The Journal of Experimental Biology 213, 2343-2353

doi:10.1242/jeb.039156

Inhibition of glutamine synthetase during ammonia exposure in rainbow trout indicates a high reserve capacity to prevent brain ammonia toxicity

L. A. Sanderson, P. A. Wright, J. W. Robinson, J. S. Ballantyne and N. J. Bernier*

Department of Integrative Biology, University of Guelph, 488 Gordon Street, Guelph, ON, Canada, N1G 2W1

*Author for correspondence (nbernier@uoguelph.ca)

Accepted 22 March 2010

SUMMARY

Glutamine synthetase (GSase), the enzyme that catalyses the conversion of glutamate and ammonia to glutamine, is present at high levels in vertebrate brain tissue and is thought to protect the brain from elevated ammonia concentrations. We tested the hypothesis that high brain GSase activity is critical in preventing accumulation of brain ammonia and glutamate during ammonia loading in the ammonia-intolerant rainbow trout. Trout pre-injected with saline or the GSase inhibitor methionine sulfoximine (MSOX, 6 mg kg^{-1}), were exposed to 0, 670 or 1000 μmol l^{-1} NH_4Cl in the water for 24 and 96 h. Brain ammonia levels were 3- to 6-fold higher in ammonia-exposed fish relative to control fish and MSOX treatment did not alter this. Brain GSase activity was unaffected by ammonia exposure, while MSOX inhibited GSase activity by ~75%. Brain glutamate levels were lower and glutamine levels were higher in fish exposed to ammonia relative to controls. While MSOX treatment had little impact on brain glutamate, glutamine levels were significantly reduced by 96 h. With ammonia treatment, significant changes in the concentration of multiple other brain amino acids occurred and these changes were mostly reversed or eliminated with MSOX. Overall the changes in amino acid levels suggest that multiple enzymatic pathways can supply glutamate for the production of glutamine *via* GSase during ammonia exposure and that alternative transaminase pathways can be recruited for ammonia detoxification. Plasma cortisol levels increased 7- to 15-fold at 24 h in response to ammonia and MSOX did not exacerbate this stress response. These findings indicate that rainbow trout possess a relatively large reserve capacity for ammonia detoxification and for preventing glutamate accumulation during hyperammonaemic conditions.

Key words: glutamate, glutamine, MSOX, alanine, tryptophan, stress.

INTRODUCTION

Ammonia, a breakdown product of amino acid catabolism, can be a potent neurotoxin in vertebrates if allowed to accumulate (Felipo and Butterworth, 2002; Randall and Tsui, 2002). This potential for neurotoxicity makes it crucial for organisms to effectively excrete ammonia. In teleosts, when exogenous ammonia concentrations are low, ammonia is passively excreted across the gills down the blood-to-water diffusion gradient (Cameron and Heisler, 1983; Wright and Wood, 1985). In contrast, when fish encounter high environmental ammonia levels, excretion of nitrogenous waste can become impaired as the concentration gradient is reduced or even reversed (e.g. Cameron and Heisler, 1983; Claiborne and Evans, 1988). In post-larval teleosts such as rainbow trout (*Oncorhynchus mykiss*) where the ornithine urea cycle is not functional, evidence suggests that the major ammonia detoxifying mechanism involves the conversion of ammonia and glutamate to glutamine *via* the enzyme glutamine synthetase (GSase; EC6.3.1.2) (reviewed by Ip et al., 2001; Randall and Tsui, 2002). In both fish and mammals, the activity of GSase is much higher in the brain than in other tissues (Cooper and Plum, 1987; Wang and Walsh, 2000; Wright et al., 2007) and it is suspected that the primary functions of GSase in the brain are to provide protection against ammonia toxicity and maintain homeostasis of the excitatory neurotransmitter glutamate (Suárez et al., 2002).

In some fish species, but not in others, brain GSase activity (Wang and Walsh, 2000; Ip et al., 2001; Wicks and Randall, 2002) and mRNA levels (Wright et al., 2007) have been demonstrated to increase in response to ammonia exposure. However, regardless of whether GSase activity is stimulated or not, brain glutamine concentrations in ammonia-exposed teleosts generally increase (Levi et al., 1974; Wang and Walsh, 2000; Wicks and Randall, 2002; Ip et al., 2004; Veauvy et al., 2005; Wee et al., 2007). In some species, the increase in brain glutamine is accompanied by a decrease in glutamate (Arillo et al., 1981; Vedel et al., 1998; Wicks and Randall, 2002; Veauvy et al., 2005). Interestingly, despite the overall reduction in brain glutamate, there is evidence suggesting that ammonia toxicity in some fish species is at least partly mediated by an overactivation of the glutamate NMDA receptors (Walsh et al., 2007).

Glutamate can also stimulate the hypothalamic–pituitary–adrenal (HPA) stress axis in mammals and cause the release of corticotropin-releasing factor (CRF) from hypophysiotropic neurons (Joanny et al., 1997). CRF stimulates the secretion of adrenocorticotropin hormone from the pituitary, which in turn stimulates the synthesis and release of glucocorticoids from the adrenal cortex of mammals and the interrenal tissue of fish (Wenderlaar Bonga, 1997; Bernier et al., 2009). In rainbow trout, while chronic ammonia exposure leads to an increase in plasma cortisol concentrations and an increase in brain preoptic area CRF mRNA expression (Wicks and Randall, 2002; Ortega et al., 2005; Bernier et al., 2008), it is not known whether glutamate also plays a role in the regulation of the hypothalamic–pituitary–interrenal (HPI) stress axis. The increase in plasma cortisol during ammonia exposure is transient, with levels returning to baseline by 96 h (Ortega et al., 2005; Bernier et al., 2008). It has been suggested that the decrease in cortisol over time is due to the conversion of ammonia and glutamate to glutamine,

catalysed by GSase (Ortega et al., 2005). Indeed, several teleost studies have shown an increase in tissue-specific GSase activity or an upregulation in GSase mRNA expression with elevations in plasma cortisol (Walsh et al., 1994; Hopkins et al., 1995; Aluru and Vijayan, 2007).

In this study, we hypothesized that high brain GSase activity is critical in preventing the accumulation of brain ammonia and glutamate during episodes of hyperammonia in the ammonia-intolerant rainbow trout. If so, then inhibition of brain GSase will result in decreased survival and increased brain ammonia and glutamate concentrations and plasma cortisol when fish are exposed to elevated external ammonia levels. To test this hypothesis, we used the GSase inhibitor methionine sulfoximine (MSOX), which has been employed effectively in previous studies on fish (Ip et al., 2005; Veauvy et al., 2005; Wee et al., 2007). Four treatment groups were established in order to investigate the role of brain GSase during ammonia exposure and the influence of MSOX: saline-control, MSOX-control, saline-ammonia and MSOX-ammonia. Finally, experiments were performed over 96 h in order to determine the response over time.

MATERIALS AND METHODS

Animals

Juvenile rainbow trout (*Oncorhynchus mykiss* Walbaum 1792) were obtained from Rainbow Springs Trout Farm (Thamesford, ON, Canada). Fish were housed in 758 l tanks in the Hagan Aqualab (Guelph, ON, Canada) under a 12 h:12 h light:dark photoperiod (16°C), and were fed 1% body mass of commercial trout pellets three times a week. At least 2 weeks before experimentation, fish were transferred into 125 l tanks in groups of 8–10 and allowed to acclimate. Fish were fasted for 48 h prior to commencement of the experiments to reduce the effects of feeding history on the experimental results. The University of Guelph's Animal Care Committee approved care and use of the animals, as per the principles of the Canadian Council for Animal Care.

Experimental protocol

A series of trial dosages of the GSase inhibitor MSOX (Sigma-Aldrich Chemical Co., St Louis, MO, USA) were tested on rainbow trout to determine the quantity required to chronically reduce brain GSase activity by 75% for a 96 h period. Fish (N=2–6 per dosage) were anaesthetized with buffered ($NaHCO_3$, 0.2 g l^{-1}) tricaine methanesulphonate (MS-222; 0.1 g l^{-1}), weighed, and injected intraperitoneally (i.p.) with either saline (0.9% NaCl) or MSOX dosages ranging from 3 to 150 mg kg^{-1} body mass (2.6 µl g^{-1} body mass). Fish mass ranged between 56.2 and 74.6 g. After each trial, surviving fish were terminally anaesthetized with 2-phenoxyethanol (2 ml l^{-1}), and their brains were surgically removed, wrapped in foil and flash frozen in liquid nitrogen for later analysis of brain GSase activity. In a final trial, a dosage of 6 mg kg^{-1} was tested in fish exposed to elevated environmental ammonia (740±10 µmol l^{-1} NH_4Cl) for 96 h.

To determine the effects of NH_4Cl exposure with or without prior treatment with MSOX, rainbow trout were divided into 16 groups of 8–10 fish. The experiment consisted of four treatment groups: (1) fish in control water and injected with saline, (2) fish in control water and injected with MSOX, (3) fish exposed to NH_4Cl and injected with saline and (4) fish exposed to NH_4Cl and injected with MSOX. An experiment was conducted on fish exposed to 670 µmol l^{-1} NH_4Cl (fish mass 80.7±1.0 g, N=160) and then the experiment was repeated on a separate group of fish exposed to 1000 µmol l^{-1} NH_4Cl (fish mass 103.8±3.1 g; N=128). The lower ammonia concentration (670 µmol l^{-1} NH_4Cl) was chosen to match that in our previous study on GSase gene expression in trout brain (Wright et al., 2007). The second experiment at a higher ammonia concentration (1000 µmol l^{-1}) was performed to maximize the ammonia load to the fish while not exceeding the toxic level (Ortega et al., 2005). It should be noted that this higher exposure level was close to the lethal limit of these fish under the conditions tested, as slightly higher levels (1200 µmol l^{-1} NH_4Cl) resulted in 100% mortality.

Each treatment was replicated in duplicate tanks and the experiment lasted either 24 or 96 h. Fish were anaesthetized with buffered ($NaHCO_3$, 0.2 g l^{-1}) MS-222 (0.1 g l^{-1}), weighed, and injected i.p. with either saline (0.9% NaCl) or MSOX at a dosage of 6 mg kg^{-1} body mass (2.6 µl g^{-1} body mass) as determined from the dosage trials. Four hours later, eight groups of fish were exposed to chronic flow-through ammonia conditions and the other eight groups were maintained in control water and served as time controls. Using a prepared stock of NH_4Cl, ammonia levels in the tanks were increased instantaneously to 670 or 1000 µmol l^{-1} and maintained at these levels using a peristaltic pump (Minipuls 3, Gilson, Villiers Le Bel, France). Water samples were periodically taken throughout the exposures and frozen at –20°C for later analysis of ammonia content. Average water temperature throughout the 670 µmol l^{-1} NH_4Cl exposure was 16.4±0.2°C and pH was 8.02±0.01, whereas in the 1000 µmol l^{-1} NH_4Cl exposure the values were 15.9±0.1°C and pH was 8.42±0.01. One mortality was recorded in a control MSOX 96 h tank of the 670 µmol l^{-1} NH_4Cl exposure. In the 1000 µmol l^{-1} NH_4Cl exposure, there were three mortalities recorded in one MSOX-control tank, two mortalities in one saline-ammonia tank, and one mortality in each of the MSOX-ammonia tanks. A few fish demonstrated a loss of equilibrium in response to 1000 µmol l^{-1} NH_4Cl – they either recovered or it occurred towards the end of the treatment and they were thus kept within the experiment.

At the end of the 24 and 96 h exposures, fish were quickly netted and terminally anaesthetized with an overdose of 2-phenoxyethanol (2 ml l^{-1}). Blood was drawn *via* caudal puncture using a Na_2EDTA-treated syringe, centrifuged for 2 min at 12,000 ***g***, and the separated plasma was stored at –20°C for later analysis of plasma ammonia and cortisol concentrations. To quantify CRF mRNA levels, the brain from half of the fish in each tank was regionally dissected to isolate the pre-optic area as described previously (Bernier et al., 2008) and immediately frozen in liquid nitrogen. Whole brains were collected from the remaining fish, flash-frozen in liquid nitrogen, and stored at –80°C until analysis of ammonia levels, GSase activity and amino acid concentrations. Similarly, livers were collected and stored as above until analysis of ammonia levels and GSase activity.

In order to determine whether MSOX has an effect on the cortisol response of rainbow trout subjected to stressors other than ammonia, we performed an additional experiment, a standardized 60 s handling stress as previously described by Barton and Iwama (Barton and Iwama, 1991). Three groups of 10 fish each (107.7±1.7 g, N=30) were acclimated for at least 2 weeks in separate 125 l tanks. One group was left undisturbed and served as a control. The two other groups were anaesthetized with buffered MS-222 as above and while one group was injected i.p. with 0.9% saline the other received MSOX at a dosage of 6 mg kg^{-1} body mass (2.6 µl g^{-1} body mass). Twenty-eight hours later, in accordance with the timeline established above for a 24 h ammonia exposure, the saline- and MSOX-injected groups were each netted out of the water for 60 s and returned to their respective tanks. One hour after the handling stressor, all three groups of fish were terminally anaesthetized with 2-phenoxyethanol

(2 ml l^{-1}). Blood was drawn *via* caudal puncture, spun and the separated plasma was stored at –20°C for later analysis of plasma cortisol concentration. Whole brains were also collected, flash frozen in liquid nitrogen, and stored at –80°C until analysis of GSase activity.

Analyses

Water ammonia concentration was determined using a colorimetric assay (Verdouw et al., 1978). To measure plasma, liver and brain ammonia concentrations, tissues were prepared as described previously (Wright et al., 2007). Plasma and liver ammonia levels were analysed using the glutamate dehydrogenase (GDH) method described by Kun and Kearney (Kun and Kearney, 1974) with micro-modifications for use in a 96-well microplate (SpectraMAX 190; Molecular Devices, Sunnyvale, CA, USA). Brain ammonia concentrations were analysed using a commercial kit (Raichem Catalog No. 85446; Cliniqa, San Marcos, CA, USA) and a spectrophotometer (Cary 300 Bio UV/Visible; Varian, Palo Alta, CA, USA).

Brain and liver GSase activity was assayed based on the production of γ-glutamyl hydroxamate as previously described (Shankar and Anderson, 1985), with the following modifications. Ground brain tissue was homogenized in 700 volumes of ice-cold homogenization buffer and the final reaction was performed over 5 min at 15°C. Ground liver tissue was homogenized in 9 volumes of ice-cold homogenization buffer and the final reaction was performed over 15 min at 15°C. Samples were read at 500 nm in a spectrophotometer (Ultrospec 3100 pro UV/Visible; Biochrom Ltd, Cambridge, UK).

Brain amino acid concentrations were quantified using the EZ:faast amino acid analysis kit (Phenomenex, Torrance, CA, USA) run on a Waters Micromass Quattro Micro API tandem mass spectrometer (Waters, Milford, MA, USA). Samples (80 mg) were ground to a fine powder under liquid nitrogen and homogenized in 4 volumes of 0.5% trifluoroacetic acid in methanol. A buffer (final concentrations, 0.11 mmol l^{-1} sodium acetate, 0.056 mmol l^{-1} NaOH) was added to bring the final volume to 1150 μl prior to centrifugation at 10,000 ***g*** (10 min, 4°C). Further details are provided in a previous publication (Speers-Roesch et al., 2006). Plasma cortisol was assayed using a validated RIA as described elsewhere (Bernier et al., 2008).

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and cDNA synthesis was carried out as previously described (Wright et al., 2007). mRNA expression of CRF was quantified from the above cDNA products using SYBR Green and an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA) as outlined before (Ortega et al., 2005). To correct for variations in template input and transcriptional efficiency, each sample was normalized to the expression of the reference gene, elongation factor 1α (EF1α). The primers used were as follows: EF1α forward 5′-GGG CAA GGG CTC TTT CAA GT- 3′ and reverse 5′-CGC AAT CAG CCT GAG AGG T- 3′ (GenBank accession no. AF498320), CRF forward 5′-ACA ACG ACT CAA CTG AAG ATC TCG-3′ and reverse 5′-AGG AAA TTG AGC TTC ATG TCA GG-3′ (GenBank accession no. AF296672).

Statistical analyses

Statistical analyses were performed using SigmaStat 3.0 (SPSS Inc., Chicago, IL, USA). Differences among treatments at a given time were analysed using one-way analysis of variance (ANOVA) followed by a Tukey *post-hoc* test if differences were found. If the data for a given variable were not normally distributed, the data were log transformed and retested. If data were not normalized after transformation, a Kruskal–Wallis one-way ANOVA on ranks was used. Differences among times for a given treatment were analysed using a *t*-test or a Mann–Whitney rank sum tests if log transformation failed to normalize the data. The significance level for all statistical tests was set at $P<0.05$. All data are presented as means ± s.e.m.

RESULTS

Preliminary experiment aimed at determining an effective MSOX dosage

Concentrations of MSOX over 30 mg kg^{-1} body mass were lethal within 24 h post-injection without the addition of ammonia. MSOX dosages of 3 and 6 mg kg^{-1} resulted in 71% (24 h post-injection) and 88% (96 h post-injection) decreases in brain GSase activity relative to saline-injected controls, respectively, and caused no mortalities even with exposure to 740 μmol l^{-1} NH_4Cl for 96 h.

Exposure to 670 and 1000 μmol l^{-1} NH_4Cl

The average water ammonia concentration in the control tanks was higher at 96 h (13±3 and 12±3 μmol l^{-1} in the 670 and 1000 μmol l^{-1} NH_4Cl experiments, respectively) than that at 24 h (2±0 and 2±1 μmol l^{-1} in the 670 and 1000 μmol l^{-1} NH_4Cl experiments, respectively), although overall water ammonia levels in the control tanks were very low relative to the ammonia-treated tanks. Water ammonia concentrations remained elevated throughout the experiment (24 h: 668±29 and 1040±31 μmol l^{-1} in the 670 and 1000 μmol l^{-1} NH_4Cl experiments, respectively; and 96 h: 678±9 and 934±10 μmol l^{-1} in the 670 and 1000 μmol l^{-1} NH_4Cl experiments, respectively) and there were no significant differences between duplicate treatment tanks or between time periods.

The physiological responses of fish to ammonia exposure and/or MSOX injection between 670 and 1000 μmol l^{-1} NH_4Cl exposures were very similar. The magnitude of the increases in plasma, brain and liver ammonia concentrations were more pronounced during the 1000 μmol l^{-1} NH_4Cl exposure relative to the 670 μmol l^{-1} NH_4Cl exposure. We chose to present data from both exposures to demonstrate the overall consistency of the changes in brain chemistry and highlight a few dissimilar results with different concentrations of external ammonia.

Ammonia concentrations in the plasma, brain and liver were all significantly elevated in fish exposed to ammonia relative to control fish and there were no differences between the saline- and MSOX-injected fish after either 24 or 96 h of ammonia exposure (Fig. 1). On average, plasma ammonia concentrations increased 16-fold, brain ammonia concentrations increased 4.6-fold and liver ammonia concentrations increased 4.1-fold in response to ammonia exposure. Within a given treatment, there was no change in brain ammonia concentrations between exposure durations, increases in liver ammonia concentrations between the 24 h and 96 h exposures in the 670 μmol l^{-1} NH_4Cl treatments, and no change in liver ammonia concentrations in the 1000 μmol l^{-1} NH_4Cl exposures. Finally, while plasma ammonia levels increased between 24 h and 96 h of exposure to 670 μmol l^{-1} NH_4Cl in the saline–ammonia treatment, the levels decreased between exposure durations in the fish exposed to 1000 μmol l^{-1} NH_4Cl and pre-injected with MSOX.

Exposure to NH_4Cl had little effect on either brain or liver GSase activity in the saline-injected fish (Fig. 2) but a small increase in brain GSase after 96 h of exposure to 670 μmol l^{-1} NH_4Cl was detected (Fig. 2A). In contrast, brain GSase activity was inhibited by 71–78% in fish injected with MSOX relative to saline-injected fish (Fig. 2A,B). In the liver, the effects of MSOX were less dramatic.

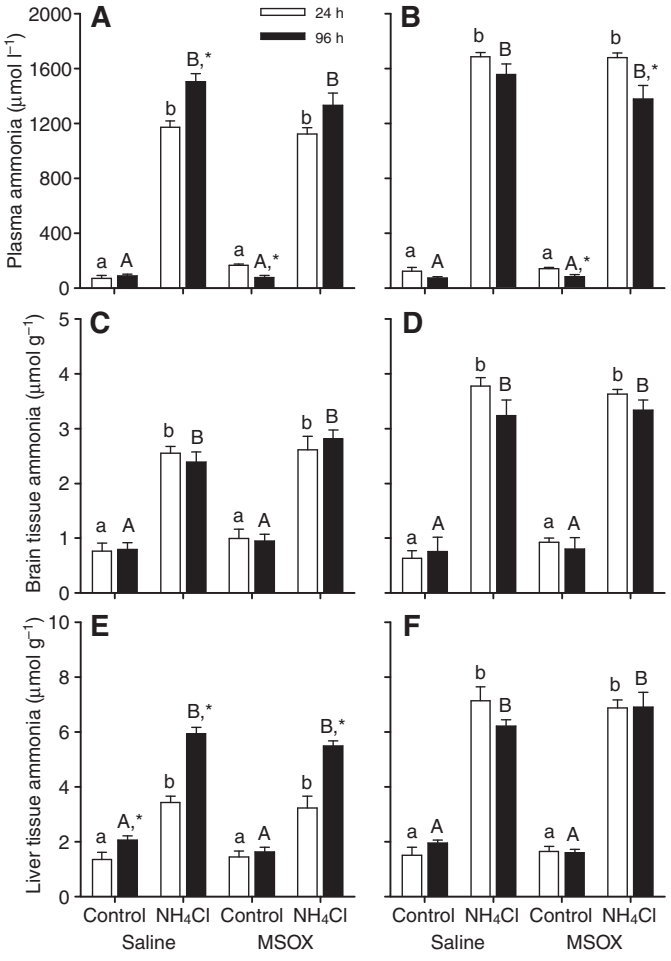

Fig. 1. Plasma (A and B), brain (C and D) and liver (E and F) ammonia concentrations in saline-injected or MSOX-injected (6 mg kg^{-1}) rainbow trout held in control water or exposed to 670 μmol l^{-1} (A,C,E) or 1000 μmol l^{-1} (B,D,F) NH_4Cl for 24 h or 96 h. At a given time, values that do not share a common letter (lowercase, 24 h; uppercase, 96 h) are significantly different from each other. An asterisk indicates a significant difference between times for a given treatment. Values are means ± s.e.m. (*N*=8).

MSOX inhibited liver GSase activity in the control and ammonia-exposed fish by 40–60% relative to the saline-injected fish at 24 h, but in most cases MSOX no longer had an inhibitory affect after 96 h (Fig. 2C,D).

In saline-injected fish, brain glutamate concentrations (Fig. 3A,B) were generally lower and glutamine concentrations (Fig. 3C,D) higher after the NH_4Cl treatments compared with control conditions. Brain glutamine levels during ammonia exposure were consistently higher after 96 h relative to 24 h (Fig. 3C,D). Pre-injection of MSOX had little impact on the changes in brain glutamate concentrations associated with hyperammonia (Fig. 3A,B). In contrast, by 96 h, the MSOX treatment was characterized by significant reductions in brain glutamine levels in both the control and ammonia-exposed fish (Fig. 3C,D).

In addition to glutamate and glutamine levels, we also quantified the impact of ammonia exposure and MSOX treatment on the brain concentration of another 20 amino acids. Elevated brain ammonia was associated with a decrease in the concentration of four amino acids (alanine, aspartate, GABA and serine; Fig. 4A,B; Tables 1 and 2) and an increase in the concentration of six amino acids (arginine, phenylalanine, threonine, tyrosine, tryptophan and valine; Fig. 4C,D; Fig. 5A,B; Tables 1 and 2). In fish pre-injected with MSOX prior to ammonia exposure, the above changes in brain amino acid concentrations were reversed or eliminated (Figs 4 and 5; Tables 1 and 2).

In saline-injected fish, ammonia exposure elicited a transient increase in plasma cortisol at 670 μmol l^{-1} NH_4Cl (Fig. 5C), but a more prolonged increase at 1000 μmol l^{-1} NH_4Cl (Fig. 5D). In fish pre-treated with MSOX, this increase in plasma cortisol with NH_4Cl exposure was eliminated at 670 μmol l^{-1} NH_4Cl (Fig. 5C) but not at 1000 μmol l^{-1} NH_4Cl (Fig. 5D). Accompanying these changes, brain CRF mRNA levels were significantly elevated in fish exposed to 1000 μmol l^{-1} NH_4Cl, especially in the MSOX-treated fish (Table 3).

Effect of MSOX on cortisol production

Pre-treatment of fish with MSOX resulted in significantly lower brain GSase activity (5-fold) relative to both unhandled fish and fish handled for 60 s and injected with saline (Fig. 6A). Independent of whether the fish were injected with saline or MSOX, the handling stressor resulted in significantly higher plasma cortisol concentrations relative to the unhandled fish 1 h after the disturbance (Fig. 6B).

DISCUSSION

The findings of this study demonstrate that rainbow trout have a considerable reserve capacity to prevent brain ammonia toxicity. Trout survived an inhibition of brain GSase by ~75% coupled to 96 h of exposure to high external ammonia concentrations (1000 μmol l^{-1}), very close to their lethal limit. We predicted that if fish survived under these conditions, then brain ammonia and glutamate concentrations would increase over the exposure time. Remarkably, brain ammonia and glutamate levels were unchanged. This suggests that alternative mechanisms exist in the brain to maintain low levels of the neurotoxin ammonia and appropriate levels of the neurotransmitter glutamate under extreme conditions of ammonia loading. In other words, although GSase appears to be the key enzyme that catalyses glutamine synthesis, it is not the only effective mechanism to regulate brain ammonia and glutamate concentrations. Our results also confirm that exposure to elevated external ammonia is stressful to fish but, counter to our prediction, inhibition of GSase with MSOX does not result in a more dramatic stress response. Although our biochemical analysis suggests that the fish treated with MSOX were able to maintain glutamate homeostasis and prevent hyperammonia toxicity, we cannot rule out the possibility that GSase inhibition affected the overall health and the neural/behavioural repertoire of the fish.

To understand more fully the biochemical changes in the brain during ammonia loading, we measured the full suite of amino acids under all experimental conditions. Although the overall changes in brain amino acid concentrations in the 670 and 1000 μmol l^{-1} NH_4Cl exposures were consistent, the concentrations of some of the individual amino acids were variable between the two exposures. Since the fish used in the two ammonia exposures were derived from the same stock, fed the same diet and reared under a common feeding regime, we suggest that seasonal differences may account for these differences. Overall, the interpretation of the changes in amino acids has been performed with caution, as changes in concentration provide only a ‘snapshot’ at a specific time point rather than a comprehensive view of changes in flux through a pathway. Nevertheless, the changes in brain amino acid concentrations with

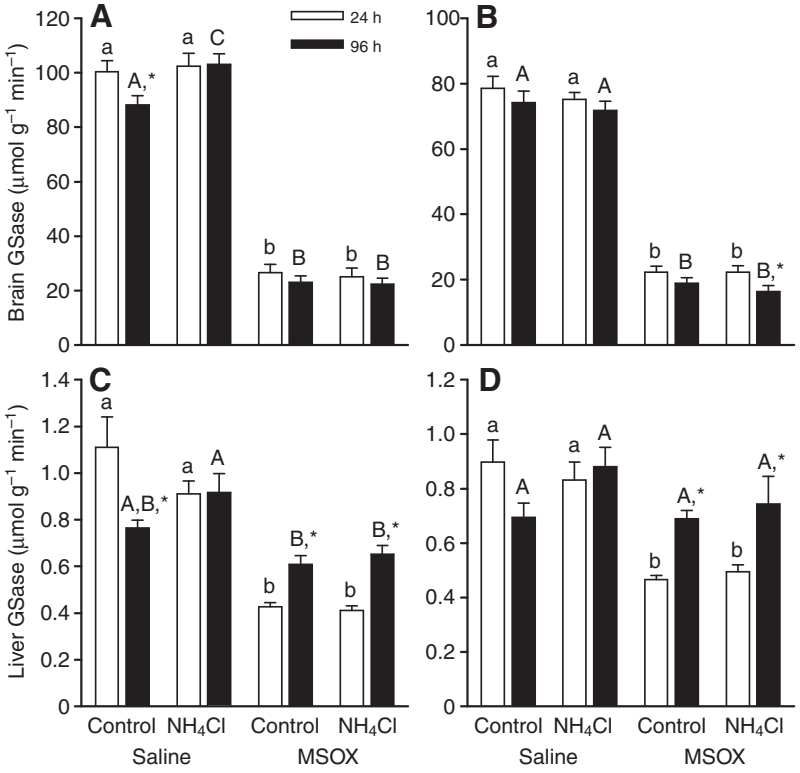

Fig. 2. Brain (A and B) and liver (C and D) glutamine synthetase (GSase) activity in saline-injected or MSOX-injected (6 mg kg^{-1}) rainbow trout held in control water or exposed to 670 μmol l^{-1} (A and C) or 1000 μmol l^{-1} (B and D) NH_4Cl for 24 h or 96 h. At a given time, values that do not share a common letter (lowercase, 24 h; uppercase, 96 h) are significantly different from each other. An asterisk indicates a significant difference between times for a given treatment. Values are means ± s.e.m. (*N*=8).

ammonia exposure with or without MSOX provide some valuable insights.

During exposure to elevated water ammonia concentrations, our results confirm that brain glutamine levels are increased similar to previous reports (Levi et al., 1974; Arillo et al., 1981; Peng et al., 1998; Ip et al., 2004; Wee et al., 2007), presumably because of increased flux through the GSase reaction. At the same time the lower concentration of brain glutamate, also shown by others (Arillo et al., 1981; Vedel et al., 1998; Wicks and Randall, 2002; Veauvy et al., 2005), implies that under high ammonia conditions the supply of glutamate is not perfectly matched to the demand. The pathways outlined in Fig. 7 provide a possible explanation of the data and explain

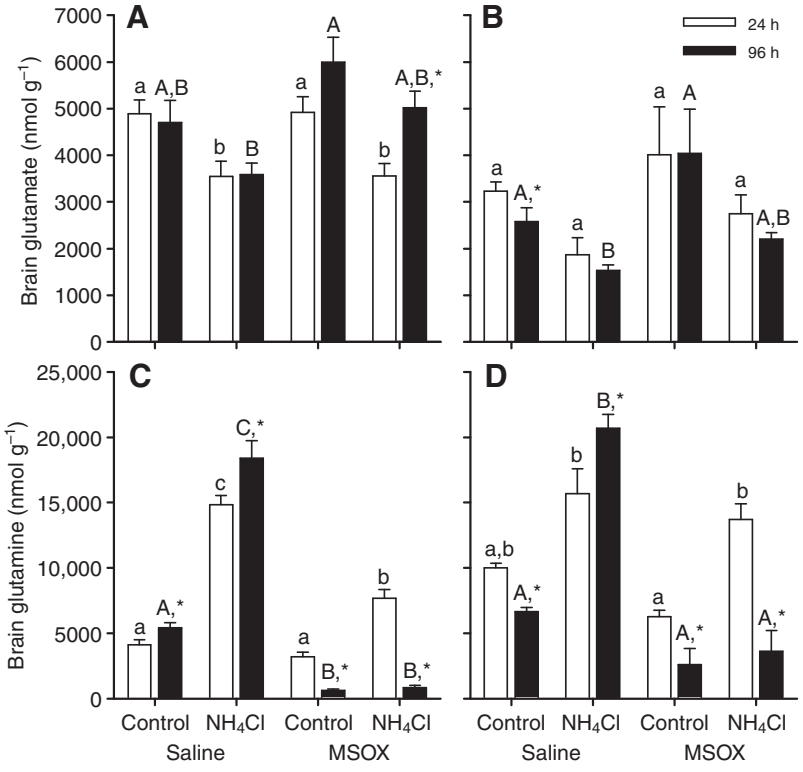

Fig. 3. Brain glutamate (A and B) and glutamine (C and D) concentrations in saline-injected or MSOX-injected (6 mg kg^{-1}) rainbow trout held in control water or exposed to 670 μmol l^{-1} (A and C) or 1000 μmol l^{-1} (B and D) NH_4Cl for 24 h or 96 h. At a given time, bars that do not share a common letter (lowercase, 24 h; uppercase, 96 h) are significantly different from each other. An asterisk indicates a significant difference between times for a given treatment. Values are means ± s.e.m. (*N*=8).

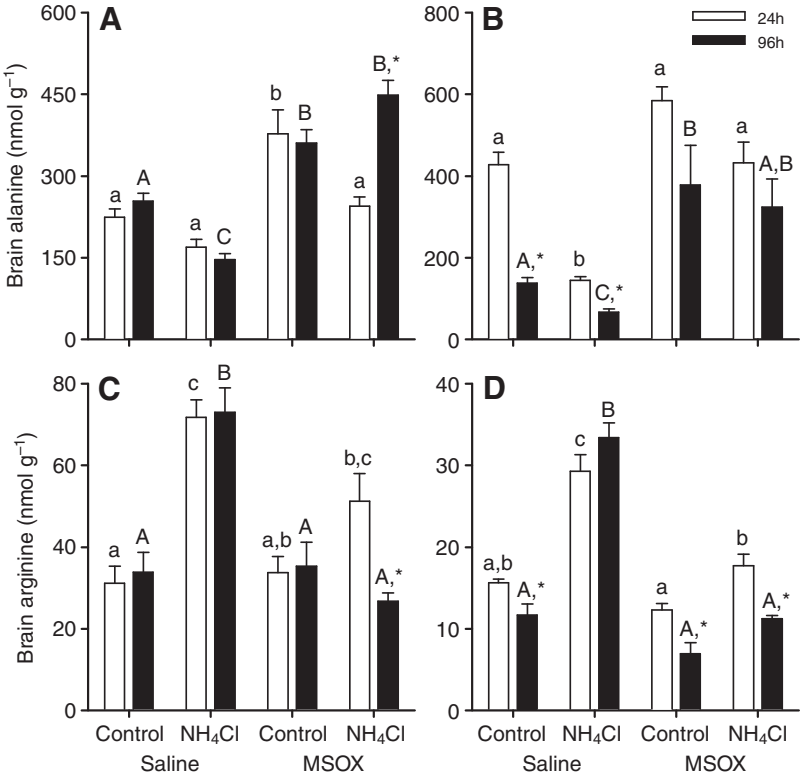

Fig. 4. Brain alanine (A and B) and arginine (C and D) concentrations in saline-injected or MSOX-injected (6 mg kg^{-1}) rainbow trout held in control water or exposed to 670 μmol l^{-1} (A and C) or 1000 μmol l^{-1} (B and D) NH_4Cl for 24 h or 96 h. At a given time, bars that do not share a common letter (lowercase, 24 h; uppercase, 96 h) are significantly different from each other. An asterisk indicates a significant difference between times for a given treatment. Values are means ± s.e.m. (*N*=8).

how glutamate levels in the trout brain may be regulated. During exposure to elevated external ammonia, brain alanine, aspartate and GABA concentrations were significantly lower. These three amino acids can provide glutamate *via* transaminase reactions, along with the GDH reaction (Fig. 7) (Nelson and Cox, 2000). The transaminase and GDH reactions deplete α-ketoglutarate, a tricarboxylic acid cycle (TCA) intermediate, and reduce the provision of ATP needed for glutamine synthesis. The depletion of α-ketoglutarate must be addressed by supplying TCA cycle intermediates, such as acetyl-CoA. Acetyl-CoA can be supplied *via* the transaminases that have pyruvate as a product, for example alanine and serine (Fig. 7). Indeed, brain alanine and serine were significantly reduced in response to elevated ammonia levels. The significantly higher levels of brain arginine, phenylalanine, tyrosine and valine during ammonia exposure were also predicted as these amino acids are produced as by-products of pyruvate synthesis (Fig. 7). The proposed pathways above and in Fig. 7

Table 1. Brain amino acid concentrations (nmol g^{-1}) in saline-injected or MSOX-injected (6 mg kg^{-1}) rainbow trout held in control water or exposed to 670 μmol l^{-1} NH_4Cl for 24 or 96 h

	24 h exposure				96 h exposure			
	Saline		MSOX		Saline		MSOX	
Amino acid	Control	NH_4Cl	Control	NH_4Cl	Control	NH_4Cl	Control	NH_4Cl
Asparagine	58.1±7.6^{a}	47.5±9.5^{a,b}	57.8±6.2^{a,b}	30.5±3.6^{b}	44.1±5.7	49.6±3.3	75.2±17.4	66.7±14.1*
Aspartate	1568.9±166.7^{a}	816.7±130.5^{b}	1285.9±96.0^{a,b}	1311.6±141.8^{a,b}	1438.9±128.5^{A}	964.8±194.0^{A,B}	1482.4±388.0^{B,*}	1204.0±66.2^{A,B}
Citrulline	7.9±1.4	8.1±1.4	5.8±0.6	8.3±2.0	8.8±1.6	12.5±1.7	7.1±1.7	8.7±1.9
GABA	1323.3±92.4^{a,b}	1196.0±73.7^{a}	1494.3±53.2^{b}	1111.4±66.3^{a}	1560.8±78.7^{A}	1184.5±54.2^{B}	999.3±57.1^{B,*}	1036.7±60.0^{B}
Glycine	184.7±30.5	163.0±45.9	204.6±33.4	106.6±14.7	140.7±28.4	151.7±28.2	234.7±52.2	269.9±62.3
Histidine	366.9±73.8	432.8±42.9	352.0±53.5	326.0 ±39.2	413.5±34.3	404.0±48.3	460.6±68.8	353.2±46.0
Isoleucine	105.7±11.5	108.4±24.6	115.7±15.5	119.8±16.4	127.3±9.0	140.5±18.1	100.2±13.9	148.9±15.1
Leucine	23.2±2.7	31.3±5.4	29.7±4.8	39.5±7.6	29.8±4.4	41.5±6.9	30.7±4.0	40.3±2.2
Lysine	128.9±23.6	51.4±3.5	154.5±26.7	93.0±9.7	86.9±8.9	93.5±11.2	184.3±15.6	290.0±29.1*
Methionine	40.1±6.4	88.4±11.4	45.5±5.3	50.3±5.8	50.7±8.2	58.5±8.6	33.0±4.7	32.1±2.1
Ornithine	13.0±1.5	14.6±2.1	11.6±1.4	14.8±1.4	14.1±1.5	15.1±0.9	11.5±1.5	13.4±1.8
Phenylalanine	26.2±3.8^{a}	72.8±10.5^{b}	24.1±4.6^{a}	46.2±7.3^{a,b}	22.6±2.0^{A}	42.8±2.4^{B,*}	19.8±2.9^{A}	15.5±1.6^{A,*}
Proline	37.1±3.9	42.9±4.4	39.7±8.1	38.9±1.2	31.5±3.7^{A,B}	32.6±2.5^{A,B}	41.1±5.3^{A}	22.2±1.5^{B,*}
Serine	336.2±1.4^{a,b}	184.6±12.3^{c}	385.7±21.4^{a}	260.0±15.4^{b}	337.8±23.3^{A}	138.5±10.7^{B,*}	381.4±21.9^{A}	283.2±9.2^{A,B}
Threonine	354.0±57.5	534.3±145.5	368.0±58.3	235.0±38.7	307.6±51.6^{A}	814.2±180.0^{B}	153.9±18.8^{A,*}	272.2±73.3^{A}
Tyrosine	13.3±2.4^{a}	35.7±5.6^{c}	13.6±1.0^{a,b}	21.7±3.0^{b,c}	9.7±2.0^{A}	22.4±2.0^{B,*}	8.1±1.1^{A,*}	8.6±1.4^{A,B,*}
Valine	12.7±1.6	17.0±2.0	13.5±2.4	16.2±1.5	12.8±1.2^{A}	28.0±4.2^{B,*}	20.4±4.0^{A,B}	22.6±0.5^{A,B,*}

At a given time, values that do not share a common letter (lowercase, 24 h; uppercase, 96 h) are significantly different from each other. An asterisk indicates a significant difference between times for a given treatment. Values are means ± s.e.m. (*N*=8).

Table 2. Brain amino acid concentrations (nmol g^{-1}) in saline-injected or MSOX-injected (6 mg kg^{-1}) rainbow trout held in control water or exposed to 1000 μmol l^{-1} NH_4Cl for 24 or 96 h

	24 h exposure				96 h exposure			
	Saline		MSOX		Saline		MSOX	
Amino acid	Control	NH_4Cl	Control	NH_4Cl	Control	NH_4Cl	Control	NH_4Cl
Asparagine	58.4±3.3	85.5±18.6	77.0±21.1	67.2±9.3	31.4±4.8*	30.0±2.5*	27.0±5.2*	21.2±3.0*
Aspartate	642.3±56.0^{a}	194.2±57.1^{c}	478.4±73.4^{a,b}	363.6±21.8^{b,c}	553.6±90.6^{A}	181.2±23.5^{B}	506.2±143.6^{A,B}	274.2±55.6^{A,B}
Citrulline	23.9±2.1	19.5±5.4	25.2±3.2	34.0±5.5	8.4±1.8*	18.8±3.5	12.1±2.6*	8.3±1.1*
GABA	1332.5±24.9^{a}	543.9±66.7^{c}	1211.9±85.4^{a}	882.9±80.6^{b}	906.6±38.0^{A,*}	647.0±51.3^{B}	738.4±116.3^{A,B,*}	656.7±66.7^{A,B,*}
Glycine	725.1±46.3	767.3±132.3	1080.4±326.1	841.4±113.8	384.1±79.6*	258.9±20.4*	433.5±78.4*	272.6±21.3*
Histidine	253.5±17.7	321.5±76.5	410.8±166.6	329.9±51.5	163.2±16.7*	153.6±16.9	156.4±33.4	172.2±13.5*
Isoleucine	65.8±3.4^{a}	36.6±4.7^{b}	58.7±7.7^{a,b}	64.4±7.7^{a}	22.0±4.5*	38.0±4.1	20.6±9.2*	34.2±0.7*
Leucine	26.0±3.5	18.0±3.6	45.5±21.0	27.3±3.9	14.7±2.7*	12.8±1.4	17.5±4.9	12.5±0.9*
Lysine	191.9±13.5^{a}	82.1±14.0^{b}	230.9±65.6^{a}	145.6±18.2^{a,b}	129.3±11.4^{A,B,*}	116.4±9.2^{B}	199.6±31.6^{A}	181.3±16.2^{A}
Methionine	91.7±4.6	94.1±6.5	77.7±2.9	82.3±3.5	64.1±3.3^{A,B,*}	78.3±3.3^{A,*}	40.0±5.4^{B,*}	46.1±6.7^{B,*}
Ornithine	58.3±2.6^{a}	18.6±5.6^{b}	56.1±5.0^{a}	54.5±5.5^{a}	31.7±3.6*	27.7±4.3	25.2±6.6*	30.9±5.8*
Phenylalanine	49.6±2.7^{a}	77.4±12.7^{a}	29.1±1.9^{b}	52.1±2.2^{a}	27.8±1.8^{A,B,*}	30.8±2.0^{A,*}	24.8±8.5^{A,B}	19.7±2.6^{B,*}
Proline	4.5±0.5^{a}	10.2±2.3^{b}	6.1±0.6^{a,b}	5.2±0.4^{a,b}	1.2±0.2*	1.9±0.2*	1.6±0.3*	1.3±0.2*
Serine	556.3±27.1^{a}	96.2±14.5^{c}	652.5±64.3^{a}	292.4±41.3^{b}	197.0±12.9^{A,*}	64.6±13.0^{C}	257.8±19.6^{A,*}	123.6±16.4^{B,*}
Threonine	397.3±108.2	2556.1±1013.4	358.1±86.9	658.1±221.8	228.7±73.4^{A}	1028.2±275.6^{B}	176.4±65.5^{A}	121.0±53.0^{A,*}
Tyrosine	10.1±1.6	12.8±3.0	8.1±3.0	9.0±1.2	5.1±0.8^{A,*}	3.6±0.3^{B,*}	6.0±0.6^{A}	1.9±0.3^{B,*}
Valine	10.2±0.8	9.0±1.7	12.5±1.1	12.6±1.6	4.5±0.7*	5.0±0.7	6.0±1.7*	4.2±0.3*

At a given time, values that do not share a common letter (lowercase, 24 h; uppercase, 96 h) are significantly different from each other. An asterisk indicates a significant difference between times for a given treatment. Values are means ± s.e.m. (*N*=8).

are consistent with the observed changes in 10 of the amino acids measured. Four amino acids showed no changes in concentration and changes in the remaining amino acids do not fit a recognizable pattern, except for tryptophan, discussed below.

Given the proposed scenario for the regulation of brain glutamate, how do fish respond to an inhibition of GSase and high external ammonia? The elevation of brain glutamine concentrations after 24 h of MSOX and ammonia treatment implies that glutamine continues to be synthesized despite the fact that GSase activities were only ~25% of normal levels. By 96 h, however, glutamine concentrations in the MSOX-treated fish were only 5–16% of levels found in saline-injected fish exposed to elevated ammonia. This apparent delayed effect of MSOX in reducing brain glutamine stores is likely to be independent of the mechanism by which MSOX inhibits GSase. MSOX is a competitive inhibitor of GSase that binds irreversibly to the active site of the enzyme, permanently inactivating it (Cooper

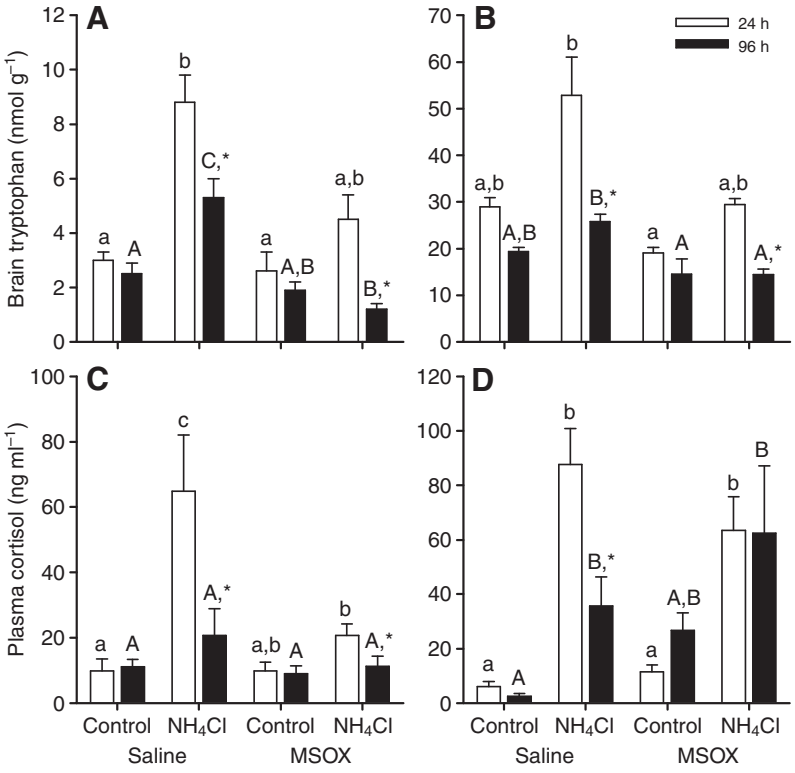

Fig. 5. Brain tryptophan (A and B) and plasma cortisol concentrations (C and D) in saline-injected or MSOX-injected (6 mg kg^{-1}) rainbow trout held in control water or exposed to 670 μmol l^{-1} (A and C) or 1000 μmol l^{-1} (B and D) NH_4Cl for 24 h or 96 h. At a given time, bars that do not share a common letter (lowercase, 24 h; uppercase, 96 h) are significantly different from each other. An asterisk indicates a significant difference between times for a given treatment. Values are means ± s.e.m. (*N*=8).

Table 3. The ratio of corticotropin-releasing factor (CRF) to elongation factor 1α (EF1α) mRNA levels in the brain preoptic area of saline-injected or MSOX-injected (6 mg kg^{-1}) rainbow trout held in control water or exposed to NH_4Cl (670 or 1000 μmol l^{-1}) for 24 or 96 h

Time (h)	NH_4Cl (μmol l^{-1})	Saline		MSOX	
		Control	NH_4Cl	Control	NH_4Cl
24	670	1.00±0.17	1.16±0.21	1.12±0.17	1.92±0.55
96		0.66±0.15	1.25±0.20	1.39±0.21	1.14±0.26
24	1000	1.00±0.11^{a}	2.05±0.22^{b}	1.31±0.16^{a}	1.97±0.19^{b}
96		0.97±0.17^{a}	2.06±0.40^{b,c}	1.16±0.12^{a,b}	3.35±0.89^{c}

At a given time, values that do not share a common letter are significantly different from each other. For comparative purposes, the expression ratios are presented relative to the respective 24 h saline-control treatment. Values are means ± s.e.m. (*N*=8).

et al., 1976). Also, the predicted increase in brain glutamate concentrations in the MSOX-ammonia treatment did not occur, consistent with a study on the marine toadfish (Veauvy et al., 2005). It is possible that excess glutamate was used in the reversible transaminase reactions and the significant increase in alanine, aspartate and GABA concentrations in fish pre-injected with MSOX and exposed to ammonia supports this notion. It is also possible that brain GDH was downregulated; however, in the African sharptooth catfish *Clarias gariepinus*, injection with MSOX (100 mg kg^{-1}) did not alter brain GDH or alanine aminotransferase *in vivo* (Wee et al., 2007). In terms of the other amino acids presented in the model (Fig. 7), GSase inhibition by MSOX treatment during ammonia exposure for the most part reversed or eliminated the changes in brain amino acid concentrations discussed above. These results are consistent with a reduced depletion of glutamate and α-ketoglutarate during hyperammonaemic conditions and the recruitment of transaminase pathways to prevent glutamate excitotoxicity and brain ammonia toxicity during MSOX treatment (Fig. 7). In fact, the reversal of transaminase reactions with MSOX treatment was more pronounced after 96 h of ammonia exposure than after 24 h. Therefore, we suggest that the reduction in brain glutamine in the 96 h MSOX-ammonia treatment results from a gradual shift away from the condensation of glutamate to glutamine towards the transamination of glutamate to alternative amino acid products.

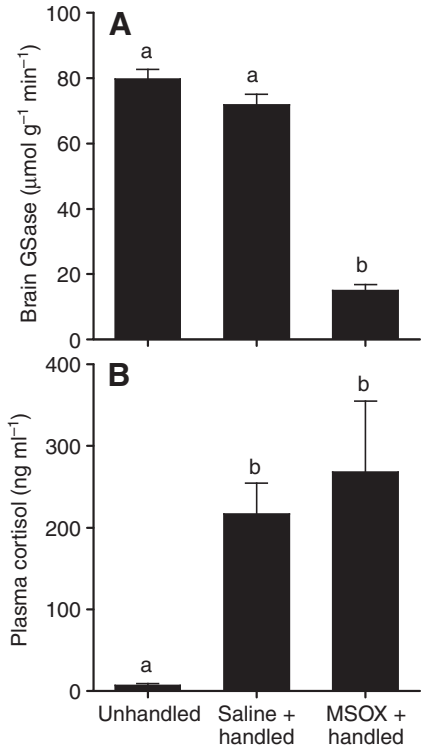

Fig. 6. Brain GSase activity (A) and plasma cortisol concentration (B) in either unhandled or handled rainbow trout. Handled trout were given an intraperitoneal injection of either saline or MSOX (6 mg kg^{-1}). Fish were handled through air exposure in a net for 60 s and allowed to recover for 1 h prior to sampling. Bars that do not share a common letter are significantly different from each other. Values are means ± s.e.m. (*N*=9–10).

Trout exposed to elevated external ammonia in this study had significantly higher plasma and tissue ammonia concentrations that were constant between 24 and 96 h. The concentration of brain ammonia depends on the plasma-to-brain tissue ammonia gradient, the permeability of the blood–brain barrier and neural cells to ammonia and the rate of ammonia incorporation into glutamate/glutamine. Under these conditions of continuous ammonia exposure, GSase does not act as a 'barrier' against ammonia influx (Cooper and Plum, 1987) and this is reinforced by the fact that inhibition of brain GSase by MSOX did not affect brain ammonia concentrations in the rainbow trout (present study) or the gulf toadfish (Veauvy et al., 2005). The classic diffusion trapping model (Pitts, 1973) predicts that the distribution of ammonia between extracellular and intracellular compartments will be entirely influenced by the H^+ gradient, if cells are impermeable. However, in the lemon sole (*Parophrys vetulus*) (Wright et al., 1988) brain ammonia concentrations under various physiological conditions far exceed those predicted by transmembrane pH gradients and were consistent with the membrane potential, indicating that NH_4^+ must be permeable across the blood–brain barrier and/or neural cell membranes. Brain ammonia permeability may also depend on the density of Rhesus (Rh) glycoproteins, the putative ammonia transporter identified in the gills of rainbow trout (Nawata et al., 2007) and other species (Nakada et al., 2007a; Nakada et al., 2007b; Hung et al., 2007) (reviewed by Wright and Wood, 2009). It is interesting to note that mRNA expression of Rhcg1 levels is downregulated in the brain of rainbow trout 48 h after exposure to 1500 μmol l^{-1} NH_4HCO_3 (Nawata et al., 2007). Although brain Rh glycoprotein expression was not quantified in this study, the results of Nawata and colleagues (Nawata et al., 2007) suggest that brain ammonia concentrations in rainbow trout may plateau after 24 h of hyperammonia exposure due in part to a decrease in the permeability of neural cells to ammonia. Similarly, the gradual recovery and increase in gill ammonia excretion rates during chronic exposure to high external ammonia may contribute to the stabilization of brain and plasma ammonia (Cameron and Heisler, 1983; Clairborne and Evans, 1988; Wilson and Taylor, 1992; Nawata et al., 2007). Here also Rh glycoproteins may be playing an important role, as the recovery of ammonia excretion in rainbow trout exposed to high

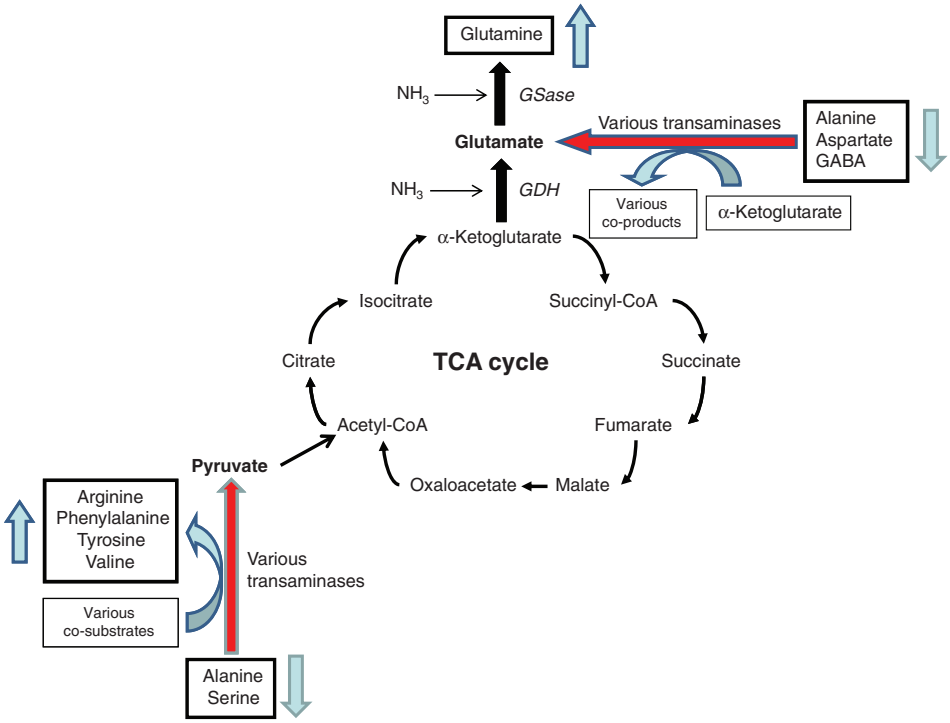

Fig. 7. Proposed model of the principal pathways involved in the synthesis of glutamine during ammonia exposure in the brain of rainbow trout. The enzymes glutamate dehydrogenase (GDH) and glutamine synthetase (GSase) catalyse the primary reactions that consume ammonia in the brain. During chronic ammonia exposure, the amino acids alanine, aspartate and γ-aminobutyric acid (GABA) are transaminated to glutamate to prevent depletion of the glutamate pool. A co-substrate of these aminotransferase reactions and of the GDH reaction is α-ketoglutarate, a tricarboxylic acid (TCA) cycle intermediate. Transaminases that have pyruvate as a product can supply acetyl-CoA to the TCA cycle and maintain the production of α-ketoglutarate. This second group of transaminase reactions reduce alanine and serine concentrations, and increase arginine, phenylalamine, tyrosine and valine concentrations. Inhibition of GSase with MSOX reverses the above reactions and changes in brain amino acid concentrations.

external ammonia is accompanied by an upregulation in Rhcg2 mRNA levels (Nawata et al., 2007).

Brain GSase activity was not altered by exposure to elevated external ammonia, in contrast to previous reports in rainbow trout (Wicks and Randall, 2002; Wright et al., 2007). It is possible that the higher resting brain GSase activity in the present study provided more than enough glutamine synthetic capacity. As well, the assay conditions for GSase between studies were not the same. In the present study, we chose to perform the GSase assay at the same temperature as the fish (16°C), in contrast to our previous study where the assay temperature was 12°C higher than the fish (26°C) (Wright et al., 2007), to enable comparison of the results with those of numerous other studies (e.g. Shankar and Anderson, 1985; Korte et al., 1997; Anderson et al., 2002; Felskie et al., 1998). Induction of brain GSase activity in response to ammonia loading is not a consistent response in fish (see Introduction) and the present data also indicate that excess GSase capacity may eliminate the need for additional enzyme activity under these conditions.

MSOX in this study was significantly more potent at inactivating GSase in the brain than in the liver. Although we do not know the proximal cause for the tissue-specific effects of MSOX in rainbow trout, the difference may result from the isoform-specific inhibitory actions of MSOX on GSase. In both mammals (Shin and Park, 2004) and bacteria (Shatters et al., 1993), the concentration of MSOX needed to inhibit different GSase isoforms varies greatly. Trout have four different GSase isoforms which are differentially expressed across tissues (Murray et al., 2003; Wright et al., 2007). The brain expresses *Onmy-GS01* over the other forms, but the liver primarily expresses *Onmy-GS02* (Murray et al., 2003; Essex-Fraser et al., 2005).

GSase and the ammonia-induced stress response

We predicted that ammonia exposure would result in an increase in plasma cortisol concentrations, consistent with previous rainbow trout studies (Wicks and Randall, 2002; Ortega et al., 2005; Bernier et al., 2008). Indeed, plasma cortisol concentrations were 7- to 15-fold higher after 24 h of NH_4Cl exposure, and at the higher ammonia level (1000 μmol l^{-1} NH_4Cl) the surge in cortisol was also present at 96 h. The prolonged elevation of plasma cortisol at 96 h was correlated with an increase in brain CRF mRNA levels, underlining the severity of the stress under these conditions. With MSOX treatment, we predicted that plasma cortisol levels would be higher relative to the saline-injected fish exposed to ammonia based on the assumption that inhibition of GSase would result in elevated brain glutamate levels. In contrast, brain glutamate levels were not substantially elevated and overall plasma cortisol levels were not enhanced in MSOX-treated fish exposed to ammonia. In fact, we were surprised to observe that MSOX treatment significantly reduced the 24 h cortisol peak at 670 μmol l^{-1} NH_4Cl. Therefore we performed a standard handling stress test (Barton and Iwama, 1991) to determine whether MSOX had a direct effect on cortisol release. Saline- and MSOX-injected trout had similar marked increases in plasma cortisol levels relative to the unhandled control fish, leading to the conclusion that MSOX does not directly influence the release of cortisol and is therefore not singularly responsible for the reduction in the cortisol response seen in the MSOX-ammonia fish. The reduction instead required the combination of MSOX and ammonia and coincided with the reduction in brain GSase.

In mammals, it has been shown that the increase in brain glutamine concentrations during an ammonia challenge results in the uptake of the large neutral amino acids (LNAAs) tryptophan, phenylalanine and tyrosine across the blood–brain barrier (Cangiano et al., 1983; Cardelli-Cangiano et al., 1984; Rigotti et al., 1985; Gorgievski-Hrisoho et al., 1986; Hilgier et al., 1992) (for a review, see Bachmann, 2002). The driving force for the increase in brain LNAAs are amino acid exchangers with 1:1 stoichiometries that transfer glutamine out of the brain at the same time as LNAAs are transported in. Tryptophan, phenylalanine and tyrosine can be synthesized into neurotransmitters implicated in HPI and HPA stress axis activation: tryptophan into serotonin, and phenylalanine and tyrosine into noradrenaline (Dinan, 1996; Winberg et al., 1997; Chaouloff, 2000; Herman et al., 2003; Pepels et al., 2004). This has

not been studied previously in fish, but results from this study suggest that LNAA movement into the brain may occur in fish exposed to ammonia. Fish without MSOX treatment had higher overall concentrations of tryptophan, phenylalanine and tyrosine relative to MSOX-treated fish during both ammonia exposures. Changes in phenylalanine and tyrosine concentrations may also be the result of alterations in brain amino acid concentrations (see above and Fig. 7). We suggest that the higher concentrations of brain glutamine may have been the catalyst for the transport of LNAAs into the brain.

By promoting the uptake of LNAAs such as tryptophan during hyperammonaemia, GSase may be indirectly involved in the regulation of the HPI stress axis. In fish, as in other vertebrates, the rate-limiting step in brain serotonin synthesis is the availability of tryptophan (Aldegunde et al., 1998; Aldegunde et al., 2000). Ammonia exposure in teleosts is associated with an increase in brain serotonergic activity (Atwood et al., 2000; Ortega et al., 2005; Ronan et al., 2007), and after 24 h of hyperammonaemic conditions in rainbow trout there is a strong correlation between hypothalamic serotonergic activity and plasma cortisol levels (Ortega et al., 2005). The end product of HPI axis activation, cortisol, has also been shown to upregulate GSase expression in the gulf toadfish (Walsh et al., 1994; Hopkins et al., 1995), the sea raven (*Hemitripterus americanus*) (Vijyan et al., 1996) and the rainbow trout (Aluru and Vijayan, 2007) in a tissue-specific manner (Esbaugh and Walsh, 2009). Although the specific roles of LNAAs and cortisol in preventing brain ammonia toxicity in fish remain to be defined, the above observations suggest complex interactions between the ammonia detoxifying pathways regulated by GSase and the components of the HPI stress axis.

ACKNOWLEDGEMENTS

The authors wish to thank Sarah Alderman and Meghan Fuzzen for their assistance with tissue collection, Bob Frank and Matt Cornish for their technical assistance in the Hagen Aqualab, Lori Ferguson for typographical help with the manuscript, and two anonymous reviewers for constructive comments. This research was funded by NSERC Discovery grants to J.S.B., P.A.W. and N.J.B.

REFERENCES

Aldegunde, M., Garcia, J., Soengas, J. L. and Rozas, G. (1998). Uptake of tryptophan into brain of rainbow trout (*Oncorhynchus mykiss*). *J. Exp. Zool.* **282**, 285-289.

Aldegunde, M., Soengas, J. L. and Rozas, G. (2000). Acute effects of L-tryptophan on tryptophan hydroxylation rate in brain regions (hypothalamus and medulla) of rainbow trout (*Oncorhynchus mykiss*). *J. Exp. Zool.* **286**, 131-135.

Aluru, N. and Vijayan, M. M. (2007). Hepatic transcriptome response to glucocorticoid receptor activation in rainbow trout. *Physiol. Genomics* **31**, 483-491.

Anderson, P. M., Broderius, M. A., Fong, K. C., Tsui, K. N. T., Chew, S. F. and Ip, Y. K. (2002). Glutamine synthetase expression in liver, muscle, stomach and intestine of *Bostrichthys siensis* in response to exposure to a high exogenous ammonia concentration. *J. Exp. Biol.* **205**, 2053-2065.

Arillo, A., Margiocco, F., Melodia, F., Mensi, P. and Schenone, G. (1981). Ammonia toxicity mechanism in fish: studies on rainbow trout (*Salmo gairdneri* Rich.). *Ecotox. Environ. Safe.* **5**, 316-328.

Atwood, H. L., Tomasso, J. R., Ronan, P. J., Barton, B. A. and Renner, K. J. (2000). Brain monoamine concentrations as predictors of growth inhibition in channel catfish exposed to ammonia. *J. Aquat. Anim. Health* **12**, 69-73.

Bachmann, C. (2002). Mechanisms of hyperammonemia. *Clin. Chem. Lab. Med.* **40**, 653-662.

Barton, B. A. and Iwama, G. K. (1991). Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. *Ann. Rev. Fish Dis.* **1**, 3-26.

Bernier, N. J., Alderman, S. L. and Bristow, E. N. (2008). Heads or tails? Stressor-specific expression of corticotropin-releasing factor and urotensin 1 in the preoptic area and caudal neurosecretory system of rainbow trout. *J. Endocrinol.* **196**, 637-648.

Bernier, N. J., Flik, G. and Klaren, P. H. M. (2009). Regulation and contribution of the corticotropic, melanotropic and thyrotropic axes to the stress response in fishes. In *Fish Physiology*, vol. 28, *Fish Neuroendocrinology* (ed. N. J. Bernier, G. Van Der Kraak, A. P. Farrell and C. J. Brauner), pp. 235-311. Burlington: Academic Press.

Cameron, J. N. and Heisler, N. (1983). Studies of ammonia in the rainbow trout: physico-chemical parameters, acid-base behaviour, and respiratory clearance. *J. Exp. Biol.* **105**, 107-125.

Cangiano, C., Cardelli-Cangiano, P., James, J. H., Rossi-Fanelli, F., Patrizi, M. A., Brackett, K. A., Strom, R. and Fischer, J. E. (1983). Brain microvessels take up large amino acids in exchange for glutamine. *J. Biol. Chem.* **258**, 8949-8954.

Cardelli-Cangiano, P., Cangiano, C., James, J. H., Ceci, F., Fischer, J. E. and Strom, R. (1984). Effect of ammonia on amino acid uptake by brain microvessels. *J. Biol. Chem.* **259**, 5295-5300.

Chaouloff, F. (2000). Serotonin, stress, and corticoids. *J. Psychopharmacol.* **14**, 139-151.

Claiborne, J. B. and Evans, D. H. (1988). Ammonia and acid-base balance during high ammonia exposure in a marine teleost (*Myoxocephalus octodecimspinosus*). *J. Exp. Biol.* **140**, 89-105.

Cooper, A. J. and Plum, F. (1987). Biochemistry and physiology of brain ammonia. *Physiol. Rev.* **67**, 440-519.

Cooper, A. J., Stephani, R. A. and Meister, A. (1976). Enzymatic reactions of methionine sulfoximine. Conversion to the corresponding α-imino and α-keto acids and to α-ketobutyrate and methane sulfinimide. *J. Biol. Chem.* **251**, 6674-6682.

Dinan, T. G. (1996). Serotonin and the regulation of hypothalamic-pituitary-adrenal axis function. *Life Sci.* **58**, 1683-1694.

Esbaugh, A. J. and Walsh, P. J. (2009). Identification of two glucocorticoid response elements in the promoter region of the ubiquitous isoform of glutamine synthetase in gulf toadfish, *Opsanus beta*. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **297,** R1075-R1081.

Essex-Fraser, P. A., Steele, S. L., Bernier, N. J., Murray, B. W., Stevens, E. D. and Wright, P. A. (2005). Expression of four glutamine synthetase genes in the early stages of development of rainbow trout (*Oncorhynchus mykiss*) in relationship to nitrogen excretion. *J. Biol. Chem.* **280**, 20268-20273.

Felipo, V. and Butterworth, R. F. (2002). Neurobiology of ammonia. *Prog. Neurobiol.* **67**, 259-279.

Felskie, A. K., Anderson, P. M. and Wright, P. A. (1998). Expression and activity of carbamoyl phosphate synthetase III and ornithine urea enzymes in various tissues of four fish species. *Comp. Biochem. Physiol.* **119B**, 355-364.

Gorgievski-Hrisoho, M., Colombo, J. P. and Bachmann, C. (1986). Stimulation of tryptophan uptake into brain microvessels by D-glutamine. *Brain Res.* **367**, 395-397.

Herman, J. P., Figueiredo, H., Mueller, N. K., Ulrich-Lai, Y., Ostrander, M. M., Choi, D. C. and Cullinan, W. E. (2003). Central mechanism of stress integration: hierarchical circuitry controlling hypothalamo-pituitary-adrenocortical responsiveness. *Front. Neuroendocrinol.* **24**, 151-180.

Hilgier, W., Puka, M. and Albrecht, J. (1992). Characteristics of large neutral amino acid-induced release of preloaded L-glutamine from rat cerebral capillaries in vitro: effects of ammonia, hepatic encephalopathy, and γ-glutamyl transpeptidase inhibitors. *J. Neurosci. Res.* **32**, 221-226.

Hopkins, T. E., Wood, C. M. and Walsh, P. J. (1995). Interactions of cortisol and nitrogen metabolism in the ureogenic gulf toadfish (*Opsanus beta*). *J. Exp. Biol.* **198**, 2229-2235.

Hung, C. Y. C., Tsui, K. N. T., Wilson, J. M., Nawata, M., Wood, C. M. and Wright, P. A. (2007). Molecular cloning, characterization and tissue distribution of the Rhesus glycoproteins Rhbg, Rhcg1 and Rhcg2 in the mangrove killifish *Rivulus marmoratus* exposed to elevated environmental ammonia levels. *J. Exp. Biol.* **210**, 2419-2429.

Ip, Y. K., Chew, S. F. and Randall, D. J. (2001). Ammonia toxicity, tolerance, and excretion. In *Fish Physiology*, vol. 20 (ed. P. A. Wright and P. M. Anderson), pp. 109-148. San Diego: Academic Press.

Ip, Y. K., Tay, A. S. L., Lee, K. H. and Chew, S. F. (2004). Strategies for surviving high concentrations of environmental ammonia in the swamp eel *Monopterus albus*. *Physiol. Biochem Zool.* **77**, 390-405.

Ip, Y. K., Leong, M. W. F., Sim, M. Y., Goh, G. S., Wong, W. P. and Chew, S. F. (2005). Chronic and acute ammonia toxicity in mudskippers, *Periophthalmodon schlosseri* and *Boleophthalamus boddaerti*: brain ammonia and glutamine contents, and effects of methionine sulfoximine and MK801. *J. Exp. Biol.* **208**, 1993-2004.

Joanny, P., Steinberg, J., Oliver, C. and Grino, M. (1997). Glutamate and N-Methyl-D-Aspartate stimulate rat hypothalamic corticotropin-releasing factor secretion *in vitro*. *J. Neuroendocrinol.* **9**, 93-97.

Korte, J. J., Salo, W. L., Cabrera, V. M., Wright, P. A., Felskie, A. K. and Anderson, P. M. (1997). Expression of carbamoyl-phospate synthetase III mRNA during the early life stages of development and in muscle of adult rainbow trout (*Oncorhynchus mykiss*). *J. Biol. Chem.* **272**, 6270-6277.

Kun, E. and Kearney, E. B. (1974). Ammonia. In *Methods of Enzymatic Analysis,* vol. 4 (ed. H. U. Bergmeyer), pp. 1802-1806. New York: Academic Press.

Levi, G., Morisi, G., Coletti, A. and Catanzaro, R. (1974). Free amino acids in fish brain: normal levels and changes upon exposure to high ammonia concentrations *in vivo*, and upon incubation of brain slices. *Comp. Biochem. Physiol.* **49A**, 623-636.

Murray, B. W., Busby, E. R., Mommsen, T. P. and Wright, P. A. (2003). Evolution of glutamine synthetase in vertebrates: multiple glutamine synthetase genes expressed in rainbow trout (*Oncorhynchus mykiss*). *J. Exp. Biol.* **206**, 1511-1521.

Nakada, T., Hoshijima, K., Esaki, M., Nagayoshi, S., Kawakami, K. and Hirose, S. (2007a). Localization of ammonia transporter Rhcg1 in mitochondrion rich cells of yolk sac, gill and kidney of zebrafish and its ionic strength-dependent expression. *Am. J. Physiol.* **293**, R1743-R1753.

Nakada, T., Westoff, C. M., Kato, A. and Hirose, S. (2007b). Ammonia secretion from fish gill depends on a set of Rh glycoproteins. *FASEB J.* **21**, 1067-1074.

Nawata, C. M., Hung, C. C. Y., Tsui, T. K. N., Wilson, J. M., Wright, P. A. and Wood, C. M. (2007). Ammonia excretion in rainbow trout (*Oncorhynchus mykiss*): evidence of Rh glycoprotein and H^+-ATPase involvement. *Physiol. Genomics* **31**, 463-474.

Nelson, D. L. and Cox, M. M. (2000). *Lehninger Principles of Biochemistry*, 3rd edn. New York: Worth Publishers.

Ortega, V. A., Renner, K. J. and Bernier, N. J. (2005). Appetite-suppressing effects of ammonia exposure in rainbow trout associated with regional and temporal activation of brain monoaminergic and CRF systems. *J. Exp. Biol.* **208**, 1855-1866.

Peng, K. W., Chew, S. F., Lim, C. B., Kuah, S. S. L., Kok, W. K. and Ip, Y. K. (1998). The mudskippers *Periophthalmodon schlosseri* and *Boleophthalmus boddaerti* can tolerate environmental NH_3 concentrations of 446 and 36 μM respectively. *Fish Physiol. Biochem.* **19**, 59-69.

Pepels, P. P. L. M., Wendelaar Bonga, S. E. and Balm, P. H. M. (2004). Bacterial lipopolysaccharide (LPS) modulates corticotropin-releasing hormone (CRH) content and release in the brain of juvenile and adult tilapia (*Oreochromis mossambicus*; Teleostei). *J. Exp. Biol.* **207**, 4479-4488.

Pitts, R. F. (1973). Production and excretion of ammonia in relation to acid-base regulation. In *Handbook of Physiology, Renal Physiology* (ed. J. Orloff and R. W. Berliner), pp. 455-496. Washington, DC: American Physiology Society.

Randall, D. J. and Tsui, T. K. N. (2002). Ammonia toxicity in fish. *Mar. Pollut. Bull.* **45**, 17-23.

Rigotti, P., Jonung, T., Peters, J. C., James, J. H. and Fischer, J. E. (1985). Methionine sulfoximine prevents the accumulation of large neutral amino acids in brains of portacaval-shunted rats. *J. Neurochem.* **44**, 929-933.

Ronan, P. J., Gaikowski, M. P., Hamilton, S. J., Buhl, K. J. and Summers, C. H. (2007). Ammonia causes decreased brain monoamines in fathead minnows (*Pimephales promelas*). *Brain Res.* **1147**, 184-191.

Shankar, R. A. and Anderson, P. M. (1985). Purification and properties of glutamine synthetase from liver of *Squalus acanthias*. *Archiv. Physiol. Biochem.* **239**, 248-259.

Shatters, R. G., Liu, Y. and Kahn, M. L. (1993). Isolation and characterization of a novel glutamine synthetase from *Rhizobium meliloti*. *J. Biol. Chem.* **268**, 469-475.

Shin, D. and Park, C. (2004). N-terminal extension of canine glutamine synthetase created by splicing alters its enzymatic property. *J. Biol. Chem.* **279**, 1184-1190.

Speers-Roesch, B., Robinson, J. W. and Ballantyne, J. S. (2006). Metabolic organization of the spotted ratfish, *Hydrolagus colliei* (Holocephali: Chimaeriformes): insight into the evolution of energy metabolism in the Chondrichthyan fishes. *J. Exp. Zool.* **305**, 631-644.

Suárez, I., Bodega, G. and Fernández, B. (2002). Glutamine synthetase in brain: effects of ammonia. *Neurochem. Int.* **41**, 123-142.

Veauvy, C. M., McDonald, M. D., Audekerke, J. V., Vanhoutte, G., Van Camp, N., Van der Linden, A. and Walsh, P. J. (2005). Ammonia affects brain nitrogen metabolism but not hydration status in the Gulf toadfish (*Opsanus beta*). *Aquatic Tox.* **74**, 32-46.

Vedel, N. E., Korsgaard, B. and Jensen, F. B. (1998). Isolated and combined exposure to ammonia and nitrite in rainbow trout (*Oncorhynchus mykiss*): effects on electrolyte status, blood respiratory properties and brain glutamine/glutamate concentrations. *Aquatic Tox.* **41**, 325-342.

Verdouw, H., van Echted, C. J. A. and Dekkers, E. M. J. (1978). Ammonia determination based on indophenol formation with sodium salicylate. *Water Res.* **12**, 325-402.

Vijayan, M. M., Mommsen, T. P., Glémet, H. C. and Moon, T. W. (1996). Metabolic effects of cortisol treatment in a marine teleost, the sea raven. *J. Exp. Biol.* **199**, 1509-1514.

Walsh, P. J., Tucker, B. C. and Hopkins, T. E. (1994). Effects of confinement/crowding on ureogenesis in the gulf toadfish (*Opsanus beta*). *J. Exp. Biol.* **191**, 195-206.

Walsh, P. J., Veauvy, C. M., McDonald, M. D., Pamenter, M. E., Buck, L. T. and Wilkie, M. P. (2007). Piscine insights into comparisons of anoxia tolerance, ammonia toxicity, stroke, and hepatic encephalopathy. *Comp. Biochem. Physiol. A Physiol.* **147**, 332-343.

Wang, X. and Walsh, P. J. (2000). High ammonia tolerance in fishes of the family Batrachoididae (Toadfish and Midshipmen). *Aquatic Tox.* **50**, 205-219.

Wee, N. L. J., Tng, Y. Y. M., Cheng, H. T., Lee, S. M. L., Chew, S. F. and Ip, Y. K. (2007). Ammonia toxicity and tolerance in the brain of the African sharptooth catfish, *Clarias gariepinus*. *Aquatic Tox.* **82**, 204-213.

Wendelaar Bonga, S. E. (1997). The stress response in fish. *Physiol. Rev.* **77**, 591-625.

Wicks, B. J. and Randall, D. J. (2002). The effect of sub-lethal ammonia exposure on fed and unfed rainbow trout: the role of glutamine in regulation of ammonia. *Comp. Biochem. Physiol. A Physiol.* **132**, 275-285.

Wilson, R. W. and Taylor, E. W. (1992). Transbranchial ammonia gradients and acid-base responses to high external ammonia concentration in rainbow trout (*Oncorhynchus mykiss*) acclimated to different salinities. *J. Exp. Biol.* **166**, 95-112.

Winberg, S., Nilsson, A., Hylland, P., Soderstom, V. and Nilsson, G. E. (1997). Serotonin as a regulator of hypothalamic-pituitary-interrenal activity in teleost fish. *Neurosci. Lett.* **230**, 113-116.

Wright, P. A. and Wood, C. M. (1985). An analysis of branchial ammonia excretion in the freshwater rainbow trout: effects of environment pH changes and sodium uptake blockade. *J. Exp. Biol.* **114**, 329-353.

Wright, P. A. and Wood, C. M. (2009). A new paradigm for ammonia excretion in aquatic animals: role of Rhesus (Rh) glycoproteins. *J. Exp. Biol.* **212**, 2303-2312.

Wright, P. A., Randall, D. J. and Wood, C. M. (1988). The distribution of ammonia and H+ between tissue compartments in lemon sole (*Parophrys vetulus*) at rest, during hypercapnia and following exercise. *J. Exp. Biol.* **136**, 149-175.

Wright, P. A., Steele, S. L., Huitema, A. and Bernier, N. J. (2007). Induction of four glutamine synthetase genes in brain of rainbow trout in response to elevated environmental ammonia. *J. Exp. Biol.* **210**, 2905-2911.