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Chronic and acute ammonia toxicity in mudskippers, *Periophthalmodon schlosseri* and *Boleophthalmus boddaerti*: brain ammonia and glutamine contents, and effects of methionine sulfoximine and MK801

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Summary

The objective of this study was to elucidate if chronic and acute ammonia intoxication in mudskippers, *Periophthalmodon schlosseri* and *Boleophthalmus boddaerti*, were associated with high levels of ammonia and/or glutamine in their brains, and if acute ammonia intoxication could be prevented by the administration of methionine sulfoximine [MSO; an inhibitor of glutamine synthetase (GS)] or MK801 [an antagonist of N-methyl D-aspartate type glutamate (NMDA) receptors]. For *P. schlosseri* and *B. boddaerti* exposed to sublethal concentrations (100 and 8 mmol l^{-1} NH_4Cl, respectively, at pH 7.0) of environmental ammonia for 4 days, brain ammonia contents increased drastically during the first 24 h, and they reached 18 and 14.5 μmol g^{-1}, respectively, at hour 96. Simultaneously, there were increases in brain glutamine contents, but brain glutamate contents were unchanged. Because glutamine accumulated to exceptionally high levels in brains of *P. schlosseri* (29.8 μmol g^{-1}) and *B. boddaerti* (12.1 μmol g^{-1}) without causing death, it can be concluded that these two mudskippers could ameliorate those problems associated with glutamine synthesis and accumulation as observed in patients suffering from hyperammonemia. *P. schlosseri* and *B. boddaerti* could tolerate high doses of ammonium acetate (CH_3COONH_4) injected into their peritoneal cavities, with 24 h LC_{50} of 15.6 and 12.3 μmol g^{-1} fish, respectively. After the injection with a sublethal dose of CH_3COONH_4 (8 μmol g^{-1} fish), there were significant increases in ammonia (5.11 and 8.36 μmol g^{-1}, respectively) and glutamine (4.22 and 3.54 μmol g^{-1}, respectively) levels in their brains at hour 0.5, but these levels returned to normal at hour 24. By contrast, for *P. schlosseri* and *B. boddaerti* that succumbed within 15–50 min to a dose of CH_3COONH_4 (15 and 12 μmol g^{-1} fish, respectively) close to the LC_{50} values, the ammonia contents in the brains reached much higher levels (12.8 and 14.9 μmol g^{-1}, respectively), while the glutamine level remained relatively low (3.93 and 2.67 μmol g^{-1}, respectively). Thus, glutamine synthesis and accumulation in the brain was not the major cause of death in these two mudskippers confronted with acute ammonia toxicity. Indeed, MSO, at a dosage (100 μg g^{-1} fish) protective for rats, did not protect *B. boddaerti* against acute ammonia toxicity, although it was an inhibitor of GS activities from the brains of both mudskippers. In the case of *P. schlosseri*, MSO only prolonged the time to death but did not reduce the mortality rate (100%). In addition, MK801 (2 μg g^{-1} fish) had no protective effect on *P. schlosseri* and *B. boddaerti* injected with a lethal dose of CH_3COONH_4, indicating that activation of NMDA receptors was not the major cause of death during acute ammonia intoxication. Thus, it can be concluded that there are major differences in mechanisms of chronic and acute ammonia toxicity between brains of these two mudskippers and mammalian brains.

Key words: ammonia, brain, glutamine, glutamine synthetase, mudskipper, methionine sulfoximine, MK801, NMDA receptors.

Introduction

Ammonia is toxic to fish for many reasons. At the organismal level, ammonia causes hyperventilation (Hillaby and Randall, 1979; McKenzie et al., 1993), hyper-excitability, coma, convulsions and finally death. Ammonia also affects the ionic balance in fish, because NH_4^+ can substitute for K^+ in Na^+, K^+-ATPase and in $Na^+/K^+/2Cl^-$ co-transport (see Wilkie, 1997, 2002 for reviews; Person Le Ruyet et al., 1997). NH_4^+ can substitute for H^+ in Na^+/H^+ exchanger (NHE), probably NHE2 and/or NHE3 (Randall et al., 1999). At the cellular level, ammonia can interfere with energy metabolism through impairment of the tricarboxylic acid cycle in fish (Arillo et al., 1981). In addition, NH_4^+ can substitute for K^+

(Binstock and Lecar, 1969) affecting the membrane potential. Smart (1978) suggested that the mechanism of ammonia toxicity in fish might be similar to the action of ammonia in mammals during hepatic encephalopathy. However, in spite of recent advances in research on mammalian brains, there is a dearth of knowledge on the effects of ammonia on fish brains at present.

In recent years, several theories, i.e. glutamatergic dysfunction, glutamine accumulation leading to astrocyte swelling, activation of N-methyl-D-aspartate-type glutamate (NMDA) receptors, have been proposed as mechanisms involved in chronic and/or acute ammonia toxicity in mammalian brains (Felipo et al., 1994; Margulies et al., 1999; Hermenegildo et al., 2000; Desjardins et al., 2001; Brusilow, 2002; Felipo and Butterworth, 2002; Rose, 2002); however, evidence that these mechanisms occur in fish brains is lacking. Unlike mammals, some tropical air-breathing fishes can tolerate high levels of ammonia (see Ip et al., 2001, 2004a,b, and Chew et al., 2005b for reviews), and/or synthesize and accumulate high levels of glutamine in their brains and extracranial tissues (Peng et al., 1998; Anderson et al., 2002; Tsui et al., 2002; Tay et al., 2003; Ip et al., 2004b,d). Thus, it is possible that the mechanisms of ammonia toxicity and adaptation to defend against them in the brains of fish species with high ammonia tolerance are different from those of mammalian brains.

Mudskippers are gobioid teleosts (Order: Perciformes, Family: Gobiidae), which are euryhaline and amphibious. They are highly adaptable to different environmental conditions, with high tolerance of aerial exposure (Ip et al., 1993), environmental hypoxia (Bandurski et al., 1968; Gordon et al., 1978; Chew et al., 1990; Ip et al., 1991; Chew and Ip, 1992a,b), environmental ammonia (Ip et al., 1993; Peng et al., 1998; Randall et al., 1999, 2004; Ip et al., 2004b,c) and alkaline pH (Chew et al., 2003). The giant mudskipper, *Periophthalmodon schlosseri* (Pallas 1770), is carnivorous and is the only genus of mudskipper not found outside the tropics. It inhabits muddy shores in estuaries and in the tidal zone of rivers in South East Asia. *P. schlosseri* has a very high tolerance of environmental ammonia. Unlike many other teleosts, it can survive in 100 mmol l^{-1} NH_4Cl for more than one week (Ip et al., 1993; Peng et al., 1998), and has a 96 h LC_{50} of 120 mmol l^{-1} total ammonia at pH 7.0 or 536 μmol l^{-1} NH_3 (Peng et al., 1998). Living in the vicinity of the habitat of *P. schlosseri* is the herbivorous Boddart's goggle-eyed mudskipper, *Boelophthalmus boddaerti* (Pallas 1770). *B. boddaerti* is also capable of tolerating high levels of environmental ammonia, albeit inferior to *P. schlosseri*, and has a 96 h LC_{50} of 13.5 mmol l^{-1} total ammonia at pH 7.0 or 60.2 μmol l^{-1} NH_3 (Peng et al., 1998).

So, how do these two mudskippers tolerate such high levels of environmental ammonia? Do they also have high tolerance of ammonia at the cellular and subcellular levels in their brains? In the first series of experiments in this study, *P. schlosseri* and *B. boddaerti* were exposed chronically to sublethal concentrations of environmental ammonia (100 and 8 mmol l^{-1} NH_4Cl, respectively, at pH 7.0) for 4 days. The objective was to determine how fast and how high the levels of ammonia and glutamine would build up in the brains of these two mudskippers confronted with chronic ammonia intoxication.

In the second series of experiments, various doses of ammonium acetate (CH_3COONH_4) were injected intraperitoneally into *P. schlosseri* and *B. boddaerti* to establish the 24 h LC_{50} of injected ammonia. Subsequently, by injecting into these mudskippers either a dose of CH_3COONH_4 close to the LC_{50} value or a sublethal dose of CH_3COONH_4, we aimed to determine the levels of ammonia and glutamine in the brains of fish that succumbed to and fish that survived the acute ammonia intoxication. The objective was to elucidate whether ammonia and/or glutamine accumulations were the major cause of death in these mudskippers after being injected with CH_3COONH_4 (acute ammonia intoxication) as compared with results obtained from the first series of experiments (chronic ammonia intoxication).

Although astrocyte swelling can be related to a loss in expression of aquaporin 4 (Margulies et al., 1999) and glucose transporter GLUT-1 (Desjardins et al., 2001), and glutamine retention does not universally cause volume change in brain cells (Zielinska et al., 2004), it has been proposed that increased glutamine synthesis and accumulation causes swelling of astrocytes, leading to cellular dysfunction, brain edema and death in patients with hyperammonemia (Albrecht and Dolinska, 2001; see Brusilow, 2002 for a review). It has been demonstrated that administration of L-methionine S-sulfoximine (MSO), an inhibitor of glutamine synthetase (GS), to rats delays or even eliminates the fatal effects of ammonia toxicity (Warren and Schenker, 1964; Takahashi et al., 1991; Willard-Mack et al., 1996; Brusilow, 2002). However, the protective effect of MSO in rats injected with CH_3COONH_4 could be due to other mechanisms different from the inhibition of GS, because it has been shown that MSO prevents glutamate release and therefore prevents the activation of NMDA receptors (Kosenko et al., 1994, 1999, 2003). Thus, in the third series of experiments, we made an effort to confirm the inhibitory effects of MSO on activities of GS from the brains of *P. schlosseri* and *B. boddaerti*. Subsequently, MSO was injected into the peritoneal cavities of these two mudskippers prior to the injection with a lethal dose of CH_3COONH_4. We aimed to evaluate if the administration of MSO would exacerbate or ameliorate ammonia toxicity. The hypothesis tested was that the synthesis and accumulation of glutamine and the release of glutamate did not have significant contribution to ammonia toxicity in brains of *P. schlosseri* and *B. boddaerti*, and therefore MSO would not reduce the mortality of fish confronted with acute ammonia toxicity.

In patients suffering from acute liver failure, glutamatergic dysfunction (Hilgier et al., 1999; Michalak et al., 1996) resulted from high levels of brain ammonia (1–3 mmol l^{-1}; Kosenko et al., 1994) remains the leading candidate in the pathogenesis of hepatic encephalopathy. Glutamate is the principle excitatory neurotransmitter in the brain. Inhibition of glutamate uptake

(Oppong et al., 1995) or increased glutamate release from neurons (Rose, 2002) can cause an increase in extracellular glutamate (Michalak et al., 1996). Extracellular glutamate binds with and activates NMDA receptors (Marcaida et al., 1992; Hermenegildo et al., 1996), which are coupled with the nitric oxide-cyclic GMP signal transduction pathway (Hermenegildo et al., 2000), leading to extensive destruction of proteins in the neurons (Kosenko et al., 1993, 1994, 1995, 1997, 1999, 2000). On the other hand, it has been demonstrated in rats injected intraperitoneally with CH_3COONH_4 that an activation of NMDA receptors precedes the increase in extracellular glutamate (Hermenegildo et al., 2000). Thus, acute ammonia intoxication in rats involves the activation of NMDA receptors (Marcaida et al., 1992, 1995), and NMDA receptor activation is likely to be initiated by a depolarization of the neuronal membrane (Sugden and Newsholme, 1975; Fan and Szerb, 1993) instead of an increase in extracellular glutamate (Hermenegildo et al., 2000). When (5R, 10S)-(+)-methyl-10, 11-dihydro-5H-dibenzo[a, d]cyclohepten-5,10-imine hydrogen maleate (MK801; a NMDA receptor antagonist) is injected into rats before the injection of CH_3COONH_4, it can delay or eliminate the fatal effects of acute ammonia toxicity (Marcaida et al., 1992; Hermenegildo et al., 1996). MK801 binds to NMDA receptors and prevents their activation by glutamate, thereby preventing the cascade of processes that eventually lead to neuronal damage. Therefore, in the fourth series of experiments, MK801 was injected into the peritoneal cavities of *P. schlosseri* and *B. boddaerti* prior to the injection of a lethal dose of CH_3COONH_4. The objective was to determine if acute ammonia toxicity in these mudskippers were mediated through NMDA receptor activation as in rats.

Materials and methods

Collection and maintenance of specimens

Specimens of *P. schlosseri* (80–120 g body mass) and *B. boddaerti* (10–18 g body mass) were captured from Benut, Malaysia, and transferred to the National University of Singapore. They were maintained in 50% seawater (15‰) in individual plastic aquaria at room temperature (27–32°C) under a 12 h:12 h light:dark regime in the laboratory. During this period, 50% seawater was changed daily and the fish were fed live guppies or an artificial diet. No attempt was made to separate the sexes. Mudskippers were acclimated to these laboratory conditions for at least 1 week before experimentation. Food was withdrawn 48 h prior to experiments, which gave sufficient time for the gut to be emptied. The wet mass of the fish was obtained to the nearest 0.1 g with a Shimadzu animal balance (Shimadzu, Kyoto, Japan).

Effects of exposure to sublethal concentrations of environmental ammonia on brain ammonia, glutamine and glutamate contents

Based on the report of Peng et al. (1998), the sublethal concentrations of NH_4Cl for *P. schlosseri* and *B. boddaerti* were chosen at 100 mmol l^{-1} (446 μmol l^{-1} NH_3) and 8 mmol l^{-1} (36 μmol l^{-1} NH_3), respectively, in 50% seawater buffered with 10 mmol l^{-1} Tris-HCl (pH 7.0). A total of 35 fish each were exposed to environmental ammonia according to the methods of Peng et al. (1998) for a period of 96 h (4 days). The external medium was changed daily, and the ammonia concentration in the medium was verified using the method of Anderson and Little (1986). At hours 0, 3, 6, 12, 24, 48 and 96, fishes (N=5 for each species) were killed with a blow to the head. Brain samples were quickly dissected out and freeze-clamped with liquid nitrogen-precooled aluminum tongs. Samples were stored at –80°C until analyses of ammonia and free amino acids (FAAs), which were performed within a month.

The frozen brain samples were weighed, ground to a powder in liquid nitrogen, and homogenized three times in 5 volumes (w/v) of 6% trichloroacetic acid at 24,000 rpm for 20 s each using an Ultra-Turrax homogenizer with intervals of 10 s between each homogenization. The homogenate was centrifuged at 10,000 ***g*** at 4°C for 15 min, and the supernatant was analyzed for ammonia and FAAs. For ammonia analysis, the pH of the deproteinized sample was adjusted to between 6.0–6.5 with 2 mol l^{-1} $KHCO_3$. The ammonia content was determined using the enzymatic method of Bergmeyer and Beutler (1985). The change in absorbance at 25°C and 340 nm was monitored using a Shimadzu UV-160A spectrophotometer. Freshly prepared NH_4Cl solution was used as the standard for comparison. For FAA analysis, the supernatant obtained was adjusted to pH 2.2 with 4 mol l^{-1} lithium hydroxide and diluted appropriately with 0.2 mol l^{-1} lithium citrate buffer (pH 2.2). FAA analyses were performed using analyzed using a Shimadzu LC-6A amino acid analysis system (Kyoto, Japan) with a Shim-pack ISC-07/S1504 Li-type column. Although a complete FAA analysis was applied to every sample, only results of glutamine and glutamate were reported. Results were expressed as μmol g^{-1} wet mass tissue.

Determination of 24 h LC_{50} of CH_3COONH_4

To determine the 24 h LC_{50} of CH_3COONH_4 injected into the peritoneal cavity, five groups of 10 specimens each of *P. schlosseri* and *B. boddaerti* (a total of 50 fish each) were anaesthetized for approximately 10 min in 0.12% phenoxyethanol (Sigma Chemical Co., St Louis, MO, USA) in 50% SW and injected intraperitoneally with 8, 12, 15, 20 and 25 μmol CH_3COONH_4 g^{-1} fish. Control groups (N=10 for each species) were injected with 0.9% NaCl; zero mortality was recorded at hour 24. The volume of medium injected was 0.4 ml 100 g^{-1} fish. Prior to the experiment, all equipments were sterilized to minimize the chance of infection. Fishes were returned to 10 volumes (w/v) of strongly aerated 50% seawater, and they usually recovered and responded to mechanical stimulation within 7–10 min. Mortality was monitored during the subsequent 24 h. Fish were regarded as dead when there was no respiratory activity and no reaction to mechanical stimulation. The 24 h LC_{50} value was determined according to the methods of Litchfield and Wilcoxon (1949) on log-probit paper.

Effects of injection with sublethal, LC_{50}, and lethal dosages of CH_3COONH_4

Specimens of *P. schlosseri* and *B. boddaerti* (*N*=15 each) were injected with a sublethal dose of CH_3COONH_4 (8 μmol g^{-1} fish). Five fishes were killed at hours 0.5, 2 and 24 with a blow to their heads. Brain samples were quickly excised and freeze-clamped with liquid nitrogen-precooled aluminum tongs. Levels of ammonia, glutamine and glutamate were determined in these samples as described above.

To determine if ammonia and/or glutamine were the cause of death in fish that had succumbed to acute ammonia toxicity, specimens of *P. schlosseri* and *B. boddaerti* (*N*=10 each) were injected with a LC_{50} dose of CH_3COONH_4 (15 and 12 μmol g^{-1} fish, respectively). Fishes were regarded as dead when they showed no respiratory activity and no response to mechanical stimulation. The time to death was recorded, and the brains of the dead fishes were immediately excised at the point of death and freeze-clamped. For fishes that did not succumb to ammonia intoxication, they were killed at hour 24 and their brains excised and frozen. Brain samples were analyzed for ammonia, glutamine and glutamate as described above.

Because fishes succumbed to a LC_{50} dose of CH_3COONH_4 at different time (normally within 1 h after injection), it was essential to evaluate the levels of ammonia and glutamine in brains of mudskippers right before they succumbed to ammonia toxicity. Therefore, specimens of *P. schlosseri* and *B. boddaerti* (*N*=5 each) were injected with a lethal dose (20 μmol g^{-1} fish) of CH_3COONH_4 or 0.9% NaCl (control, *N*=5 each), returned to strongly aerated 50% SW, and killed exactly 15 min later (death naturally occurred between 15 and 40 min). Brain samples were collected, and subsequently, ammonia and glutamine contents were determined.

Effects of MSO and MK801

To determine if MSO (Sigma-Aldrich Chemical Co., St Louis, MI, USA) acted as an inhibitor of GS from the brains of *P. schlosseri* and *B. boddaerti*, brain samples of control fishes (*N*=4 each) kept in 50% seawater were collected and frozen in liquid nitrogen. They were homogenized three times in 5 volumes (w/v) of ice-cold extraction buffer containing 50 mmol l^{-1} immidazole-HCl (pH 7.0) and 3 mmol l^{-1} EDTA at 24,000 rpm for 20 s each with a 10 s off interval using an Ultra-Turrax homogenizer. The homogenates were centrifuged at 10,000 ***g*** at 4°C for 15 min. The supernatant obtained was passed through a 5 ml Econo-Pac 10DG desalting column (Bio-Rad Laboratorories Inc., CA, USA) equilibrated with the same buffer. The resulting eluent was used for the determination of GS activity. GS activity was determined according to the method of Shanker and Anderson (1985) as modified by Tay et al. (2003), and expressed as μmol γ-glutamylhydroximate formed min^{-1} mg^{-1} protein. Protein was determined according to the method of Bradford (1976). The effect of MSO on GS activity *in vitro* was determined by including 0.1 ml of 2 mmol l^{-1} MSO (pH 7.0) in the assay medium.

To evaluate if MSO had a protective effect against acute ammonia toxicity on these two mudskippers, specimens of *P. schlosseri* and *B. boddaerti* (*N*=10 each) were injected intraperitoneally with 100 μg MSO g^{-1} fish (pH 7.0) 10 min prior to the injection with a lethal dose of CH_3COONH_4 (20 and 15 μmol g^{-1} fish, respectively). Preliminary results obtained indicated that injection of 100 μg MSO g^{-1} fish alone had no observable effects on these two mudskippers. Fishes (*N*=10 each) injected with 0.9% NaCl solution in place of MSO served as references for comparison. The mortality rate and time of death for succumbed fish were recorded during the subsequent 24 h.

Similarly, the possible protective effect of MK801 (Sigma-Aldrich Chemical Co., St Louis, MI, USA) was evaluated by injecting 2 μg MK801 g^{-1} fish into the peritoneal cavities of *P. schlosseri* and *B. boddaerti* (*N*=10 each) 10 min prior to the injection with a lethal dose of CH_3COONH_4. Preliminary results obtained indicated that injection of 2 μg MK801 g^{-1} fish alone had no observable effects on these two mudskippers. Controls (*N*=10 each) were injected with 0.9% NaCl solution in place of MK801 before the injection with CH_3COONH_4. Subsequently, due to the lack of a protective effect of MK801 on *P. schlosseri* and *B. boddaerti*, efforts were made to demonstrate that the same batch of MK801 indeed had a protective effect on another fish species, the goldfish *Carassius auratus*.

Statistical analyses

Results were presented as means ± standard errors of the mean (S.E.M.). Student's *t*-test and one-way analysis of variance followed by Duncan's multiple range test were used to evaluate differences between means where applicable. Differences were regarded as statistically significant at $P<0.05$.

Results

Effects of exposure to a sublethal level of environmental ammonia

No mortality was observed in *P. schlosseri* and *B. boddaerti* exposed to sublethal concentrations of environmental NH_4Cl (100 and 8 mmol l^{-1}, respectively) during the 4 day period. For *P. schlosseri*, the ammonia content in the brain of fish exposed to 100 mmol l^{-1} NH_4Cl increased significantly by 4.8-fold at hour 24, and reached very high level (18.0 μmol g^{-1} tissue) at hour 96 (Fig. 1A). By comparison, a significant increase in the brain glutamine content occurred much earlier; it increased by 6.2-fold at hour 6 (Fig. 1B). At hour 96, the brain glutamine content reached an exceptionally high level of 29.8 μmol g^{-1} tissue. In spite of a significant increase in glutamine content in the brain of *P. schlosseri*, there was no significant change in the brain glutamate level throughout the 96 h period (Fig. 1C). Similar observations were made on *B. boddaerti* (Fig. 2), except that significant increases in brain ammonia (Fig. 2A) and glutamine (Fig. 2B) contents occurred earlier (at hour 6) and later (at hour 12), respectively, in comparison with *P. schlosseri*. At hour 96, the ammonia and glutamine levels in

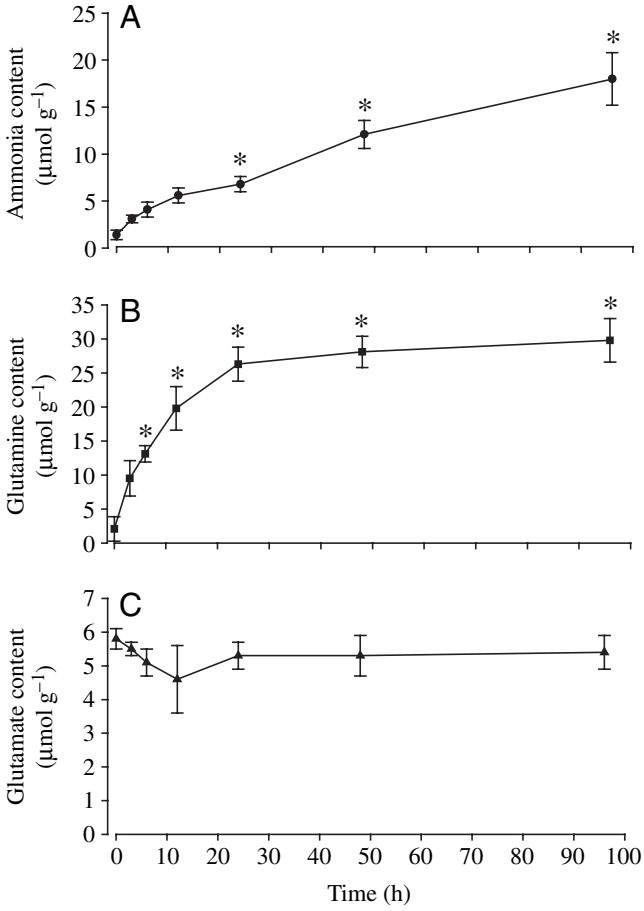

Fig. 1. Effects of 4 days of chronic exposure to a sublethal concentration of environmental ammonia (100 mmol l^{-1} NH_4Cl in 15‰ water at pH 7.0) on contents (μmol g^{-1}) of (A) ammonia, (B) glutamine and (C) glutamate in the brain of *Periophthalmodon schlosseri*. *Significantly different from the time 0 value.

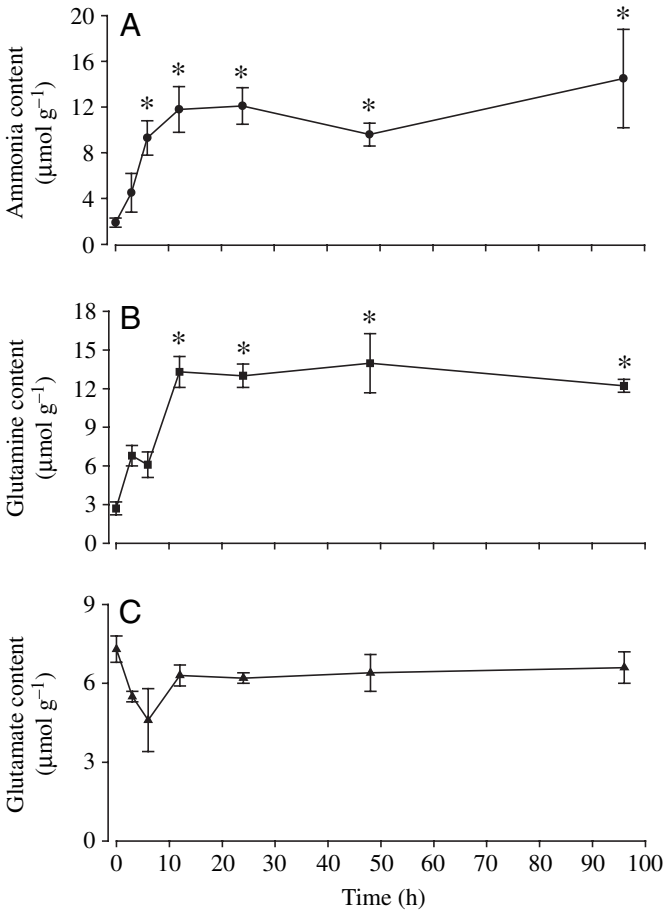

Fig. 2. Effects of 4 days of chronic exposure to a sublethal concentration of environmental ammonia (8 mmol l^{-1} NH_4Cl in 15‰ water at pH 7.0) on contents (μmol g^{-1}) of (A) ammonia, (B) glutamine and (C) glutamate in the brain of and *Boleophthalmus boddaerti*. *Significantly different from the time 0 value.

the brain of *B. boddaerti* exposed to 8 mmol l^{-1} NH_4Cl reached 14.5 and 12.1 μmol g^{-1} tissue, respectively.

24 h LC_{50} of injected CH_3COONH_4 and effects of injections with a sublethal, LC_{50} or lethal dose of CH_3COONH_4

The 24 h LC_{50} of injected CH_3COONH_4 for *P. schlosseri* and *B. boddaerti* were determined as 15.6 and 12.3 μmol g^{-1}, respectively. Subsequently, these two mudskippers were challenged with a sublethal dose of CH_3COONH_4 at 8 μmol g^{-1} fish, which caused no mortality during the 24 h experimental period. For *P. schlosseri*, the injection with a sublethal dose of CH_3COONH_4 led to significant increases in ammonia (16.5-fold) and glutamine (2.2-fold) contents in the brain within the first 0.5 h (Fig. 3). However, the brain ammonia and glutamine contents returned to control levels at hour 24 (Fig. 3). By contrast, there was no significant change in the glutamate level in the brain through the 24 h (Fig. 3). Similar observations were made on *B. boddaerti* injected with a sublethal dosage of CH_3COONH_4 (Fig. 4).

When *P. schlosseri* was injected with a LC_{50} dose (15 μmol g^{-1} fish) of CH_3COONH_4, 5 out of the 10 fish died within 15–47 min. The brain ammonia content of fish that succumbed to acute ammonia toxicity (Fig. 5) increased to a level (12.8 μmol g^{-1} tissue; N=5) much higher than that of fish killed at hour 0.5 (or 30 min) after being injected with saline (Fig. 5) or a sublethal dose of CH_3COONH_4 (Fig. 3). There was also a greater level of glutamine in the brain of the fish that had succumbed, but the brain glutamate content was not significantly different from that of fish injected with a sublethal dose of CH_3COONH_4 and killed at hour 0.5 (compared between Fig. 5 and Fig. 3). For fish that survived the challenge of a LC_{50} dose of CH_3COONH_4 (N=5), the brain ammonia and glutamine contents returned to control levels (fish injected with saline and killed after 0.5 h) at hour 24 (Fig. 5). For *B. boddaerti* injected with a LC_{50} dose (12 μmol g^{-1} fish) of CH_3COONH_4, six out of the 10 fish died within 20–40 min. The brain ammonia content of fish that succumbed to ammonia toxicity increased to 15 μmol g^{-1} (N=6), but both the brain glutamine and glutamate contents were comparable to those of fish injected with saline and killed after 0.5 h (Fig. 5).

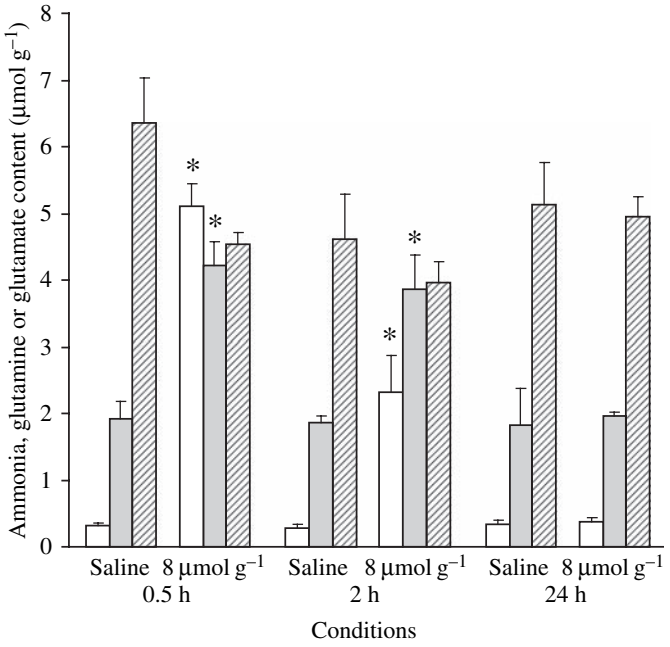

Fig. 3. Effects of an intraperitoneal injection with saline (0.9% NaCl, control) or a sublethal dose (8 μmol g^{-1} fish) of CH_3COONH_4 on the contents (μmol g^{-1}) of ammonia (open), glutamine (filled) and glutamate (hatched) in the brains of *Periophthalmodon schlosseri*. *Significantly different from the corresponding saline-injected control value.

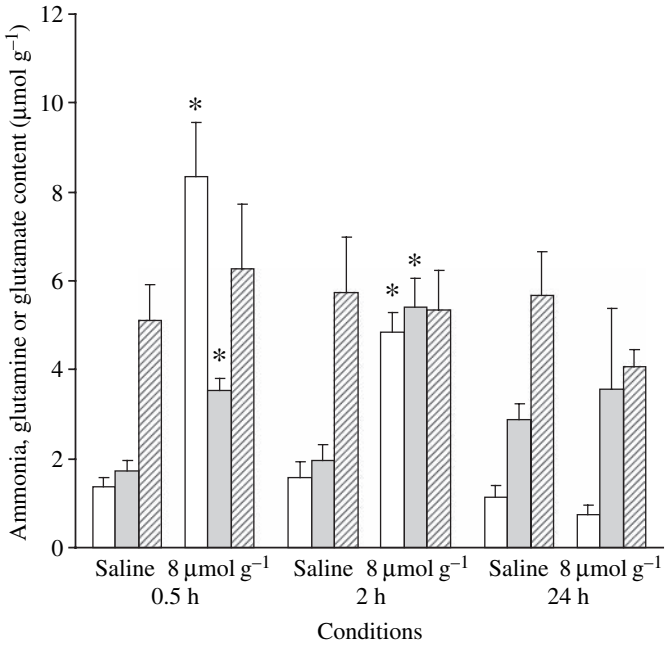

Fig. 4. Effects of an intraperitoneal injection with saline (0.9% NaCl, control) or a sublethal dose (8 μmol g^{-1} fish) of CH_3COONH_4 on the contents (μmol g^{-1}) of ammonia (open), glutamine (filled) and glutamate (hatched) in the brains of *Boleophthalmus boddaerti*. *Significantly different from the corresponding saline-injected control value.

For *P. schlosseri* injected with a lethal dose (20 μmol g^{-1} fish) of CH_3COONH_4 and killed 15 min later, the ammonia and glutamine levels were 13.5±1.13 and 4.53±0.32 μmol g^{-1} tissue, respectively, which were significantly higher than the corresponding values of the saline-injected control killed at the same time (0.31±0.14 and 1.89±0.27 μmol g^{-1} tissue, respectively). Similarly, for *B. boddaerti* injected with 20 μmol CH_3COONH_4 g^{-1} fish and killed 15 min later, the ammonia content (15.0±0.9 μmol g^{-1} tissue) was significantly greater than that of the saline-injected control (1.54±0.11 μmol g^{-1} tissue). However, the brain glutamine content of the former (2.87±0.37 μmol g^{-1} tissue) was not significantly different from that of the latter (2.34±0.28 μmol g^{-1} tissue).

Effects of MSO or MK801

MSO was an inhibitor of GS (transferase) activities determined *in vitro* from the brains of *P. schlosseri* and *B. boddaerti*. For *P. schlosseri*, the brain GS activity was 0.77± 0.02 μmol min^{-1} mg^{-1} protein. In the presence of MSO, the activity decreased significantly to 0.53±0.04 μmol min^{-1} mg^{-1} protein, exhibiting an inhibition of 30.8±3.5%. In the case of *B. boddaerti*, the brain GS activity was 0.56± 0.07 μmol min^{-1} mg^{-1} protein. The activity decreased significantly to 0.36±0.02 μmol min^{-1} mg^{-1} protein in the presence of MSO, with a 36.5±3.4% inhibition. However, MSO (100 μg g^{-1} fish) had no protective effect on *B. boddaerti* injected with a lethal dose (15 μmol g^{-1} fish) of CH_3COONH_4 (Table 1). As for *P. schlosseri*, MSO did not decrease the mortality of fish injected with 20 μmol CH_3COONH_4 g^{-1} fish, but delayed the time to death significantly (Table 1).

MK801 (2 μg g^{-1} fish) did not have any protective effect on *P. schlosseri* or *B. boddaerti* injected with a lethal dose of CH_3COONH_4 (20 and 15 μmol g^{-1} fish, respectively) (Table 1). However, the same batch of MK801 at the same dosage decreased the mortality (from 80% to 50%) and delayed significantly the time to death of goldfish injected with 20 μmol CH_3COONH_4 g^{-1} fish (Table 1).

Discussion

Accumulations of high levels of ammonia and glutamine during 4 days of exposure to sublethal environmental ammonia

Ammonia is toxic and is produced within cells (Campbell, 1973). Therefore fishes usually adopt strategies to ameliorate ammonia toxicity at the cellular and sub-cellular levels when exposed to low concentrations of environmental ammonia or during aerial exposure (Ip et al., 2001, 2004a). By contrast, when the ammonia concentration in the environment reaches levels that reverse the normal NH_3 partial pressure gradient (ΔP_{NH3}), the excretion of endogenous ammonia would be completely impeded. Simultaneously, there would be a net influx of exogenous ammonia from the environment (ammonia-loading). Hence, in high concentrations of environmental ammonia, the fish is confronted with accumulation of both endogenous and exogenous ammonia

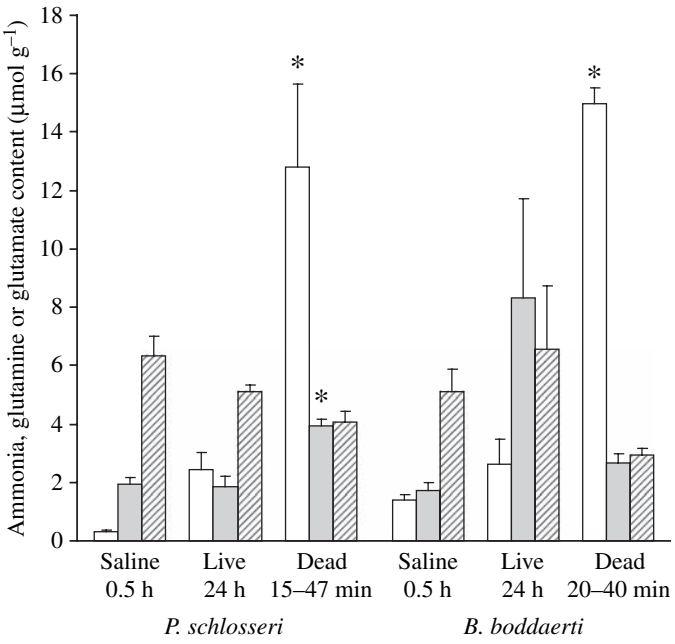

Fig. 5. Contents (μmol g^{-1}) of ammonia (open), glutamine (filled) and glutamate (hatched) in the brains of *Periophthalmodon schlosseri* or *Boleophthalmus boddaerti* killed 15 min after the injection with saline (0.9% NaCl; control), succumbed within 15–50 min to an intraperitoneal injection with a sublethal dose (15 and 12 μmol g^{-1} fish, respectively) of CH_3COONH_3 (dead), or survived a sublethal dose of CH_3COONH_3 (15 and 12 μmol g^{-1} fish, respectively) and killed at hour 24. *Significantly different from values of the saline-injected control and fish survived for 24 h.

and has to defend against ammonia toxicity at both the cellular/subcellular and the branchial/epithelial levels (Ip et al., 2004b).

For *P. schlosseri* and *B. boddaerti* exposed to sublethal concentrations (100 and 8 mmol l^{-1}, respectively) of environmental ammonia, drastic increases in ammonia contents in the brains occurred during the first 24 h. In comparison with *B. boddaerti*, the build up of ammonia in the brain of *P. schlosseri* was less abrupt, but spanned through a longer period (48 h as compared with 12 h in *B. boddaerti*). These could be related to differences in gill morphology and morphometry, and also in the mode of aquatic respiration, between these two mudskippers. *P. schlosseri* has greater terrestrial affinity and is more active on land than *B. boddaerti* (Ip et al., 1993; Kok et al., 1998), because its gills are specialized for aerial exposure (Low et al., 1988, 1990; Wilson et al., 1999, 2000). The gills of *P. schlosseri* have branched filaments, and the filaments have intrafilamentous interlamellar fusions. These fusions form fenestrae that trap water and prevent desiccation of the branchial surfaces when the fish is on land (Low et al., 1988; Ip et al., 2001); however, they render aquatic respiration ineffective. Thus, *P. schlosseri*, but not *B. boddaerti*, often holds air in its buccal cavity during immersion (Y.K.I., unpublished), and this in effect prevented the branchial and buccal respiratory surfaces from having direct contact with the external medium. Consequently, this behavioral adaptation would contribute in part to a less abrupt build up of ammonia in its body, and hence the brain, during environmental ammonia exposure. The apparent 'saturation' of ammonia levels in the brains of *P. schlosseri* and *B. boddaerti* during prolonged exposure to environmental ammonia indicate that adaptations that defend against ammonia toxicity had been initiated. These adaptations might involve a reduction in the net influx of exogenous ammonia at the branchial and epithelial surfaces (Ip et al., 2004b, 2004c), a suppression of endogenous ammonia production (Ip et al., 2004c; Chew et al., 2005b), and detoxification of ammonia to glutamine (Peng et al., 1998; Ip et al., 2004a,b).

In mammals, glutamine synthesis *via* GS is activated in the brain to remove the excess ammonia present when there is an increase in ammonia level (Suárez et al., 2002). Also, an increase in GS expression occurs in the central nervous system of carp exposed to environmental ammonia for 7–60 days (Hernández et al., 1999), and the areas that show induction of GS expression coincide with regions of high glutamate receptor density in the fish brain (Tong et al., 1992; Barnes and Henley, 1994). In patients with urea cycle disorders, hyperammonemic encephalopathy is a consequence of astrocyte swelling and dysfunction resulting from the osmotic effects of astrocyte glutamine synthesis (activated by ammonia) and accumulation (Brusilow, 2002) and the loss in expression of aquaporin 4 and GLUT-1 (Margulies et al., 1999; Desjardins et al., 2001). Cell swelling may be so severe as to cause raised intracranial pressure and, as a consequence, brain herniation, which is the major cause of mortality in patients with acute liver failure. The magnitude of the increase in arterial ammonia concentrations in patients with acute liver failure predicts the evolution of brain herniation in these patients (Clemmensen et al., 1999). In addition, chronic hyperammonemia (unlike acute ammonia intoxication) leads to a depression of excitatory neurotransmission by impairment of NMDA receptor function and by inhibition of NMDA receptor-mediated signal transduction pathways (Felipo and Butterworth, 2002), as demonstrated by a decrease in binding of ^{3}H-MK801 to membranes from rat hippocampus (Marcaida et al., 1995).

In the brains of *P. schlosseri* and *B. boddaerti* exposed to environmental ammonia chronically for 4 days, increases in ammonia levels were also associated with increases in glutamine contents. The increase in brain glutamine content was more abrupt in *P. schlosseri* than in *B. boddaerti*, which correlated well with the presence of a greater activity of GS in the former (185 μmol min^{-1} g^{-1}) than in the latter (119 μmol min^{-1} g^{-1}) (Peng et al., 1998). This could also explain in part the relatively slower build up of ammonia in the brain of *P. schlosseri*. Because glutamine accumulated to extraordinarily high levels in *P. schlosseri* (18.0±2.8 μmol g^{-1}) and *B. boddaerti* (14.5±4.3 μmol g^{-1}) without causing death during the 4 day period, it can be concluded that increased synthesis and accumulation of glutamine resulting in astrocyte swelling and increased ammonia level leading to the impairment of NMDA receptor function were not the main

Table 1. *Effects of a prior injection with methionine sulfoximine or MK801 on the mortality rate and time to death in fish injected with a lethal dose of CH_3COONH_4*

	Conditions	Mortality (%)	Time to death (min)
P. schlosseri	Saline + CH_3COONH_4	100	29.7±1.5
	MSO + CH_3COONH_4	100	50.6±2.6*
	MK801 + CH_3COONH_4	100	26.8±1.2
B. boddaerti	Saline + CH_3COONH_4	70	29.4±5.2
	MSO + CH_3COONH_4	90	14.1±1.7
	MK801 + CH_3COONH_4	100	11.2±0.8*
Carassius auratus	Saline + CH_3COONH_4	80	23.5±3.4
	MK801 + CH_3COONH_4	50	40.3±1.7*

*Significantly different from fish injected with saline + CH_3COONH_4.

Methionine sulfoximine (100 μg g^{-1} fish); MK801 (2 μg g^{-1} fish); time to death (min) in *P. schlosseri* and *B. boddaerti* injected with a lethal dose of CH_3COONH_4 (20 and 15 μmol g^{-1} fish, respectively). Also, effects of MK801 (2 μg g^{-1} fish) on *Carassius auratus* injected with 20 μmol CH_3COONH_4 g^{-1} fish.

causes of chronic ammonia toxicity in these two mudskippers, and that these two mudskippers were able to overcome these deleterious effects as observed in mammals.

How these supposedly deleterious effects were ameliorated in the brains of these two mudskippers awaits future studies, but something can be learned from the crucian carp and common carp. In general, fish brains are imbedded in a jelly-like tissue called meninx, and the cranial room surrounding the brain is much larger than the brain volume. Using *in vivo* T_2 and diffusion-weighed magnetic resonance imaging, Van der Linden et al. (2001) studied anoxia-induced changes in brain volume, free water content and water homeostasis in the crucian carp and common carp. The anoxic crucian carp shows no signs of brain swelling. By contrast, the common carp brain suffers from cellular edema, net water gain and a volume increase (by 6.5%). However, the common carp recovers from anoxia, proving that the changes are reversible and suggesting that the oversized brain cavity allows brain swelling during energy deficiency without a resultant increase in intracranial pressure (Van der Linden et al., 2001). Because mudskippers have high tolerance of environmental hypoxia (Bandurski et al., 1968; Chew et al., 1990, 1992a,b; Ip et al., 1991), adaptations similar to those of crucian carp confronted with hypoxia might occur in the brains of *P. schlosseri* and *B. boddaerti* during chronic environmental ammonia intoxication, and thus there may be a subtle relationship between hypoxia tolerance and ammonia tolerance in certain fishes as suggested by Chew et al. (2005a). On the other hand, if brain swelling indeed occurred in these two mudskippers during ammonia exposure, like the common carp (Van der Linden et al., 2001), the oversized brain cavity would allow for the accumulation of glutamine without causing a build up of cranial pressure.

Surprisingly, the accumulation of glutamine was not accompanied by a depletion of glutamate in brains of both mudskippers throughout the 4 day period. Thus, there must be a simultaneous increase in the rate of glutamate formation, probably through the amination of α-ketoglutarate catalyzed by glutamate dehydrogenase. Because depletions of NADH and α-ketoglutarate in relation to glutamate synthesis could also contribute to ammonia toxicity (see Cooper and Plum, 1987 for a review), it is apparent that *P. schlosseri* and *B. boddaerti* were adapted to overcome these problems during environmental ammonia exposure.

24 h LC_{50} of injected CH_3COONH_4 and effects of injection with a sublethal dose of CH_3COONH_4

In addition to high tolerance of environmental ammonia, *P. schlosseri* and *B. boddaerti* could tolerate high doses of CH_3COONH_4 injected into their peritoneal cavities. The 24 h LC_{50} of injected CH_3COONH_4 for *P. schlosseri* (15.6 μmol g^{-1}) was slightly higher than that for *B. boddaerti* (12.3 μmol g^{-1}). Therefore, it can be concluded that the exceptionally high environmental ammonia tolerance in *P. schlosseri* is a result of not only its high capacity of active NH_4^+ excretion (up to at least 30 mmol l^{-1} NH_4Cl pH 7; Randall et al., 1999; Chew et al., 2003; Ip et al., 2004c), but also its ability to tolerate high doses of ammonia introduced into its body. While the capability of *B. boddaerti* to actively excrete NH_4^+ is inferior to that of *P. schlosseri* (8 mmol l^{-1} NH_4Cl pH 7; Y.K.I., unpublished; Chew et al., 2003), the ability to tolerate high doses of injected-ammonia was apparently adequate to provide it with an environmental ammonia tolerance greater than those of many fish species.

After a sublethal dose of CH_3COONH_4 (8 μmol g^{-1} fish) was injected into *P. schlosseri* or *B. boddaerti*, there were significant increases in ammonia and glutamine levels in their brains at hours 0.5 and 2. Similar to environmental ammonia exposure, glutamine synthesis was associated with no glutamate depletion. The highest levels of ammonia and glutamine (at hour 0.5) obtained for *P. schlosseri* and *B. boddaerti* injected with 8 μmol g^{-1} CH_3COONH_4 were lower than those for fishes exposed to sublethal concentrations of environmental ammonia. This can be explained by the lack of a reversed ΔP_{NH3} to impede ammonia excretion in the former, and ammonia could be excreted immediately after the experimental fish were returned to water. By hour 24, both

ammonia and glutamine contents returned to control levels, indicating that these two mudskippers were able to effectively tolerate and/or defend against ammonia toxicity before the complete excretion of the injected ammonia.

At hour 0.5, the ammonia levels in the brains of *P. schlosseri* and *B. boddaerti* injected with 8 μmol CH_3COONH_4 g^{-1} fish reached 5.11 and 8.36 μmol g^{-1}, respectively. By contrast, mammalian brains can rarely tolerate >1 μmol g^{-1} of ammonia; encephalopathy occurs usually at 3 μmol g^{-1} (Cooper and Plum, 1987; Felipo and Butterworth, 2002). These results suggest that the brains of these two mudskippers could tolerate very high levels of ammonia, at least for a short period. Because the brain ammonia levels of these experimental fishes drastically decreased during the first 2 h and because there was no mortality at hour 24, it can be concluded that the effectiveness in removal of ammonia from, and/or detoxification of ammonia in, their brains were crucial factors in determining if the fish would survive or die.

Effects of injection with a LC_{50} or lethal dose of CH_3COONH_4

After the injection of a LC_{50} dose (15 and 12 μmol g^{-1} fish, respectively) of CH_3COONH_4 into *P. schlosseri* and *B. boddaerti*, 50–60% of fishes succumbed to ammonia toxicity within 15–50 min. For *P. schlosseri* and *B. boddaerti* that died, the ammonia contents in the brains reached levels (12.8 and 14.9 μmol g^{-1}, respectively) much greater than those obtained (5.11 and 8.36 μmol g^{-1}, respectively) from fish injected with a sublethal (8 μmol g^{-1} fish) dose of CH_3COONH_4 at hour 0.5. Similar observations were made on fish killed 15 min after the injection with a lethal dose (20 μmol g^{-1} fish) of CH_3COONH_4. Apparently, *P. schlosseri* and *B. boddaerti* could tolerate higher levels of ammonia in their brains (18 and 14.5 μmol g^{-1}, respectively, on day 4) during chronic environmental ammonia exposure than during acute ammonia intoxication. Thus, it can be concluded that the rate of ammonia build up in the brain, which was much greater in fish injected with a sublethal or lethal dose of CH_3COONH_4 (death occurred within 15–50 min), was the determining factor of ammonia toxicity in these two mudskippers. In addition, our results suggest that some adaptive adjustments would have occurred at the cellular and subcelluar levels in the brains of these two mudskippers when the rate of ammonia increase was relatively slower during the 4 day period of exposure to environmental ammonia.

Surprisingly, the glutamine levels in the brains of fishes succumbed to a lethal dose of CH_3COONH_4 and fish killed 0.5 h after being injected with a sublethal dose of CH_3COONH_4 were comparable. Therefore, it can be concluded once again that glutamine synthesis and accumulation in the brain was not the major cause of death in *P. schlosseri* and *B. boddaerti* confronted with acute ammonia toxicity. Because the brain ammonia contents were extraordinarily high, membrane depolarization (Sugden and Newsholme, 1975) resulting from high concentrations of ammonia (NH_4^+) could be the main cause of ammonia toxicity in these two mudskippers. Since membrane depolarization can lead to the activation of NMDA receptors (Fan and Szerb 1993; Hermenegildo et al., 2000), efforts were made in subsequent experiments to determine if MK801 had a protective effect on these two mudskippers against acute ammonia toxicity (see below).

The glutamine synthetic capacities in the brains of *P. schlosseri* and *B. boddaerti* were high, because there were high GS activities in their brains (Peng et al., 1998) that could accumulate glutamine to exceptionally high levels during 4 days of exposure to environmental ammonia. However, the glutamine contents in the brains of *P. schlosseri* and *B. boddaerti* succumbed to acute ammonia toxicity were relatively low. Why would there be such a discrepancy? The reason became obvious when we evaluated the rates at which glutamine synthesis and accumulation had occurred. The rates of glutamine synthesis in these two mudskippers succumbed within a 15–50 min period were 3–10-fold greater than those in fishes exposed to sublethal concentrations of environmental ammonia for a period of 4 days. So, for fish confronted with acute ammonia toxicity, there was a drastic increase in the rate of glutamine synthesis in fish succumbed to ammonia toxicity within a short period (15–50 min), but it was apparently inadequate to remove ammonia at a rate that could prevent the ammonia content from increasing to an intolerable level. Thus, it can be concluded that ammonia *per se*, and not glutamine synthesis and its accumulation, was the major cause of death in *P. schlosseri* and *B. boddaerti* injected intraperitoneally with CH_3COONH_4.

MSO had no protective effects on B. boddaerti *but delayed the time of death in* P. schlosseri

MSO is an irreversible inhibitor (Folbergrova, 1964) of GS in the mammalian system. In patients suffering from hyperammonemic encephalopathy, MSO ameliorates ammonia toxicity by decreasing the levels of glutamine synthesized, and therefore prevents cell swelling due to glutamine accumulation; hence, death is prevented *via* the prevention of brain edema (Brusilow, 2002; Butterworth, 2002). In the case of rats injected intraperitoneally with CH_3COONH_4, the protective effects of MSO can be a result of the prevention of glutamate release and the avoidance of activation of NMDA receptors (Kosenko et al., 1994). Our results confirm that MSO inhibited GS activities from the brains of *P. schlosseri* and *B. boddaerti*. However, MSO, at a dosage (100 μg g^{-1} fish) that has protective effects on mammals, did not protect *B. boddaerti* against the ammonia toxicity caused by the injection with 15 μmol CH_3COONH_4 g^{-1} fish. In fact, the mortality increased from 70% to 90% with the administration of MSO. In the case of *P. schlosseri* injected with 20 μmol CH_3COONH_4 g^{-1} fish, the administration of MSO did not reduce the mortality rate (100%) but prolonged the time to death significantly. These results are in support of the above-mentioned conclusion that glutamine synthesis and accumulation was not the major cause of death in these two mudskippers exposed to acute ammonia intoxication, although glutamine synthesis and accumulation

might have a slight exacerbating effect on ammonia toxicity in *P. schlosseri*.

MK801 did not have protective effects on both mudskippers

In rats, the mortality due to acute ammonia toxicity *in vivo* is prevented by the administration of a wide range of NMDA receptor antagonists (Marcaida et al., 1992; Hermenegildo et al., 1996). Studies using *in vivo* cerebral microdialysis show that acute ammonia exposure results in the activation of NMDA receptor-coupled nitric oxide-cyclic GMP signal transduction pathway in the rat brain (Hermenegildo et al., 2000). Administration of MK801, a NMDA receptor antagonist, at a dosage of 2 $\mu g\ g^{-1}$ leads to significant improvement in clinical grading and slowing of electroencephalogram activity and provide significant protection in mouse given a lethal dose of ammonia (Marcaida et al., 1991). Recently, Tsui et al. (2004) reported (as unpublished results) that MK801 (2 $\mu g\ g^{-1}$ fish) injected 15 min prior to the injection of CH_3COONH_4 (21 $\mu mol\ g^{-1}$ fish) had a protective effect on the weatherloach *Misgurnus anguillicaudatus*, as reflected by a decrease in mortality from 60% to 0%. By contrast, MK801 (2 $\mu g\ g^{-1}$ fish) had no protective effect on *P. schlosseri* and *B. boddaerti* injected with a lethal dose of CH_3COONH_4 (15 and 20 $\mu mol\ g^{-1}$ fish, respectively). Rather, MK801 appears to exacerbate ammonia toxicity in *B. boddaerti* because the mortality increased from 70% to 100%. Since negative results were obtained, it became imperative to prove that the same batch of MK801 used in this study had a positive effect on another fish species. Indeed, when the same batch of MK801 was injected into the goldfish *Carassius auratus* at the same dosage (2 $\mu g\ g^{-1}$ fish) prior to the injection of CH_3COONH_4 (20 $\mu mol\ g^{-1}$ fish), there was a decrease in mortality (from 80% to 50%) and a significant delay in the time of death for fish that succumbed to ammonia toxicity.

Thus, our results reveal for the first time that activation of NMDA receptors might not be the explanation for acute ammonia toxicity in the brains of *P. schlosseri* and *B. boddaerti*. Indirectly, these results are in support of the proposition that astrocyte swelling might not have occurred in the brains of these two mudskippers. This is because NMDA receptors are activated by extracellular glutamate, and astrocyte swelling can lead to an increase in extracellular glutamate concentration under cell-culture conditions (Kimelberg et al., 1990) due to increase in glutamate release and/or decrease in glutamate uptake. In animals, an increase in intracellular NH_4^+ would lead to changes in membrane potential (Sugden and Newsholme, 1975) that would result in the reversal of glutamate transport and hence an increase in the extracellular glutamate concentration (Szatkowski et al., 1990). In addition, membrane depolarization can lead to the removal of the Mg^{2+} block on NMDA receptors and result in their activation (Fan and Szerb, 1993). Therefore, our results also suggest that *P. schlosseri* and *B. boddaerti* could have special abilities to control the intracellular ammonia level in their brains despite drastic increases in brain ammonia contents (intracellular + extracellular). NH_4^+ can replace K^+ in the facilitated diffusion of K^+ through K^+ channels and/or active transport of K^+ through Na^+, K^+-ATPase; both these processes have direct or indirect deleterious effects on the membrane potential of a cell. In view of the high levels of ammonia in the brains of *P. schlosseri* and *B. boddaerti* exposed to chronic and acute ammonia toxicity and the lack of protective effect from MK801, it can be deduced that either membrane depolarization occurred but did not lead to activation of NMDA receptors, or membrane potentials were resilient to NH_4^+ interference due to the presence of K^+ channels and Na^+, K^+-ATPase with high substrate specificities for K^+, in the brains of these two mudskippers.

In conclusion, glutamine synthesis and accumulation in the brain was not the major cause of death in *P. schlosseri* and *B. boddaerti* confronted with chronic or acute ammonia toxicity. In addition, activation of NMDA receptors was not the major cause of death during acute ammonia intoxication. Thus, our results suggest for the first time that the mechanisms of and adaptations to defend against ammonia toxicity in the brains of these two mudskippers (and possibly other fish species with exceptionally high ammonia tolerance) are different from those in mammalian brains.

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