clamp recordings were initiated by first applying tetrodotoxin (TTX; 1 μmol l^{-1}) to the slice using a fast-step drug perfusion system (VC-6 Perfusion System, Warner Instruments) to block action potentials that would obscure NMDA receptor currents. Immediately following TTX application, NMDA receptor currents were initiated by a 1–10 s administration of NMDA (300 μmol l^{-1}), which is a highly specific agonist of the receptor. Whole-cell patch-clamp recordings were made using 2–5 MΩ pipettes, into which an Ag/AgCl electrode was connected to a CV-4 headstage and AxoPatch-1D amplifier (Axon Instruments, Sunnyvale, CA, USA). In experimental slices, a recording was initially made in ammonia-free aCSF. Twenty minutes later, the aCSF was switched from the ammonia-free solution to the ammonia-enriched solution, and whole-cell patch-clamp recordings subsequently made at 20 min and 40 min, before switching back to the ammonia-free solution to monitor recovery at 60 min and 80 min. Control whole-cell recordings (aCSF only) were measured every 20 min for up to 80 min using unmodified aCSF. As an additional control, 10 mmol l^{-1} sodium acetate was added to aCSF and NMDA receptor currents recorded after 20 min and 40 min, before switching back to the unmodified aCSF. The resting membrane potential was also monitored to test the hypothesis that ammonia exposure resulted in a similar depolarization of goldfish neurons as reported in mammals (Fan and Szerb, 1993). Data were collected using a TL-1 DMA interface (Axon Instruments Inc.) connected to the amplifier, and digitized and stored on a personal computer with Clampex 6 software (Axon Instruments Inc.). All experiments were done at room temperature (20–22°C).

Series 5 – quantification of NMDA receptor NR1 subunit abundance during exposure to high external ammonia

To further elucidate how NMDA receptor function was affected by ammonia, western blot analysis was performed on whole-brain goldfish extracts collected after 1, 3 or 5 days of exposure to HEA, and following 3 days of post-ammonia exposure recovery. The fish were exposed to a nominal NH_4Cl concentration of 5 mmol l^{-1} in darkened containers (1 liter) housed in a similar pH-statted recirculation system to that described above. Blood and brain samples were collected as described above after 1, 3 and 5 days of HEA, and 3 days of post-ammonia exposure recovery. A group of control fish (N=7), not exposed to ammonia but held under otherwise identical conditions, was sampled on day 5. The blood plasma and whole brain were preserved in liquid nitrogen and stored at –80°C as described above (Series 2). Water samples were collected at regular intervals and saved for later determination of water ammonia concentration.

Frozen brain tissue (–80°C) was processed for western blot analysis after grinding it into a fine powder under liquid nitrogen, and mixing the powder with four volumes of tissue homogenization buffer (50 mmol l^{-1} Tris-HCl, 1 mmol l^{-1} EDTA, 1 mmol l^{-1} dithiothreitol, 0.5% (vol./vol.) Tween-20; pH 7.5). The resulting slurry was further mixed with a hand-held homogenizer, immersed in ice for 10 min, and stored at –80°C until used for SDS-PAGE. For SDS-PAGE, equal amounts of protein (80 μg) were loaded onto 4% stacking, 10% resolving acrylamide gels and electrophoresed in running buffer at 15 mA. Once stacking was complete, the gels were electrophoresed at 25 mA, and the separated proteins transferred to a polyvinlydiene difluoride (PVDF) membrane using a semi-dry transfer apparatus (Transblot SD, BioRad, Mississauga, ON, Canada) at 14 V and constant current for 2 h. Membranes were stored overnight at 4°C in Tris-buffered saline (TBS). A duplicate SDS-PAGE gel was stained with Coomassie Blue to verify equal protein loading to each lane.

Following protein transfer, the PVDF membranes were incubated for 60 min in blocking solution [5% (wt/vol.) dried milk powder in TBS with 0.1% (wt/vol.) Tween-20 (TBS-T)], followed by incubation with immunoaffinity purified IgG polyclonal mouse anti-NMDAR1 (BD Biosciences, Pharmingen, San Diego, CA, USA; Catalog No. 556308) diluted 300–500 times in 1% BSA (wt/vol.) TBS-T for 90 min at room temperature or overnight at 4°C. Primary antibody incubation was followed by incubation with peroxidase conjugated affinity purified anti-mouse IgG (rabbit) (Rockland Immunochemicals, Gilbertsville, PA, USA; Catalog No. 610-4320) secondary antibody diluted 5000 times in 1% BSA (wt/vol.) TBS-T for 60 min at room temperature. The membranes were then incubated in chemiluminescent solution for 20 min, and the resulting images captured using a DNR Bio-imaging system 303 PC (DNR Bio-Imaging Systems, Jerusalem, Israel). Densitometric quantification of NR1 subunit abundance was done with ImageJ software (Abramoff et al., 2004) by measuring the mean gray value of all bands on the blot and standardizing the values relative to control. Total protein in the brain tissue homogenates was quantified colorimetrically by Bradford assay (Bradford, 1976) at 595 nm using a commercial kit (Bio-Rad, Hercules, CA, USA; Catalog No. 500-0006). Preliminary western blot comparisons were also made using rat brain homogenate and goldfish brain homogenate which confirmed that the IgG polyclonal mouse anti-NMDAR1 used in our experiments cross-reacted with the goldfish glutamate (NMDA) receptor NR1 subunit, near the appropriate molecular weight of 120 kDa (D. Carapic, M. Smith and M.P.W., unpublished results).

Analytical techniques and calculations

Quantification of ammonia, urea and glutamine

Measurements of J_{Urea} and J_{Amm} were based on changes in the concentration of water urea or ammonia when water flow was cut off to holding containers over a set time period, and correcting for the volume of water in the container and the fish's body mass. Water ammonia concentrations were determined using the salicylate–hypochlorite assay after diluting samples to fall within the linear range (Verdouw et al., 1978). Water urea concentration was determined using the diacetylmonoxime assay without dilution (Crocker, 1967).

Brain and muscle tissues were homogenized under liquid nitrogen and deproteinized using 7% ice-cold perchloric acid (Wang et al., 1994a). The resulting slurry was set on ice for 5 min, and centrifuged at 10,000 ***g*** at 4°C. The supernatant was drawn off and neutralized with 1 mol l^{-1} KOH, and frozen in liquid nitrogen until analyzed. Plasma and tissue (brain, muscle) urea concentration were determined on diluted samples using the diacetylmonoxime assay, while plasma, muscle and brain ammonia were determined

enzymatically (glutamate dehydrogenase) using a commercial assay kit (Sigma Chemical Co.; Procedure No. AA0100). Muscle glutamine concentrations were measured using GS (Mecke, 1985).

Statistical analysis

All excretion rate, blood and tissue, normalized NMDA receptor currents and NR1 abundance data were expressed as the means ± 1 standard error of the mean (s.e.m.). In the whole-cell patch-clamp recording experiments, the initial NMDA receptor current under nominally ammonia-free conditions (control) was set to 100%, and subsequent currents (either acetate or ammonia exposure) were normalized to this value (Shin and Buck, 2003). Data were analyzed using one-way analysis of variance (ANOVA), and where significant variation was observed, statistical differences between the means were determined using the Tukey–Kramer post-test at the $P \leq 0.05$ level. When the mean data compared had unequal variances, statistical analysis was performed using a Kruskal–Wallis test followed by Dunn's Multiple Comparison's post-test at the $P<0.05$ level. All statistical analysis was performed with GraphPad InStat, Version 3.02 (GraphPad Software, Inc., San Diego, CA, USA).

RESULTS

Goldfish and rainbow trout acute ammonia toxicity

We had initially intended to use the loss of equilibrium by goldfish and trout as an end-point when determining the acute ammonia toxicity, but this proved unsuitable because the fish would often lose equilibrium temporarily and then recover, and in many cases withstand the entire ammonia challenge. Instead, fish were considered to have reached their end-point when no opercular beats were evident. Using this criterion, we determined that small goldfish (3–7 g) had a 96 h LC_{50} for T_{Amm} of 4132 μmol l^{-1} which was ~4-fold greater than the 96 h LC_{50} of 1141 μmol l^{-1} determined in similarly sized (2–11 g) rainbow trout (Table 1). When toxicity was expressed as the 96 h LC_{50} for NH_3, the goldfish was 6-fold more tolerant to ammonia than the rainbow trout as indicated by respective 96 h LC_{50} values of 199 μmol l^{-1} and 36 μmol l^{-1} (Table 1).

Effects of elevated external ammonia on blood and tissue nitrogen stores and urea excretion in the goldfish

Exposure of larger goldfish (36±3 g) to a sub-lethal, nominal total ammonia concentration of 5 mmol l^{-1} HEA resulted in minimal mortality (N=2). The ammonia concentration and pH in the water averaged 4.6±0.2 mmol l^{-1} and 7.79±0.01, respectively, during the 5 days exposure. As a result of the 0.3–0.4 lower water pH than that used in the acute toxicity tests, the corresponding water NH_3 concentrations averaged 87±3.1 μmol l^{-1}.

The goldfish survived HEA despite 4-fold increases in plasma [T_{Amm}] from ~214 μmol l^{-1} under control conditions to ~800 μmol l^{-1} after 4 h at HEA. Plasma [T_{Amm}] increased a further 1.6-fold to ~1300 μmol l^{-1} after 1 day, before stabilizing near to 1000 μmol l^{-1} between 2 days and 5 days (Fig. 1A). In brain, T_{Amm} concentrations were 2300 μmol kg^{-1} WM under control conditions, increasing more than 2-fold to 5000 μmol kg^{-1} WM after 4 h (Fig. 1B). Unlike plasma, brain [T_{Amm}] continued to increase in a step-wise fashion to ~5700 μmol kg^{-1} WM after 1 day, followed by increases to 6300 μmol kg^{-1} and 6700 μmol kg^{-1} WM after 2 days and 5 days, respectively. Ammonia accumulation was also pronounced in the muscle, where the control [T_{Amm}] was ~900 μmol kg^{-1} WM. After 4 h at HEA, however, there was a marked 4-fold increase in muscle [T_{Amm}] to ~4000 μmol kg^{-1} WM (Fig. 1C). By 1 day, muscle [T_{Amm}] was ~8-fold higher than in control fish, at ~7700 μmol kg^{-1} WM. Muscle ammonia concentrations stabilized near this value for the remainder of the experiment (Fig. 1C).

The goldfish excreted ammonia under control conditions, as indicated by a net outward (positive) J_{Amm} of 136 nmol g^{-1} h^{-1} (Fig. 2A). However, HEA resulted in a significant net influx of ammonia characterized by a J_{Amm} of –2600 nmol g^{-1} h^{-1} during the

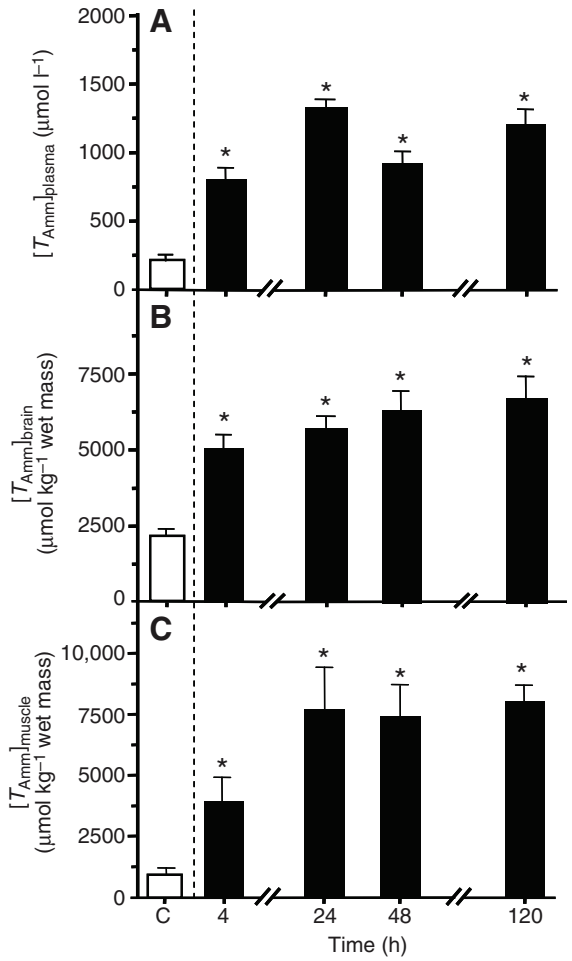

Fig. 1. Concentration of total ammonia (T_{Amm}) in (A) plasma, (B) brain and (C) muscle of goldfish exposed to high external ammonia (HEA) (nominal [T_{Amm}]=5 mmol l^{-1}) for 5 days. Data presented as the means ± 1 s.e.m. N=9–15 under control (pre-exposure) conditions, and N=6–7 after 4 h at HEA, N=3–5 after 24 h of HEA, and N=6–10 after 48 h and 120 h at HEA. Asterisks denote significant differences between the ammonia-exposed fish (solid bars) and a separate group of control fish (open bars) held under nominally ammonia-free, but otherwise identical conditions.

Table 1. Acute toxicity (96 h LC_{50}) of total ammonia (T_{Amm}=NH_3+NH_4^+) and NH_3 to rainbow trout (*Oncorhynchus mykiss*) and goldfish (*Carassius auratus*)

	T_{Amm}	NH_3
Rainbow trout	1141 (987–3249)	36 (29.4–52.5)
Goldfish	4132 (3190–5608)	199 (153.1–271.7)

The 96 h LC_{50} values were determined using Probit Analysis and the measured ammonia concentration in each container. Data displayed in μmol l^{-1} with confidence intervals in parentheses; N=5–7 per concentration tested. Mass was 3–7 g and 2–11 g for goldfish and trout, respectively. LC_{50}, median lethal concentration; T_{Amm}, total ammonia.

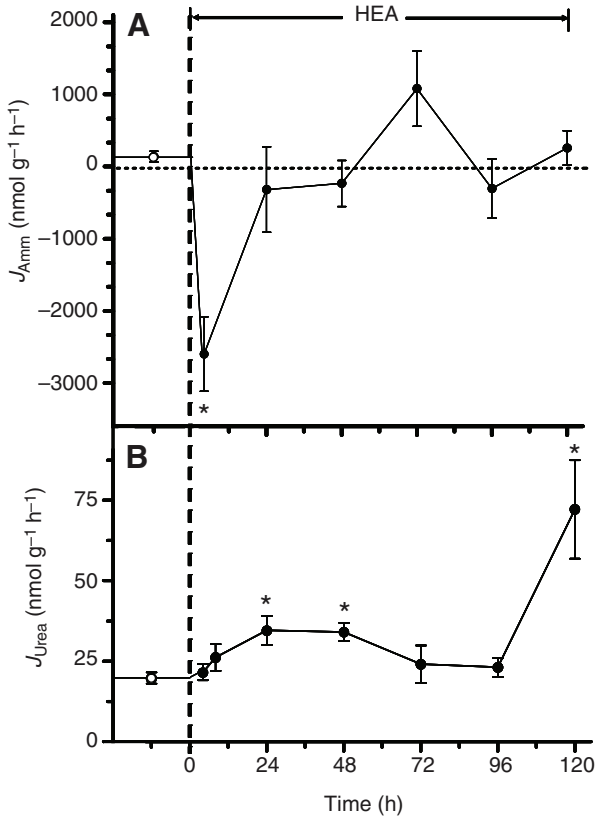

Fig. 2. Rates of (A) ammonia (J_{Amm}) and (B) urea excretion (J_{Urea}) in goldfish exposed to high external ammonia (nominal [T_{Amm}]=5 mmol l^{-1}) for 5 days. Data presented as the means ± 1 s.e.m. N=15–16 under control (pre-exposure) conditions, N=10–16 at 4 h and 24 h, and N=6–10 from 48 h to 120 h. Asterisks denote significant differences between the ammonia-exposed fish (solid circles) and the control fish (open circles) held under nominally ammonia-free, but otherwise identical conditions.

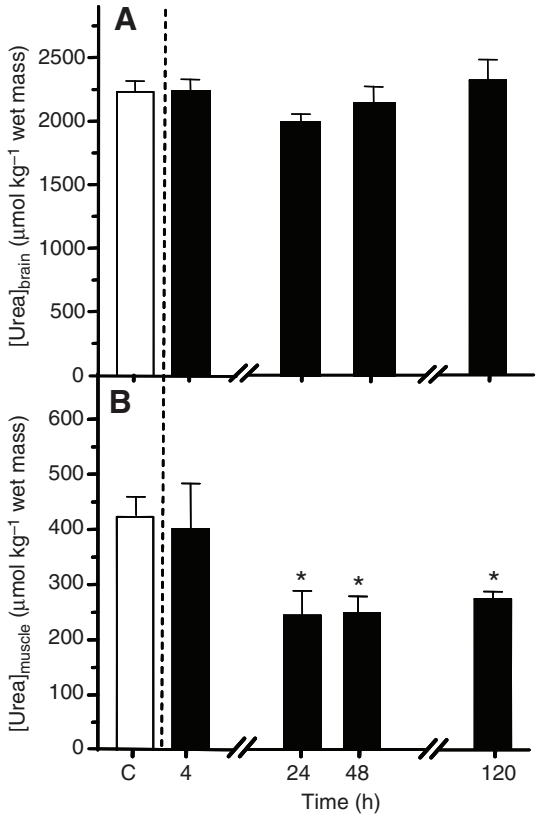

Fig. 3. Concentration of urea in the (A) brain and (B) muscle of goldfish during 5 days of high external ammonia exposure (nominal [T_{Amm}]=5 mmol l^{-1}). Data presented as the means ± 1 s.e.m. N=9–14 controls, and N=6–7 after 4 h, N=3–5 after 24 h, and N=7–10 after 48 h and 120 h. Asterisks denotes a significant difference between the ammonia-exposed fish (solid bars) and a separate group of control fish (open bars) held under nominally ammonia-free, but otherwise identical conditions.

first 4 h of exposure (Fig. 2A). The net influx of ammonia slowed to near to –300 nmol g^{-1} h^{-1} over the next 24–48 h, and was not significantly different from control values due to the high variability of the data set. Over the remaining 72 h, J_{Amm} continued to be variable, but by 120 h J_{Amm} was again outwardly directed and comparable to control values (Fig. 2A).

The retention of ammonia at HEA was accompanied by modest transient increases in J_{Urea} from 24 h to 48 h, which was about 2-fold greater than the control J_{Urea} of ~20 nmol g^{-1} h^{-1} (Fig. 2B). After 5 days of HEA, J_{Urea} had significantly increased more than 3-fold to ~70 nmol g^{-1} h^{-1} (Fig. 2B). These changes in urea excretion patterns were not accompanied by significant changes in brain urea concentration (Fig. 3A), which fluctuated around 2000 µmol kg^{-1} WM in both control and ammonia-exposed fish (Fig. 3A). In muscle, urea concentrations were much lower, near to 400 µmol kg^{-1} WM in control animals. However, HEA resulted in a significant, 40% reduction in muscle urea concentration after 1 day (Fig. 3B).

There were notable changes in brain glutamine concentration, which doubled to ~7500 µmol kg^{-1} WM after 4 h of exposure to HEA (Fig. 4A). Glutamine concentrations continued to increase in the brain, stabilizing at ~15,000 µmol kg^{-1} WM after 48 h (Fig. 4A). No significant changes in muscle glutamine were observed, which ranged between 1000 µmol kg^{-1} and 2500 µmol kg^{-1} WM during HEA (Fig. 4B).

Effects of NMDA receptor antagonists and a GS inhibitor on ammonia tolerance in the goldfish and rainbow trout

In the absence of HEA, no mortality was observed in trout or goldfish following saline administration only (data not shown). At HEA, the administration of MK801 protected trout against ammonia toxicity in a dose-dependent manner, but not goldfish. In trout exposed to a nominal NH_4Cl concentration of 2.0 mmol l^{-1} (measured water [T_{Amm}]=2.0±0.1 mmol l^{-1}) survival increased by ~75% and 100% following injection with respective MK801 doses of 4 mg kg^{-1} and 8 mg kg^{-1} (Fig. 5A). In contrast, goldfish survival was not significantly enhanced during exposure to 9.5 mmol l^{-1} NH_4Cl (measured water [T_{Amm}]=9.67±0.1 mmol l^{-1}) at any dose of MK801 (Fig. 5B).

Further evidence of NMDA receptor involvement in the neurotoxic response of rainbow trout to ammonia was evident following treatment with ethanol, which significantly enhanced trout survival at HEA (measured water [T_{Amm}]=1.76±0.2 mmol l^{-1}) by 70% following injection of an ethanol dose of 4 mmol kg^{-1} (Fig. 6A). However, goldfish survival during HEA exposure (measured water [T_{Amm}]=8.0±0.3 mmol l^{-1}) was not enhanced by ethanol administration (Fig. 6B).

Injection of the GS inhibitor MSO also decreased survival of trout and goldfish during HEA exposure but the responses were variable. In the rainbow trout challenged at HEA (measured water

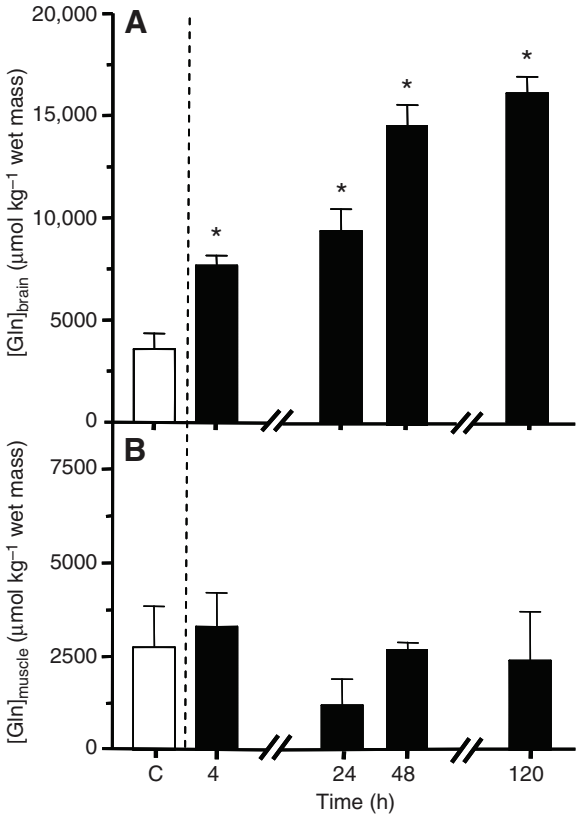

Fig. 4. Concentration of glutamine (Gln) in (A) brain and (B) muscle of goldfish during 5 days of high external ammonia exposure (nominal $[T_{Amm}]$=5 mmol l^{-1}). Data presented as the means ± 1 s.e.m. *N*=9–14 controls, and *N*=6–7 after 4 h, 3–5 after 24 h, and *N*=7–10 after 48 h and 120 h. Asterisks denote significant differences between the ammonia-exposed fish (solid bars) and a separate group of control fish (open bars) held under nominally ammonia-free, but otherwise identical conditions.

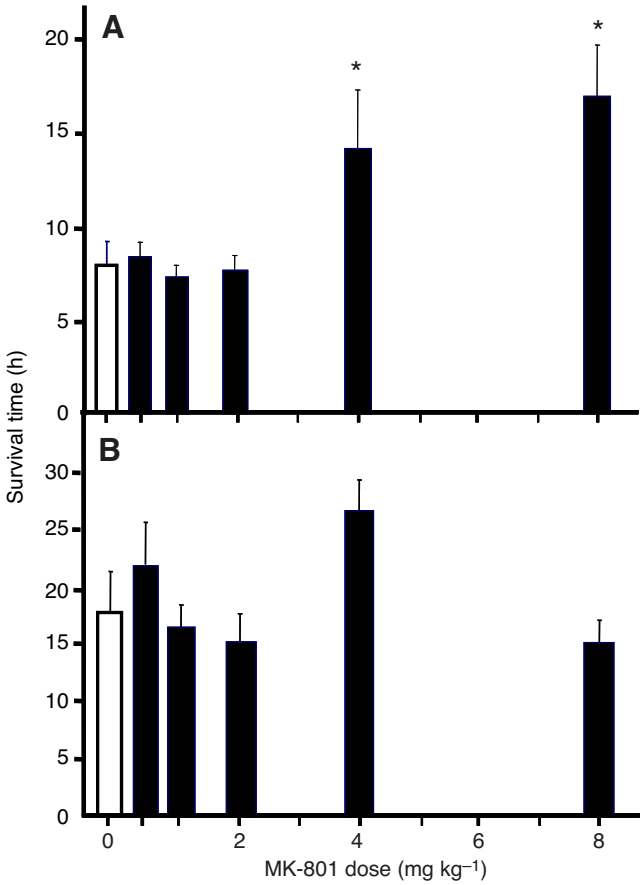

Fig. 5. Survival time of (A) rainbow trout and (B) goldfish following intraperitoneal injection of the *N*-methyl-D-aspartate (NMDA) receptor antagonist MK801 and acute exposure to toxic concentrations of ammonia (trout, 2 mmol l^{-1}; goldfish, 9.5 mmol l^{-1}). Data presented as the means ± 1 s.e.m.; *N*=10 at each dose tested. Asterisks denote significant differences between the MK801-injected fish (solid bars) and a separate group of control fish injected with the same volume of Cortland's saline only (open bars), but exposed to the same concentration of ammonia.

$[T_{Amm}]$=2.0±0.1 mmol l^{-1}), MSO administration reduced survival by almost 75% in fish injected with 5 mg kg^{-1} and 100 mg kg^{-1} of the drug (Table 2). In goldfish challenged at HEA (measured water $[T_{Amm}]$=8.0±0.3 mmol l^{-1}), survival was significantly reduced by ~50% in fish injected with doses of 10 mg kg^{-1} and 50 mg kg^{-1}, but survival time was unaffected at the lowest (5 mg kg^{-1}) and highest dose of the drug (100 mg kg^{-1}; Table 2).

NMDA receptor activity and abundance at HEA

The mean resting membrane potential of neurons exposed to ammonia-free (unmodified) aCSF was –66.9±2.7 mV (*N*=24 measurements), and the application of NMDA resulted in the generation of NMDA receptor currents with a mean amplitude of 1271.7±125.9 pA (*N*=33; Fig. 7, inset). The normalized NMDA receptor current amplitudes almost tripled after 20 min exposure to 10 mmol l^{-1} NH_4^+, but declined over the second 20 min period (Fig. 7). The NH_4^+-induced potentiation of NMDA receptor currents was completely eliminated after 20 min and 40 min (total elapsed time 60 min and 80 min) depuration in ammonia-free aCSF (Fig. 7). Similar observations were made during treatment with 5 mmol l^{-1} ammonia, but the trend was not statistically significant (Fig. 7). As a further control, the normalized NMDA receptor current amplitudes measured in slices bathed with normal aCSF (ammonia-free) and sodium acetate enriched (10 mmol l^{-1}) aCSF were unaltered over a similar time course, verifying that the potentiation of the NMDA receptor current during ammonium acetate treatment was due to the ammonia (Fig. 7). Over the course of the pre-exposure (20 min), 10 mmol l^{-1} NH_4^+ exposure (40 min) and recovery periods (20–30 min), the membrane potential of the neurons was not significantly altered, remaining stable between –65 mV and –70 mV (Fig. 8).

Longer-term ammonia exposure of whole goldfish to ammonia decreased NR1 subunit abundance in whole-brain homogenates of goldfish, likely reflecting a decrease in functional NMDA receptor quantity (Fig. 9). Similar to the Series 1 experiments, exposure to HEA resulted in a marked, 10-fold elevation of plasma ammonia, which peaked at ~1500 μmol l^{-1} and gradually declined to 800 μmol l^{-1} as the external water ammonia concentration declined from ~4.5 mmol l^{-1} to 3.0 mmol l^{-1} over the 5 days exposure (Fig. 9A). Western blots and the accompanying densitometry revealed that NR1 subunit abundance was reduced by 40% after 1 day, and by almost 60% after 3 days of HEA (Fig. 9B). Although relative NR1 subunit abundance remained suppressed for the entire period of HEA, it was restored to control (pre-exposure) levels after a 3 days recovery period in nominally ammonia-free water, when plasma ammonia concentrations had returned to control levels (Fig. 9A,B).

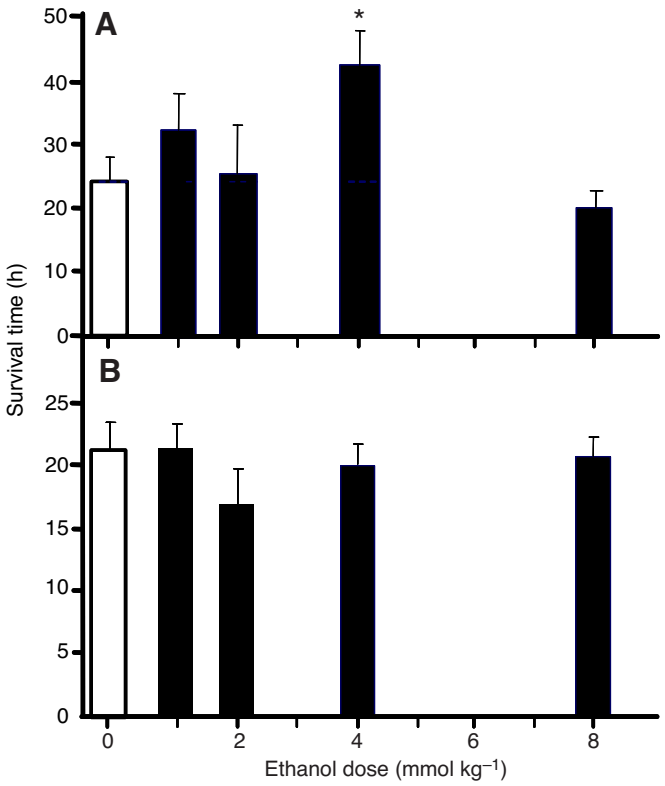

Fig. 6. Survival time of (A) rainbow trout and (B) goldfish following intraperitoneal injection of ethanol and acute exposure to toxic concentrations of ammonia (trout, ~2 mmol l^{-1}; goldfish, ~8.0 mmol l^{-1}). Data presented as the means ± 1 s.e.m.; *N*=10 at each dose tested. Asterisks denote significant differences between ethanol-injected fish (solid bars) and control fish injected with the same volume of Cortland's saline only (open bars), but exposed to the same concentration of ammonia.

Table 2. Effects of methionine sulfoximine (MSO) dose on survival time of rainbow trout and goldfish acutely exposed to a toxic concentrations of ammonia

Dose of MSO (mg kg^{-1})	Rainbow trout (water [T_{Amm}]= 2.0 mmol l^{-1})	Goldfish (water [T_{Amm}]= 8.0 mmol l^{-1})
0 (saline only)	40.1±12.0	23.9±4.7
5	9.6±1.6*	33.0±4.8
10	44.0±15.3	8.6±5.0*
25	54.0±6.5	29.0±5.0
50	35.7±4.3	12.5±2.3*
100	10.4±1.6*	35±3.6

Data shown represent the means ± 1 s.e.m. (*N*=10 per treatment). Asterisks denote statistically significant differences ($P<0.05$) from the survival times of MSO-treated fish compared with fish injected with saline only. T_{Amm}, total ammonia.

DISCUSSION

The anoxia-tolerant goldfish is ammonia tolerant

In addition to their well-known tolerance to anoxia (e.g. Nilsson, 2001; Bickler and Buck, 2007), the present study demonstrates that goldfish also exhibit high tolerance to ammonia. Contrary to our original hypothesis, NMDA receptor currents of the goldfish were reversibly potentiated by acute exposure to ammonia *in vitro*, suggesting that the NMDA receptors themselves are ammonia sensitive. However, longer-term *in vivo* exposure to elevated external ammonia resulted in significant downregulation of functional NMDA receptors, as characterized by 40–60% reductions in NR1 subunit abundance in the goldfish brain. Such a reduction in NR1 subunit abundance could lower the sensitivity of the goldfish nervous system to ammonia-induced excitotoxicity, in a similar manner to that suggested for anoxic western painted turtles exposed to long-term anoxia (Bickler et al., 2000). This conclusion is further supported by observations that administration of the NMDA receptor antagonist MK801 had no effect on survival time when goldfish were challenged with acutely lethal concentrations of ammonia but did in trout. Other possible defenses against hyperammonemia, such as glutamine synthesis, may also contribute to the high ammonia tolerance of goldfish.

Based on its NH_3 96 h LC_{50}, the goldfish is 5–10 times more tolerant to ammonia than most freshwater fishes, including the rainbow trout with which it was compared in the present study (USEPA, 1999). Although goldfish are ammonia tolerant, their ability to withstand ammonia does not match that of ureogenic fishes that possess a fully functional ornithine urea cycle (OUC), such as the gulf toadfish (*Opsanus beta*) and oyster toadfish (*Opsanus tau*), and the Lake Magadi tilapia (*Alcolapia alcalicus grahami*), which can withstand 2–3 times higher concentrations of NH_3 (Walsh et al., 1993; Wang and Walsh, 2000). The ammonia tolerance of the goldfish is also about 50% less than values reported for some air-breathing tropical fishes, such as the weatherloach (*Misgurnus anguillacaudatus*; 96 h LC_{50}=389 μmol l^{-1}) (Moreira-Silva et al., 2010) and the mudskipper (*Periophthalmodon schlosseri*; 96 h LC_{50}=536 μmol l^{-1}) (Peng et al., 1998), and well below that reported for swamp eels (*Monopterus albus*; 96 h LC_{50}=1092 μmol l^{-1}) (Ip et al., 2004a). These air breathers use different strategies to cope with increased external ammonia including active Na^+/NH_4^+ exchange, which is used by giant mudskippers to excrete ammonia while in air or during ammonia exposure (Randall et al., 1999). In contrast, the weatherloach (Tsui et al., 2002) and mangrove killifish (*Kryptolebia marmoratus*) (Frick and Wright, 2002; Litwiller et al., 2006) excrete ammonia using NH_3 volatilization *via* the skin and/or gills while in air.

Despite the wide array of adaptations to HEA, a common feature shared between the goldfish and most ammonia-tolerant fishes is a high neural tolerance to ammonia. The control brain ammonia concentration of ~2 mmol kg^{-1} WM was in the same range or slightly higher than values previously reported in goldfish (Levi et al., 1974), trout (Arillo et al., 1981), mudskippers (Ip et al., 2005), and the toadfishes *O. beta* and *O. tau* (Wang and Walsh, 2000). After 2–5 days of ammonia exposure, however, the concentration of ammonia measured in the whole brain of goldfish was 6–7 mmol kg^{-1} WM, which is in the same range measured in ammonia-tolerant fishes following sub-lethal IP injections of NH_4^+, air exposure or elevated external ammonia. For instance, the toadfishes readily withstand neural ammonia concentrations of 4–6 mmol kg^{-1} WM during HEA (Wang and Walsh, 2000) whereas immersed (4 days) climbing perch (*Anabas testudineus*) had brain ammonia concentrations near to 4 mmol kg^{-1} WM (Tay et al., 2006). The highest concentrations of ammonia, at 13–16 mmol kg^{-1} WM, were reported in the brain of swamp eel and giant mudskippers exposed to 8 mmol l^{-1} and 75 mmol l^{-1} total ammonia, respectively (Ip et al., 2004a; Ip et al., 2005). In contrast to these ammonia-tolerant fishes, overturning was reported in trout at brain ammonia concentrations near to 6 mmol l^{-1} following NH_4Cl injection (Arillo et al., 1981), and brain ammonia concentrations of 3–5 mmol kg^{-1} WM cause coma in rats injected with ammonium salts (Felipo and Butterworth, 2002).

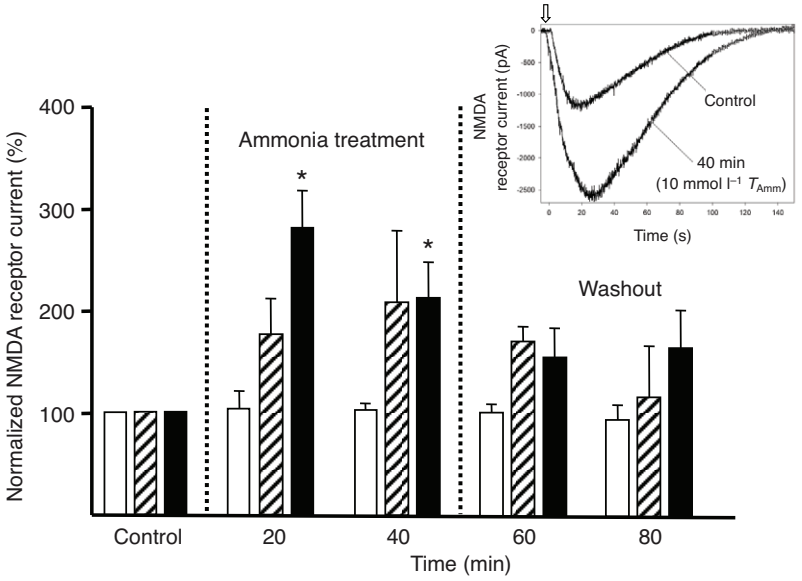

Fig. 7. Whole-cell recordings of normalized *N*-methyl-D-aspartate (NMDA) receptor current amplitudes measured in brain slices prepared from goldfish telencephalon during 40 min perfusion with modified artificial cerebrospinal fluid (aCSF) containing ammonia [nominal [T_{Amm}]=5 mmol l^{-1} (hatched bars) or 10 mmol l^{-1} (solid bars)] or 10 mmol l^{-1} sodium acetate (open bars). Inset: raw current traces recorded in a slice perfused with unmodified aCSF or aCSF containing 10 mmol l^{-1} ammonium acetate. All slices were perfused with unmodified aCSF under control conditions for 20–40 min, and two recordings of control NMDA receptor current amplitudes were made. Slices were then perfused with modified aCSF containing 5 mmol l^{-1} ammonium acetate (*N*=4–6), 10 mmol l^{-1} ammonium acetate (*N*=10–11) or 10 mmol l^{-1} sodium acetate (*N*=4–5). Treatment was followed by depuration in unmodified aCSF. Data presented as the means ± s.e.m. Asterisks denote significant differences (P<0.05) from recordings made in unmodified aCSF for each respective treatment group. T_{Amm}, total ammonia.

The role of glutamine in ammonia detoxification and toxicity

The GS-catalyzed formation of glutamine from NH_4^+ and glutamate at the expense of ATP is widely viewed as a mechanism of ammonia detoxification in vertebrates (e.g. Copper and Plum, 1987; Kosenko et al., 1994; Kosenko et al., 2003b; Felipo and Butterworth, 2002; Ip et al., 2004b). As expected, exposure to ammonia resulted in pronounced increases in brain glutamine concentration in the goldfish. Baseline (pre-exposure) glutamine levels were comparable to concentrations reported by Levi et al. (Levi et al., 1974) in goldfish, but about 2–3-fold greater than concentrations reported by Schenone et al. (Schenone et al., 1982) on the same species. This variation could be due to differences in the genetic strain of goldfish used, season, feeding status and/or diet. Nevertheless, our pre-exposure values are within previously reported measurements made in other fishes, including trout (Wicks and Randall, 2002; Sanderson et al., 2010), mudskippers (Ip et al., 2005), and the toadfishes and midshipmen (Wang and Walsh, 2000). The 3–4-fold increase in brain glutamine concentrations after 48 h of HEA was comparable to that reported in ammonia-exposed goldfish (Schenone et al., 1982), toadfish (Veauvy et al., 2005), trout (Arillo et al., 1981; Sanderson et al., 2010) and in mudskipper (*Boleophthalmus boddaerti*) exposed to 8 mmol l^{-1} total ammonia (Ip et al., 2005).

The suggestion that glutamine synthesis has a neuroprotective role against ammonia toxicity was supported by the observation that low (5–10 mg kg^{-1}) and high (50–100 mg kg^{-1}) doses of MSO decreased trout and goldfish survival times during ammonia exposure. However, other doses of MSO (10–50 mg kg^{-1}) appeared to have no effect on ammonia tolerance in either the goldfish or trout. Veauvy et al. reported a lag-time of ~16 h before maximal inhibition of brain GS activity was observed following MSO administration in toadfish (Veauvy et al., 2005). It is therefore possible that the full effects of MSO-induced inhibition of GS were not realized in the fish subjected to the ammonia challenge. More consistent decreases in survival time might have been observed had more time elapsed between MSO administration and the ammonia challenge in the present experiment. Pre-injection of MSO several hours before ammonia challenges, along with measurements of GS activity in the brain of MSO-injected fish, would shed more light on the role(s) of glutamine synthesis in ammonia detoxification in goldfish, trout and other fish species.

Although the findings support the current dogma that glutamine synthesis is an important mechanism of ammonia detoxification (Cooper and Plum, 1987; Kosenko et al., 1994; Randall and Tsui, 2002; Walsh et al., 2007), it appears that glutamine formation is not the only defense against ammonia toxicity. More recent studies on gulf toadfish (Veauvy et al., 2005), rainbow trout (Sanderson et al., 2010) and mudskippers (Ip et al., 2005) demonstrated that MSO administration does not lead to greater ammonia accumulation in the brain or plasma, despite lowering GS activity and glutamine accumulation in both the brain and liver (Veauvy et al., 2005; Sanderson et al., 2010), which would be expected if glutamine synthesis was crucial for ammonia detoxification. Such studies suggest that fish may have a ‘reserve capacity’ to detoxify ammonia (Sanderson et al., 2010). Indeed, ‘partial amino acid catabolism’ of NH_4^+ to alanine has been proposed for mudskippers (Ip et al., 2004b), the Indian air-breathing catfishes (Saha and Ratha, 2007) and for goldfish (Levi et al., 1974). This reserve capacity may also partially explain why the effects of MSO on trout and goldfish survival at HEA varied. Moreover, MSO itself is toxic (Veauvy et al., 2005), and high doses (100 mg kg^{-1}) have been known to cause excitotoxicity in rats (Shaw et al., 1999), which could further complicate interpretation.

A few researchers have suggested that glutamine accumulation within astrocytes exacerbates ammonia toxicity by promoting osmotic swelling (Brusilow, 2002; Albrecht and Norenberg, 2006). Such swelling can lead to edema, increased intracranial pressure, brain herniation and death (Ganz et al., 1989; Vaquero and Butterworth, 2006). The absence of any significant MSO-induced increase in survival time during HEA for rainbow trout and goldfish suggests that glutamine accumulation was not likely toxic to these fish during the 48 h experiment. There is limited evidence to suggest that glutamine accumulation contributes to ammonia toxicity in fish, but such conclusions should be made with caution. Although inhibition of GS (IP injection 100 mg MSO kg^{-1}) appeared to prolong survival in air-exposed sharptooth catfish injected (IP) with a lethal dose of ammonium acetate, the effect on survival time was a modest increase of only 20% (Wee et al., 2007). Similarly, in the mudskippers *B. boddaerti* and *P. schloserri*, MSO marginally

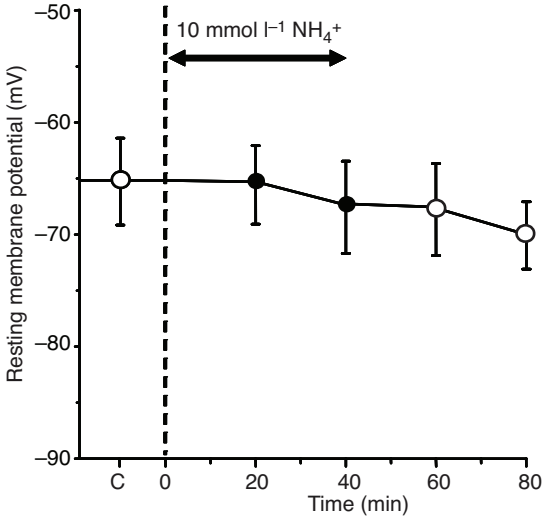

Fig. 8. Recordings of membrane potential measured in brain slices prepared from goldfish telencephalon before (open circle), during (filled circles) and after (open circles) perfusion with modified artificial cerebrospinal fluid (aCSF) containing ammonium acetate (nominal $[T_{Amm}]$=10 mmol l^{-1}; indicated by double-headed arrow). Slices were perfused with unmodified aCSF before and after perfusion with the ammonia-enriched aCSF. Data presented as the means ± 1 s.e.m.; N=8–12 at each measurement period. T_{Amm}, total ammonia.

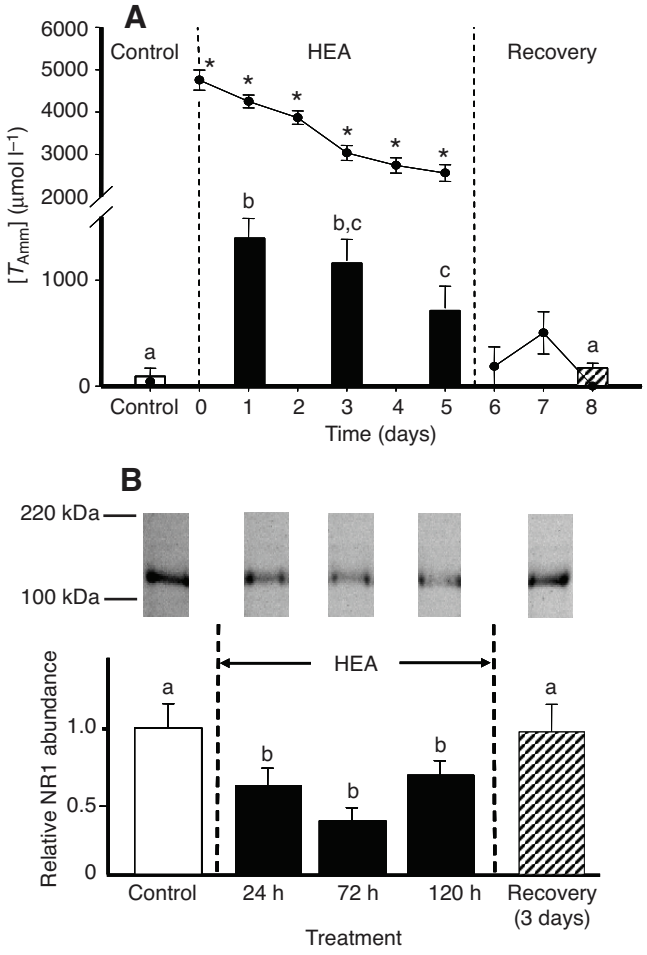

Fig. 9. (A) Plasma $[T_{Amm}]$ (bars) of goldfish exposed to high water $[T_{Amm}]$ (solid line) for 5 days and after 3 days recovery in ammonia-free water. (B) Representative western blot (upper, probed with polyclonal anti-NR1 antibody) and corresponding densitometric analysis showing goldfish *N*-methyl-D-aspartate (NMDA) receptor NR1 subunit relative abundance during and following ammonia exposure. Immunoreactive NR1 bands from different areas of the same representative gel are shown for each treatment, and positions of molecular weight markers (kDa) are indicated to the left. Densitometric data are presented as the means ± 1 s.e.m.; N=6–7 at each sample period. Asterisks denote water ammonia concentrations that are significantly different from control levels (nominally ammonia-free); respective plasma ammonia and densitometry values that are significantly different from one another are indicated with different letters ($P \leq 0.05$). T_{Amm}, total ammonia; HEA, high external ammonia.

improved survival time in the latter but did not improve overall survival in either group of fish (Ip et al., 2005). The failure of Hermenegildo et al. (Hermenegildo et al., 1996) to prolong rat survival following a lethal dose of injected (IP) ammonium acetate + MSO, also suggests that glutamine accumulation does not exacerbate ammonia toxicity.

Effects of HEA on muscle nitrogen stores and urea excretion in the goldfish

Based on an ammonia excretion rate of 136 nmol g^{-1} h^{-1} in nominally ammonia-free water (Fig. 2A), we determined that the goldfish should have excreted a total of 16.3 µmol g^{-1} body mass of ammonia, had there been no uptake of ammonia or inhibition of J_{Amm} during the 120 h of HEA (5 mmol l^{-1}; Table 3). Due to the high ammonia in the water, however, the net outward ammonia flux (or J_{Amm}) was likely inhibited or inwardly directed leading to ammonia uptake. Based on differences between the observed minus predicted ammonia flux, we therefore calculated the predicted ammonia burden, which we defined as the net gain of ammonia (in µmol g^{-1} body mass) expected in the fish during HEA exposure (Wilkie and Wood, 1995). Using this analysis, negative values represent predicted net losses of ammonia (excretion) by the animal, while positive values indicate predicted net gains of ammonia. These calculations revealed that the fish should have experienced a net ammonia burden of 37.7 µmol g^{-1} body mass during the first 24 h of HEA (Table 3), due to the very high rates of ammonia uptake in the first few hours of exposure (Fig. 2). However, this predicted burden peaked near 48 µmol g^{-1} body mass at 48 h, before dropping to near 35 µmol g^{-1} by 96 h, where it remained. This partial elimination of the burden in the final 48–72 h of the experiment was due mainly to reductions in rates of ammonia uptake from the water followed by the restoration of net outward ammonia excretion. The relatively stable ammonia burden in the latter stages of HEA also indicated that the fish were functioning at a new, elevated steady-state internal ammonia level, which coincided with the stabilization of internal ammonia concentrations in the plasma, brain and muscle of the fish (Fig. 1).

Some of the predicted ammonia burden was stored in muscle intracellular fluid (ICF). Ammonia is distributed between the extracellular and intracellular compartments of the muscle according to the combined pH and electrochemical gradient (Wright and Wood, 1988; Wang et al., 1994b). Due to the more acidic ICF of the muscle compared with the extracellular fluid (ECF) (Milligan and Wood, 1986a), most of the ammonia would have been trapped in the ICF as NH_4^+. Because the muscle represents the largest internal reservoir in which to store ammonia, we used a previously described approach (Wilkie and Wood, 1995) to determine what proportion of the ammonia burden was stored in the muscle. To quantify how much

Table 3. Ammonia burden in the whole body, extracellular fluid (ECF) and white muscle intracellular fluid (ICF) of goldfish exposed to high external ammonia (nominal [T_{Amm}]=5 mmol l^{-1})

Treatment	Ammonia flux (μmol g^{-1} body mass)		Ammonia burden[a] (μmol g^{-1} body mass)	Muscle ICF ammonia load[b,c]		Whole body ECF ammonia load[c,d] (μmol g^{-1} body mass)
	Predicted	Observed		(μmol ml^{-1} ICF)	(μmol g^{-1} body mass)	
Control	–	–	–	1.3	0.6	0.05
HEA						
4 h	–0.5	5.5	6.1	5.6	2.5 (41.4%)	0.20 (3.3%)
24 h	–3.3	34.5	37.7	10.9	4.9 (13.0%)	0.33 (0.9%)
48 h	–6.6	40.9	47.5	10.5	4.7 (10.0%)	0.23 (0.5%)
72 h	–9.8	30.9	40.8	–	–	–
96 h	–13.1	22.0	35.1	–	–	–
120 h	–16.3	22.5	38.9	11.4	5.1 (13.1%)	0.30 (0.8%)

[a]Ammonia burden equals the observed cumulative ammonia flux under control conditions minus the predicted cumulative ammonia flux expressed in μmol g^{-1} body mass, where positive values indicate net gains of ammonia, and negative values denote net losses (excretion) of ammonia. Respective T_{Amm} flux values were calculated from ammonia flux rates (J_{Amm}) under control conditions or during high external ammonia (HEA) multiplied by time (h).

[b]Muscle ICF ammonia load=(muscle [T_{Amm}]–ECFV×plasma [T_{Amm}])/ICFV, expressed as μmol ml^{-1} ICF using the muscle [T_{Amm}] data (Fig. 1C), and where ECFV and ICFV represent ECF and ICF volumes of 0.086 and 0.697 ml g^{-1} wet mass (WM), respectively (Milligan and Wood 1986a). The muscle ICF ammonia load is expressed as μmol g^{-1} body mass assuming muscle ICFV constitutes 45% of whole body mass (Wilkie and Wood 1995).

[c]Values in parentheses denote percentage (%) of total ammonia burden stored in the muscle ICF or the whole body ECF.

[d]Whole body ECF ammonia load calculated from the plasma [T_{Amm}] (Fig. 1A), multiplied by the total ECFV (0.25 ml g^{-1} body mass) (Milligan and Wood 1986b).

of the whole body ammonia burden was stored in the muscle, the ammonia concentration was expressed per milliliter of ICF, and it was assumed that the muscle comprised ~60% of the fish's body mass (Stevens, 1968) (Table 3). At 4 h, the ammonia burden measured in the muscle was 5.6 μmol ml^{-1} ICF, which was equivalent to 2.5 μmol g^{-1} body mass, or about 40% of the predicted ammonia burden (Table 3). The amount of ammonia in the muscle stabilized between 24 h and 48 h near to 5 μmol g^{-1} body mass fish, accounting for ~10–13% of the ammonia burden. Thus, some of the predicted burden was stored in the muscle. Even less was stored in the extracellular fluid (less than 1%; Table 3), which comprises 25% of the body mass. Because the muscle intracellular fluid volume (ICFV) and the extracellular fluid volume (ECFV) constitute the biggest ammonia reservoirs in the body, these findings suggest that the fish likely decreased ammonia production rates during HEA, and/or converted the ammonia to less toxic waste products (see below).

The partial elimination of the ammonia burden during the later stages of HEA (Table 3) was associated with a re-establishment of ammonia excretion over the last 72 h of exposure. This was most likely due to the restoration of blood–water NH_3 diffusion gradients due to the increased blood ammonia that was observed, as described previously (e.g. Wilson et al., 1994; Wilkie and Wood, 1995). However, further experiments, including measurements of blood pH, are required to test this hypothesis. The possibility that increases in Rhesus (Rh) glycoprotein expression in the gills promoted ammonia excretion at HEA in these fish is another intriguing possibility given the role of these proteins in teleost ammonia excretion (for reviews, see Weihrauch et al., 2009; Wright and Wood, 2009). The Rh glycoproteins Rhcgb and Rhcg are not only found on the lamella of goldfish (Perry et al., 2010) but also on the inter-lamellar cell masses (ILCM) that are formed between lamellae of cold-acclimated goldfish (Sollid et al., 2005).

Although the muscle served as a storage reservoir for ammonia, it did not appear to be a site of ammonia detoxification as reported in other fishes (Lindley et al., 1999; Iwata et al., 2000; Anderson et al., 2002; Ip et al., 2004b). There was no accumulation of glutamine or urea in the muscle, which might have been expected if the fish were converting ammonia into these less toxic end-products of nitrogen metabolism. In retrospect, the lack of glutamine and urea accumulation was not surprising given the relatively low activities of GS in the muscle of goldfish, and the likely absence of a fully functional OUC (Felskie et al., 1998). There was, however, an intriguing drop in muscle urea concentrations. These reductions coincided with increases in urea excretion, suggesting that one response of the goldfish to HEA is to unload urea-N. It seems unlikely that there was an induction of OUC activity (e.g. Levi et al., 1974), because the increases in urea excretion were relatively modest. Similar declines in urea were reported in the muscle of rainbow trout exposed to alkaline water (Wilkie et al., 1996), and in the plasma of Atlantic salmon (*Salmo salar*) exposed to ammonia (Knoph and Thorud, 1996). It seems unlikely that the unloading of such relatively modest amounts of urea by ammonia-exposed goldfish would play a role in ammonia detoxification given the 10–20-fold higher concentrations of ammonia in the muscle of the fish at HEA. A similar absence of urea accumulation in the brain suggests that urea production is not involved in ammonia detoxification by goldfish.

Effects of NMDA receptor antagonists on ammonia tolerance in the goldfish and rainbow trout

In addition to astrocyte swelling, acute ammonia toxicity is thought to cause glutamate excitotoxicity in neurons *via* the NMDA receptor (for reviews, see Felipo and Butterworth, 2002; Randall and Tsui, 2002; Ip et al., 2004a). However, excitotoxicity has been reported to be mitigated by the NMDA receptor antagonist MK801 not only in mammals (Marcaida et al., 1992; Hermenegildo et al., 2000) but in some fishes such as the weatherloach (Tsui et al., 2004; Ip et al., 2005). In goldfish brain slice preparations, MK801 irreversibly blocks NMDA-induced whole-cell currents (Wilkie et al., 2008). Despite these antagonistic effects, MK801 did not prolong goldfish survival when they were exposed to toxic concentrations of ammonia. However, MK801 did enhance survival in ammonia-sensitive trout in a dose-dependent manner, suggesting that the trout is more sensitive to glutamate-induced excitotoxicity under high ammonia, than the goldfish.

The observation that ethanol offered no protection against ammonia toxicity in the goldfish, while it did in trout, further supports the hypothesis that the goldfish is able to resist ammonia-induced excitotoxicity. In mammals, ethanol is known to inhibit

NMDA receptor activity (Lovinger et al., 1989; Popp et al., 1999), which would lower the risk of excitotoxicity during ammonia exposure (Hermenegildo et al., 1996). However, ethanol also promotes γ-aminobutyric acid (GABA) release by GABAergic neurons, which suppresses electrical activity in the brain (Wallner et al., 2006). Thus, potentiation of GABAeric activity by ethanol could also reduce the probability of NMDA receptor activation and excitotoxicity during hyperammonemia in mammals, and perhaps trout. Indeed, GABA release during anoxic episodes in western painted turtle is thought to reduce the risk of excitotoxicity by suppressing electrical activity, lowering the risk of NMDA receptor activation (Pamenter et al., 2011). The absence of a similar protective effect of ethanol against HEA in the goldfish could be due to a lack of ethanol-induced suppression of the NMDA receptor activity in goldfish. Unlike mammalian NMDA receptors, goldfish NMDA receptor currents were not inhibited by ethanol concentrations as high as 10 mmol l^{-1} in whole-cell patch-clamp experiments (Wilkie et al., 2008). This observation could be because the goldfish, and the closely related crucian carp, produce ethanol during periods of anoxia and hypoxia (Shoubridge and Hochachka, 1981; Johnston and Bernard, 1983; Nilsson, 1991), and may have simply evolved greater tolerance to ethanol-induced suppression of CNS activity.

NMDA receptor activity and abundance at HEA

Our findings suggested NMDA receptor involvement in ammonia toxicity in trout, and that goldfish might be resistant to ammonia-induced excitotoxicity. One strategy that goldfish could use to protect against ammonia toxicity would be to decrease NMDA receptor activity in the presence of ammonia. Decreased NMDA receptor activity has been suggested to protect both the anoxia-tolerant western painted turtle (*Chrysemys picta bellii*) (Bickler et al., 2000; Pamenter and Buck, 2008), and more recently the goldfish (Wilkie et al., 2008) from anoxia. In both turtles and goldfish, whole-cell recordings revealed that NMDA receptor current amplitude is reduced by 40–60% in brain slices acutely perfused with oxygen-depleted aCSF (Shin and Buck, 2003; Wilkie et al., 2008).

Unlike anoxia, whole-cell recordings revealed that NMDA receptor currents were potentiated in goldfish telencephalon neurons exposed to NH_4^+ (both 5 mmol l^{-1} and 10 mmol l^{-1}), and that the current amplitudes were quickly restored to control levels when the ammonia was removed from the perfusing media. Therefore, goldfish NMDA receptor activity is stimulated by ammonia in a manner similar to that reported in rat hippocampal slices exposed to NH_4Cl (3 mmol l^{-1}) (Fan and Szerb, 1993). Using intracellular recordings to measure NMDA receptor currents, these authors found that NH_4^+ (3 mmol l^{-1}) in the aCSF caused a reversible potentiation of NMDA receptor currents. However, unlike in rat hippocampal slices, the presence of NH_4^+ alone did not lead to any changes in neuronal membrane potential in goldfish slices, which remained steady at –65 mV to –70 mV throughout the 80–90 min experiments.

Normally, when glutamate is released from the pre-synaptic neurons, NMDA receptor activation is preceded by AMPA receptor activation, which causes a slight depolarization (or excitatory post-synaptic potential) that is required to displace the Mg^{2+} from the NMDA receptor channel pore (Mayer et al., 1984). In the mammalian model of ammonia toxicity, it has been suggested that a modest NH_4^+-induced depolarization of membrane potential (~15 mV) removes the Mg^{2+} block of the NMDA receptor, which permits activation and potentiation of the NMDA receptor currents by glutamate that is present in the synapse (Fan and Szerb, 1993). Eventually, the continual activation of the NMDA receptor is thought to lead to excitotoxicity, which is later compounded by excess glutamate in the synapse due to the ammonia-induced inhibition of glutamate re-uptake by astrocytes (Hermenegildo et al., 2000). The findings of the present study suggest that NH_4^+ causes no such depolarization in goldfish neurons, which may prevent removal of the Mg^{2+} block from the NMDA receptor and thus its over-activation in the presence of ammonia. This may also explain why MK801, an open NMDA receptor channel antagonist, affords no additional protection against ammonia toxicity when the goldfish is exposed to acutely toxic ammonia concentrations. The effectiveness of MK801 depends upon activation of the NMDA receptor, and removal of the Mg^{2+} block, to allow it to enter and block the NMDA receptor channel pore (cf. Wilkie et al., 2008).

Ammonia-induced depolarization of mammalian neurons appears related to a disruption of the K^+ gradient across neuronal membranes (Fan and Szerb, 1993). *In vivo* experiments using rat brain hippocampal and cortical slices demonstrated that ammonia treatment lowered intracellular K^+ (Benjamin and Quastel, 1975) and increased extracellular K^+ (Alger and Nicoll, 1983), indicating that ammonia interfered with K^+ movement across the plasma membrane. Similarly, plasma K^+ increased in venous blood following *in vivo* administration of ammonium salts in rat brain (Hawkins et al., 1973). Although, it seems likely that ammonia interferes with the CNS K^+ balance, the underlying mechanisms remain unclear and deserve further investigation. It is noteworthy, however, that the anoxia tolerance of the crucian carp, and presumably the closely related goldfish studied here, is reflected by their ability to maintain K^+ balance during prolonged oxygen starvation compared with more anoxia-sensitive trout and mammals which experience marked increases in extracellular K^+ and neuronal depolarization under such conditions (Johansson and Nilsson, 1995). Although, Johansson and Nilsson pointed out that this is related to the ability of the crucian carp to maintain the ATP supply needed to maintain ion balance in the nervous system (Johansson and Nilsson, 1995), it is tempting to speculate that the resistance to neuronal depolarization during anoxia might also be beneficial during periods of elevated internal ammonia. Clearly, further studies are warranted to test these hypotheses, especially using neurons isolated from animals subjected to both acute and chronic exposure to HEA.

Another strategy to lower the risk of ammonia-induced excitotoxicity would be to decrease the abundance of functional NMDA receptors. The NR1 subunit is integral for the NMDA receptor, which is thought to be a tetramer comprised of two NR1 subunits, in combination with two NR2 sub-units, of which there are several sub-types (NR2A, NR2B, NR2C, NR2D), or less commonly two subunits from the NR3 family (Wenthold et al., 2003). The consistent reductions in NR1 subunit abundance (Fig. 9) during HEA, and the reversibility of the response upon return to nominally ammonia-free water, supports the hypothesis that another protective strategy against ammonia toxicity in the goldfish is a downregulation of functional NMDA receptors. While it is true that the polyclonal antibody used (anti-NMDAR1) would be unable to distinguish functional NR1 subunits comprising functional NMDA receptors from the relatively large pool of non-functional NR1 sub-units sequestered in the cytosolic vesicles (Wenthold et al., 2003), it seems unlikely such large decreases in NR1 abundance would be restricted to the non-functional population.

Similar reductions in NR1 subunit abundance were reported during anoxia, in the anoxia-tolerant western painted turtle (Bickler et al., 2000). More recently, Ellefsen et al. noted a decrease in gene

expression for the NR1, NR2C and NR3A subunits of the NMDA receptor in crucian carp following 7 days of anoxia (Ellefsen et al., 2009). Taken together, these findings and those of the present study suggest that an overall suppression of NMDA receptor activity could be an important general defense against excitotoxicity during not only anoxia, but also during periods of hyperammonemia in anoxia-tolerant turtles and goldfish.

CONCLUSIONS

A key adaptation that may allow the goldfish CNS to withstand high concentrations of ammonia is an ability to prevent ammonia-induced neuronal depolarization. We propose that this prevents NMDA receptor over-activation and excitotoxicity, and also explain why the sensitivity of goldfish to ammonia is unaffected by NMDA receptor antagonists such as MK801 and ethanol. However, MK801 increases the ammonia tolerance of rainbow trout, suggesting that trout NMDA receptors are more sensitive to ammonia. Although the NMDA receptor of goldfish appears to be less sensitive to ammonia than that of the trout, the mere fact that high concentrations of ammonia cause NMDA receptor potentiation in goldfish brain slices suggests that this tolerance is finite. Indeed, higher concentrations of ammonia may very well cause greater potentiation of the NMDA receptor, leading to excitotoxicity. Thus, we suggest that the ability of the goldfish to decrease NMDA receptor abundance, as characterized by reduced NR1 subunit density, may be a crucial defense against toxicity during longer-term exposure to sub-lethal concentrations of ammonia. Glutamine formation in the brain also likely defends against ammonia toxicity in goldfish. The high ammonia and anoxia tolerance of the goldfish likely explains why goldfish, and their related cousins the crucian carp, thrive in marginal, shallow, eutrophic environments that are vulnerable to oxygen deprivation and increases in environmental ammonia (Nilsson, 2001; Walsh et al., 2007), and are likely uninhabitable for many of their predators.

ACKNOWLEDGEMENTS

The authors thank Dr Damian Shin (University of Toronto) for his valued expertise and instruction on the use of the patch clamp set-up used in these experiments. Dr Siddhartha Dutta and Gena Braun (Wilfrid Laurier University) provided valuable guidance and assistance with the western blot experiments.

FUNDING

This work was supported by Natural Sciences and Engineering Research Council (NSERC) of Canada Undergraduate Student Research Awards to S.D., N.S. and G.S., and by funds provided by the Wilfrid Laurier Science Technology Endowment Program (STEP). M.E.P. was the recipient of an Ontario Graduate Scholarship. This research was supported by NSERC Discovery Grants to M.P.W., M.D.S. and L.T.B.

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