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Review

Dogmas and controversies in the handling of nitrogenous wastes: Ammonia tolerance in the oriental weatherloach *Misgurnus anguillicaudatus*

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Summary

The oriental weatherloach *Misgurnus anguillicaudatus* is an extremely ammonia-tolerant fish. Many ammonia-protection mechanisms have been reported in this fish. Six strategies used by this fish to deal with the problem of excess ammonia are described. The fish can (1) reduce ammonia production through reduction in protein and/or amino acid catabolism; (2) reduce ammonia production and obtain energy through partial amino acid catabolism leading to alanine formation; (3) detoxify ammonia to glutamine; (4) tolerate very high ammonia levels in its tissues; (5) get rid of ammonia as NH_3 gas and, probably, (6) possesses background K^+ channels that are impermeable to NH_4^+. The effects of extracellular ammonia on the contraction performance of the heart from this fish were found to be the same as in rainbow trout, an ammonia-sensitive fish. It suggests that the hearts of most, if not all, fish species are protected against ammonia. MK-801, an NMDA receptor blocker, was found to have a protective effect against ammonia intoxication in the oriental weatherloach, which suggests that the NMDA receptor, as in mammals, is involved in ammonia toxicity.

Key words: oriental weatherloach, *Misgurnus anguillicaudatus*, ammonia, glutamine, alanine, K^+ channel, membrane potential, NMDA receptor.

Introduction

The oriental weatherloach *Misgurnus anguillicaudatus* (Order: Cyprinifimormes; Family: Cobitidae), is a freshwater teleost fish that inhabits streams, ditches and rice paddy fields, preferably with soft muddy bottom (Man and Hodgkiss, 1981). It is widely distributed in Asia, from China to Myanmar. It is a facultative air-breather (McMahon and Burggren, 1987). In addition to its gills, it uses its intestine for gaseous exchange. To breathe air, it comes to the water surface, gulps a mouthful of fresh air and releases bubbles of exhaled air from its vent. During a drought period, it actively buries itself in the mud to keep moist, and presumably relies solely on air-breathing for respiration.

High ammonia levels could be a problem in the natural habitat of this weatherloach, especially for those living in paddy fields, where fertilizers are heavily utilized. The fish must be very ammonia-tolerant in order to survive. In fact, its 96 h LC_{50} of NH_4Cl at pH 6.7 at 20°C was found to be around 75 mmol l^{-1} (Y. Cheung, W. K. Tang and T. K. N. Tsui, unpublished data). The fish can also survive for weeks, or even months, out of water, where ammonia excretion is impeded. Thus, study of this fish provides an opportunity for us to understand more about the mechanisms of ammonia tolerance, as well as the nature of ammonia toxicity.

Many strategies dealing with the problem of ammonia have been described (Ip et al., 2001b, 2003). Different ammonia-tolerant fishes use different strategies to protect against ammonia. It is interesting, however, that many of these strategies can be found in a single species – the oriental weatherloach. This paper is a review of our current understanding of how this fish tolerates ammonia. Six strategies, five confirmed and one suspected, that have been observed in the fish are described, and the nature of ammonia toxicity is also discussed.

Reduction in amino acid catabolism and proteolysis

Ammonia is continuously being produced in animals, mainly by general protein and amino acid catabolism as well as breakdown of muscle adenylates. Protein and amino acids

are the major energy sources for long-term muscular activities (Moon and Johnston, 1981). Endogenously produced ammonia has to be removed from the body. In fish, this is normally done *via* the gills, down a concentration gradient into the surrounding water. However, under certain conditions such as lack of water, high ambient pH or high external ammonia levels, the ammonia excretion process can be greatly impeded (for a review, see Ip et al., 2001b). As a result, ammonia can potentially be accumulated in the body and reach a level that is harmful to the fish.

One way to deal with the problem would be to reduce the amount of ammonia produced in the body. When exposed to air, the rates of ammonia and urea excretion in the oriental weatherloach decrease significantly (Chew et al., 2001; Tsui et al., 2002). Ammonia levels in the fish liver, muscle and plasma increase as a consequence of the reduced ammonia excretion (Chew et al., 2001). The amino acid profiles of the fish tissues were analyzed, and alanine and glutamine levels were found to be significantly increased in the liver and muscle. Alanine, glutamine and urea contain amino groups and therefore can be considered as ammonia-equivalents (glutamine and urea actually each contain two ammonia groups). The results could be used to construct a balance sheet on ammonia-equivalents of the fish subjected to normal and aerial conditions (Chew et al., 2001). The reduction in excretion of ammonia-equivalents was 99 μmol more than the accumulation of ammonia-equivalents in the body after 24 h of aerial exposure for a 15 g fish. The discrepancies increased with time, reaching 283 and 558 μmol after 48 h and 72 h, respectively. The levels of ammonia-equivalents accumulated in the body, however, appeared to reach a plateau after 48 h. It could therefore be deduced that there had been a reduction in amino acid catabolism and/or proteolysis, which resulted in the reduction in ammonia production.

A similar phenomenon has been reported for the air-exposed mudskippers *Periophthalmodon schlosseri* and *Boleophthalmus boddaerti* in constant darkness, as well as the air-exposed four-eyed sleeper *Bostrichthys sinensis* (Lim et al., 2001; Ip et al., 2001a). These fish species are also very ammonia-tolerant.

Partial amino acid catabolism leading to alanine formation

The oriental weatherloach actively seeks a suitable place to bury itself into the mud when there is a lack of water. Even during the initial burying period, the fish will still try to move around in the mud in order to find a position that it likes. All these muscular movements require large amount of energy. Most of the energy is probably derived from protein and amino acid catabolism (Moon and Johnston, 1981); however, there is a problem associated with this type of catabolism during aerial exposure. Ammonia is produced from complete protein and amino acid catabolism, and when there is a lack of water, ammonia excretion is greatly decreased. Therefore, complete protein and amino acid catabolism could potentially result in accumulation of ammonia to toxic levels in the body after vigorous muscular activity in air.

Partial amino acid catabolism has been proposed as a solution to this problem (Ip et al., 2001b). Many amino acids can be partially catabolized without releasing ammonia, forming alanine. For example, a mole of glutamate can be converted to alanine, yielding 10 moles of ATP; the ATP yield would be higher if the starting substrate were arginine or proline (Hochachka and Guppy, 1987). The concentration of alanine in the oriental weatherloach liver increased by more than twofold after 12 h of aerial exposure (Chew et al., 2001), which shows that partial amino acid catabolism leading to alanine formation takes place in this fish. This strategy would help alleviate the problem of ammonia and provide energy for the fish to be active when it is out of water.

In mammals, alanine can also act as a carbon carrier for inter-organ carbon transport (Felig, 1975). When delivered to the heart, for example, it can be converted to pyruvate for further oxidation. In liver, it can act as a glucose precursor in gluconeogenesis (Felig, 1975). Thus, alanine is an important alternative energy source to glycogen, which can then be spared for utilization in the future.

Partial amino acid catabolism leading to alanine formation was reported in the giant mudskipper *Periophthalmodon schlosseri*, exposed to land conditions under a 12 h:12 h light:dark regime (Ip et al., 2001c). This mudskipper spends a lot of time out of water and can be very active while on land. In contrast, another mudskipper, *Boleophthalmus boddaerti*, did not accumulate alanine in its tissue and used glycogen instead for energy. The snakehead, *Channa asiatica* is another fish that has been reported to form high levels of alanine (Chew et al., 2003).

Glutamine formation

While the above two solutions are strategies that reduce ammonia production during adverse conditions, glutamine formation is a true ammonia detoxification strategy. Glutamine is formed from glutamate and NH_4^+, catalyzed by the enzyme glutamine synthetase. Glutamate is, in turn, formed from α-ketoglutarate and NH_4^+, catalyzed by the enzyme glutamate dehydrogenase. Thus, if the starting substrate is α-ketoglutarate, by forming one mole of glutamine, two moles of NH_4^+ are effectively detoxified (Campbell, 1973). In the oriental weatherloach, the glutamine levels in the muscle, liver and brain increased significantly after 48 h of aerial exposure (Chew et al., 2001; K. C. Hiong and Y. K. Ip, unpublished data). The liver glutamate dehydrogenase activities in the deamination direction decreased significantly upon aerial exposure (Chew et al., 2001). Thus glutamate catabolism was slowed down during aerial exposure, enhancing glutamine formation in the oriental weatherloach. A problem associated with this detoxification strategy is that two moles of ATP are hydrolyzed for every one mole of glutamine formed. The oriental weatherloach can adopt this strategy during aerial

exposure probably because it stays motionless during the later stages of aerial exposure. During the early stage of aerial exposure, it has to actively bury itself into the mud and look for a suitable position. Once it has found a good position, it stays relatively quiet and so does not require much energy for muscular activities.

Glutamine accumulation has been reported in many other fish species, especially in the brain tissue. The brain glutamine levels increased significantly upon exposure to high ambient ammonia in the common carp *Cyprinus carpio* (Dabrowska and Wlasow, 1986), the goldfish *Carassius auratus* (Levi et al., 1974) and rainbow trout *Oncorhynchus mykiss* (Arillo et al., 1981). Studies on the mudskippers *Periophthalmodon schlosseri* and *Boleophthalmus boddaerti* (Peng et al., 1998), as well as the four-eyed sleeper *Bostrichthys sinensis* (Anderson et al., 2002), also revealed increased levels of glutamine in their muscle, liver and plasma, in addition to the brain, during exposure to high ambient ammonia. Furthermore, the marble goby *Oxyeleotris marmoratus* (Jow et al., 1999) and the four-eyed sleeper (Ip et al., 2001a) detoxified endogenously produced ammonia to glutamine during aerial exposure. Thus detoxification of ammonia to glutamine seems to be an important strategy utilized by fish.

Tissue ammonia tolerance

Ammonia is well known to be toxic to cells. Inside an animal body, most of the ammonia is in the form of NH_4^+ because the physiological pH is around 7.0–7.8 (pK value for ammonia is about 9.5). However, since the cell membrane is largely impermeable to NH_4^+, most ammonia enters cells in the form of NH_3. Being a weak base, NH_3 binds with a proton and raises the intracellular pH upon entering the cell. This intracellular alkalinization would affect the normal biochemical reactions, as enzymes are generally pH-dependent. In addition, NH_4^+ inhibits some important enzymes involved in metabolism, such as isocitrate dehydrogenase and α-ketoglutarate dehydrogenase (Cooper and Plum, 1987). Also, NH_4^+ stimulates phosphofructokinase-1 and, thus, glycolysis (Sugden and Newsholme, 1975). It was found that the lactate and pyruvate levels in plasma of rainbow trout exposed to high ambient ammonia increased (Fromm and Gillette, 1968).

Due to its harmful effects, the ammonia levels at the tissue level are normally kept low, especially in the brain, which is very sensitive to ammonia. The total ammonia levels in the oriental weatherloach, however, were found to be very high (Chew et al., 2001; Tsui et al., 2002), climbing up to about 5 mmol l^{-1} in the plasma upon aerial or ammonia exposure. The levels can reach >15 μmol g^{-1} in the muscle and liver, which is one of the highest tissue ammonia levels reported. It is not clear why the oriental weatherloach can tolerate such high ammonia levels in its tissues. It is suspected that its enzymes involved in metabolism are relatively insensitive to NH_4^+, when compared to enzymes from other animals. Also, it must possess mechanisms for regulating intracellular pH disturbed by ammonia entrance.

NH_3 volatilization

The oriental weatherloach is able to get rid of ammonia in the form of NH_3 gas (Tsui et al., 2002). This is indeed a very special strategy for dealing with the problem of ammonia. The ammonia level in the body, as mentioned above, became very high when the fish was exposed to aerial and high ammonia conditions (Chew et al., 2001; Tsui et al., 2002). The pH of the plasma also became significantly higher when exposed to aerial conditions. Thus, the amount of NH_3 in the plasma increased and a NH_3 concentration gradient was set up. NH_3 can pass through either the body surface or the digestive tract to reach outside and is excreted as gas. The high ammonia levels in the body tissues might be a prerequisite for NH_3 volatilization to occur. In addition, the pH of the body surface, as well as the mucosal surface of the anterior part of the digestive tract, became significantly higher upon aerial exposure (Tsui et al., 2002). These also enhance the process of volatilization because more ammonia exists as NH_3 at higher pH values.

It is interesting to note that the wall of the digestive tract of the oriental weatherloach is very thin. Also, the whole digestive tract is heavily vascularized, so it is ideal for respiratory gaseous exchange. The structure of the digestive tract allows NH_3 volatilization to take place. It was observed under the electron microscope that blood capillaries were located within the epithelia of the digestive tract, extremely close to the lumen (P. Laurent and J. M. Wilson, unpublished observation). A similar vessel arrangement has been observed in the buccal skin of the mudskipper *Periophthalmodon schlosseri* (Randall et al., 1999), which uses the buccal skin for respiratory gaseous exchange. This is very unusual as blood capillaries are normally not found within the epithelia.

A similar phenomenon has been reported in another teleost, the mangrove killifish *Rivulus marmoratus* (Frick and Wright, 2002). This process of NH_3 volatilization is actually better understood in arthropods. For example, there is periodic NH_3 volatilization in the isopod *Porcellio scaber* (Wright and O'Donnell, 1993), and NH_3 volatilization has also been reported in certain terrestrial crabs, which carry out the process by alkalinization of urine (De Vries and Wolcott, 1993; Varley and Greenaway, 1994).

Highly selective background K^+ channels

NH_4^+ and K^+ ions share similar radii. Thus, NH_4^+ can also pass through K^+ channels. It has long been known that NH_4^+ can cause depolarization in excitable tissue such as the neural tissues (Binstock and Lecar, 1969). K^+ and thus NH_4^+ ions are permeable to membranes even in the resting state (i.e. not during an action potential). There must be some pathways, or channels, in the membrane *via* which these ions pass through, although because they carry a positive charge, K^+ and NH_4^+ cannot possibly pass through the lipid bilayer of the membrane directly. The pathways have long remained a mystery, only being referred to as 'background', 'leak' or 'resting' channels. Whether this K^+ current exists as an independent entity, or as a residual flux through other pathways, remained controversial

until the cloning of KCNKØ from *Drosophila melanogaster* (Goldstein et al., 1996). Since then, many other background channels have been cloned from various animal species, including human, and the list is still growing. These background K^+ channels contribute strongly to the membrane permeability to K^+ and NH_4^+ ions. As a result, the ratio between the intracellular and extracellular K^+ concentrations determines the membrane potential according to the Nernst equation. If there were an increase in the extracellular NH_4^+ concentration, the effect would be similar to an increase in extracellular K^+ concentration, which would result in depolarization of the membrane.

The resting membrane potential of the white muscle of the brown trout *Salmo trutta* was depolarized when exposed to copper and low pH, which resulted in elevated plasma ammonia level, from a control value of –86.5 mV to –52.2 mV (Beaumont et al., 2000). Upon exposure to sublethal ammonia concentrations, the swimming performance of rainbow trout was found to have decreased significantly (Shingles et al., 2001). The critical swimming speed (U_{crit}) reduced from 2.23 *BL* s^{-1} to 1.61 *BL* s^{-1} (where *BL* is body length). The decrease in swimming performance was attributed to a partial depolarization of the white muscle.

There is no doubt that ammonia can cause depolarization in excitable cell tissues. But how can the oriental weatherloach tolerate such high ammonia levels in its plasma and its tissues? Would NH_4^+ also cause depolarization in the tissues of this fish? We hypothesize that the background K^+ channels in the neuronal and muscular tissues of the oriental weatherloach are very selective for K^+ so that NH_4^+ can barely, if at all, pass through them. If the background K^+ channels are impermeable to NH_4^+, any change in the extracellular NH_4^+ concentration would not affect the membrane potential. In order to test this hypothesis, the K^+ and NH_4^+ concentrations were measured, and the results fitted into the Nernst equation. If the background K^+ channel is also permeable to NH_4^+, the E_K value should be very similar to the E_{NH4} value. Table 1 shows that this is not the case. The E_K and E_{NH_4} values were always different for brain and muscle under the different conditions tested (Table 1), which means that the distributions of K^+ and NH_4^+ in the two tissues were different, suggesting that the background K^+ channels in these tissues were relatively impermeable to NH_4^+ under the various conditions.

According to the model presented by Wright et al. (1988), if cell membranes have a significant permeability to NH_4^+ (though not necessarily as large as their permeability to NH_3), then the membrane potential would dictate the distribution of ammonia across the membrane. In the present study, the E_{NH_4} values for both muscle and brain were too positive compared to expected membrane potential (Table 1). This means that the distribution of ammonia across the plasma membrane of the two tissues is not according to the membrane potential, which again agrees with the hypothesis that the oriental weatherloach possesses background K^+ channels that are selective against NH_4^+.

Table 1. *Nernst equilibriums of K^+ and NH_4^+ across the plasma membrane of the muscle and brain of* Misgurnus anguillicaudatus *exposed to control and 50 mmol l^{-1} NH_4Cl* conditions

	Control		NH_4Cl	
	E_K	E_{NH_4}	E_K	E_{NH_4}
Muscle	–66.9	–35.2	–67.9	–33.0
Brain	–60.7	10.4	–62.8	12.2

E_K and E_{NH_4}, Nernst equilibriums of K^+ and NH_4^+ (mV).

Animals were exposed to control conditions or 50 mmol l^{-1} NH_4Cl, pH 6.7, for 48 h at 20ºC.

To further test this hypothesis, the effects of extracellular ammonia on heart muscle contraction were studied. Ventricular trabeculae were taken from hearts of oriental weatherloach and rainbow trout. It was thought that the background K^+ channels in rainbow trout were permeable to NH_4^+ and those in oriental weatherloach were not. The extracellular ammonia was expected to cause a larger decrease in the contraction performance of ventricular trabeculae from rainbow trout than from weatherloach. NH_4^+ concentrations of up to 5 mmol l^{-1} were used for the experiments, as levels of 5 mmol l^{-1} ammonia have been reported in the plasma of oriental weatherloach (Tsui et al., 2002). However, the results were surprising. The effects of extracellular ammonia on the development of tension in the ventricular trabeculae from both species were the same (Fig. 1).

Even when exposed to a total ammonia concentration approximately 50% of the 96 h LC_{50} for ammonia (288 μmol l^{-1}) at pH 8.4, the total plasma ammonia concentration of rainbow trout was only 0.44 mmol l^{-1} after swimming at 0.75 *BL* s^{-1} overnight (Shingles et al., 2001). At similar plasma ammonia concentrations, brown trout *Salmo trutta* already showed significant depolarization in its white muscle plasma membrane (Beaumont et al., 2000). In fact, both rainbow trout and brown trout showed decreased swimming performance while having such plasma ammonia concentration (Beaumont et al., 1995, 2000; Shingles et al., 2001). Being excitable muscle tissue, one would expect the heart of rainbow trout to depolarize and contract less well than that of oriental weatherloach in 0.5 mmol l^{-1} extracellular total ammonia. However, at 0.5 mmol l^{-1} total ammonia, the isometric force developed by ventricular trabeculae of rainbow trout and weatherloach were 85% and 91% of their corresponding control value, respectively (Fig. 2), which were very similar and very close to their control values.

A possible explanation for the results is that hearts from both ammonia-sensitive and ammonia-tolerant fish species have protective mechanisms against ammonia. The observed decrease in swimming performance in rainbow trout and brown trout was probably due to depolarization in the white muscle only, possibly because the white muscle, and not the heart, of ammonia-sensitive species is depolarized by ammonia.

But why do ammonia-sensitive species have such a

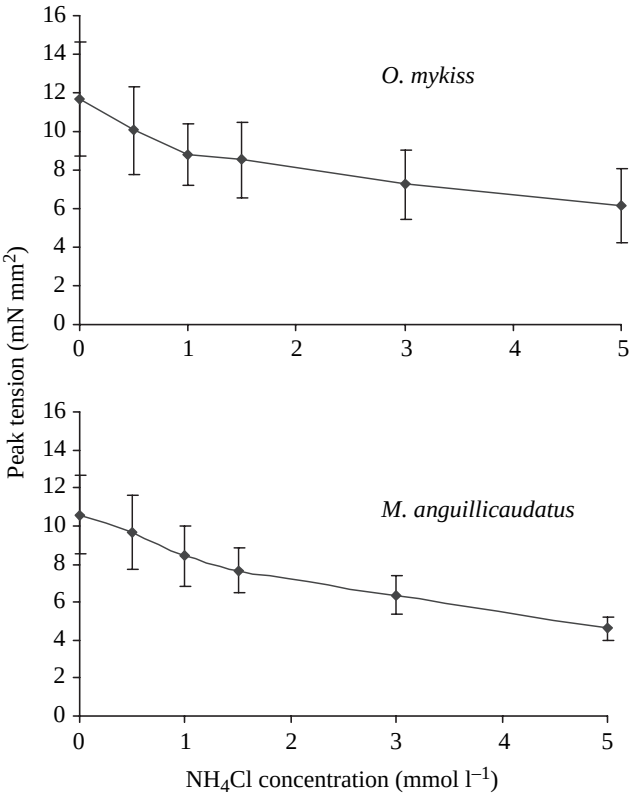

Fig. 1. Effects of different concentrations of NH_4Cl on isometric tension development (actual values) of ventricular trabeculae of *O. mykiss* and *M. anguillicaudatus*. Values are means ± S.E.M. (*N*=6).

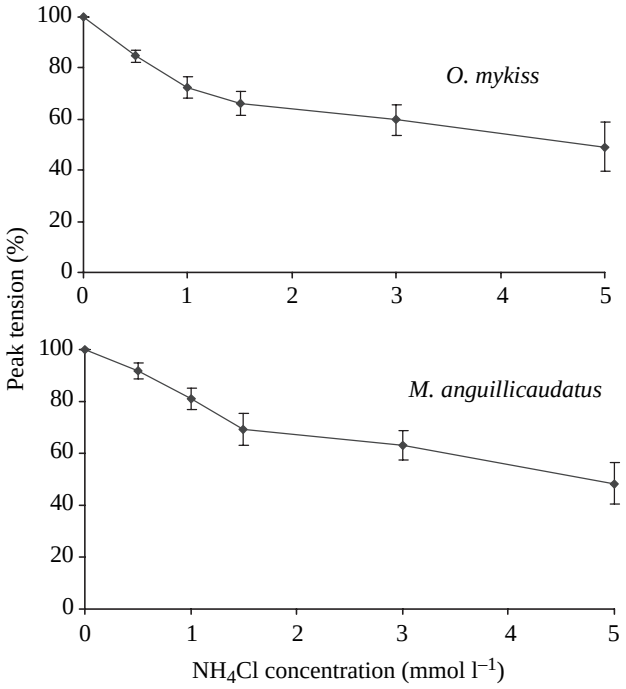

Fig. 2. Effects of different concentrations of NH_4Cl on isometric tension development (relative to control value) of ventricular trabeculae of *O. mykiss* and *M. anguillicaudatus*. Values are means ± S.E.M. (*N*=6).

protective mechanism against ammonia in the heart? It has been reported that, after feeding, the ammonia concentration in the plasma of rainbow trout increased to a level similar to that associated with death due to environmental ammonia exposure (Wicks and Randall, 2002). The fish may tolerate not protecting its white muscle from depolarization and just stop swimming around after eating, but its heart must have some means of protection against the elevated plasma ammonia levels.

An alternative explanation for the results is that there was actually no protective mechanism against ammonia in hearts from either species. The decrease in isometric force observed in the ventricular trabeculae at 0.5 mmol l^{-1} total plasma ammonia actually contributes to the decrease in swimming performance of fish exposed to external ammonia.

In either event, the heart does not seem to be the organ where ammonia toxicity acts in rainbow trout. Total plasma ammonia levels would never be higher than 1 mmol l^{-1}, and at this concentration, the heart still could function at 73% of its normal performance (Fig. 2), which would be unlikely to be low enough to kill the fish. Ammonia most likely kills rainbow trout by affecting its nervous system.

Ammonia toxicity in brain

Ammonia seems to have its effects mainly on the central nervous system of vertebrates. There is evidence that high ammonia levels in the brain lead to high levels of extracellular glutamate by increasing glutamate release or/and decreasing glutamate synaptic reuptake (Rao et al., 1992; Bosman et al., 1992; Schimdt et al., 1993). NMDA-type glutamate receptors have also been proposed to be involved during ammonia intoxication in mammals (Marcaida et al., 1992). Excessive activation of NMDA receptors during ammonia intoxication leads to excessive influx of Ca^{2+} and Na^+. The increased intracellular Ca^{2+} concentration activates Ca^{2+}-dependent enzymes and a cascade of reactions takes place that eventually result in cell death. Blocking NMDA receptor with its antagonist, MK-801, has been shown to protect mice from acute ammonia intoxication (Marcaida et al., 1992; Hermenegildo et al., 1996).

Using a microdialysis technique, Hermenegildo and co-workers (2000) showed that activation of receptors actually preceded the increase in extracellular glutamate levels during acute ammonia intoxication, and MK-801 prevented the increase in extracellular glutamate level. Thus extracellular glutamate increase was the result, rather than the cause, of excessive activation of the NMDA receptor. The resulting increase in extracellular glutamate level following NMDA receptor activation could bring about further activation of the NMDA receptor.

How does ammonia cause excessive activation of NMDA receptors? The best explanation is that NH_4^+ causes depolarization in the neuronal membrane, which results in removal of Mg^{2+} that normally blocks the NMDA receptor

channel (Hermenegildo et al., 2000; Felipo and Butterworth, 2002). Therefore, any tonic activation of NMDA receptor during normal functioning of the brain would cause a larger influx of Ca^{2+} than usual, which would eventually cause excessive NMDA receptor activation.

MK-801 can also prevent ammonia toxicity in the oriental weatherloach. By injecting MK-801 (2 mg kg^{-1}) 15 min prior to injection of ammonium acetate (21 mmol kg^{-1}), the mortalilty of the weatherloach dropped to 0%, compared to 60% when fish was not given MK-801 (T. K. N. Tsui, unpublished data). This indicates that NMDA receptors are also involved in ammonia toxicity in fish brain. If indeed this fish possesses background K^+ channels that are impermeable to NH_4^+, the mechanism of NMDA receptor activation in this fish might be different from that in mammals.

Conclusions

Six strategies used by the oriental weatherloach to deal with the problem of excess ammonia were described. This fish can (1) reduce ammonia production through reduction in protein and/or amino acid catabolism; (2) reduce ammonia production and obtain energy through partial amino acid catabolism leading to alanine formation; (3) detoxify ammonia to glutamine; (4) tolerate very high ammonia levels in its tissues; (5) get rid of ammonia as NH_3 gas and, probably, (6) possesses background K^+ channels that are impermeable to NH_4^+. More work is needed to verify the existence of the highly selective background K^+ channels in this fish. This further work may enhance our understanding on the nature and mechanisms of ammonia toxicity.

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