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Induction of four glutamine synthetase genes in brain of rainbow trout in response to elevated environmental ammonia

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Summary

The key strategy for coping with elevated brain ammonia levels in vertebrates is the synthesis of glutamine from ammonia and glutamate, catalyzed by glutamine synthetase (GSase). We hypothesized that all four GSase isoforms (*Onmy-GS01–GS04*) are expressed in the brain of the ammonia-intolerant rainbow trout *Oncorhynchus mykiss* and that cerebral GSase is induced during ammonia stress. We measured GSase activity and the mRNA expression of *Onmy-GS01–GS04* in fore-, mid- and hindbrain and liver, as well as ammonia concentrations in plasma, liver and brain of fish exposed to 9 or 48 h of 0 (control) or 670 μmol l^{-1} NH_4Cl (75% of the 96 h-LC_{50} value). The mRNA of all four GSase isoforms were detected in brain (not liver). After 9 h of NH_4Cl exposure, brain, liver and plasma ammonia content were elevated by two- to fourfold over control values. Midbrain, hindbrain and liver GSase activities were 1.3- to 1.5-fold higher in ammonia-exposed fish relative to control fish. *Onmy-GS01–GS04* mRNA levels in brain (not liver) of ammonia-exposed fish (9 h) were significantly elevated by two- to fourfold over control values. After 48 h of the NH_4Cl treatment, ammonia content and GSase activity, but not mRNA levels, in all tissues examined remained elevated compared to control fish. Taken together, these findings indicate that all four GSase isoforms are constitutively expressed in trout brain and are inducible under high external ammonia conditions. Moreover, elevation of GSase activities in fore-, mid- and hindbrain in response to environmental ammonia underlines the importance of brain GSase in the ammonia-stress response.

Key words: ammonia toxicity, liver, enzyme activity, gene expression, mRNA, nitrogen metabolism, glutamine, sublethal ammonia exposure.

Introduction

The vertebrate brain is the most vulnerable organ to the highly mobile and toxic ammonia (Felipo and Butterworth, 2002). The major end product of hepatic protein and amino acid catabolism, ammonia is normally eliminated by passive diffusion across the gills of fish down the blood-to-water gradient (Evans et al., 2005). Fish may experience environments in the wild where ammonia is elevated as a result of agricultural, industrial or sewage wastes or in culture settings where stocking density is high and/or water turnover is low. Fish vary considerably in their ability to tolerate elevated external ammonia, for example the total ammonia (NH_3+NH_4^+) 96 h LC_{50} value for adult freshwater rainbow trout is 0.89 mmol l^{-1}, pH 8.2 (Thurston et al., 1981) and for the marine toadfish *Opsanus beta* is ~9.75 mmol l^{-1} (Wang and Walsh, 2000). Elevated external ammonia reverses the branchial diffusion gradient, resulting in the uptake of ammonia and accumulation in the extra- and intracellular compartments and, if severe, can result in convulsions, coma and eventually death (Randall and Tsui, 2002). Ammonium can be combined with α-ketoglutarate to form glutamate, catalyzed by glutamate dehydrogenase (GDH; EC1.4.1.3). A second ammonium ion can then be added to glutamate to form glutamine, catalyzed by glutamine synthetase (GSase; EC6.3.1.2). GSase is a multifunctional enzyme that is involved in amino acid balance, nucleotide biosynthesis, neurotransmitter metabolism, as well as ammonia detoxification (Walsh and Mommsen, 2001).

GSase activity in fish brain is several orders of magnitude higher relative to other tissues, including liver (Webb and Brown, 1976; Chamberlin et al., 1991; Wang and Walsh, 2000; Wicks and Randall, 2002; Mommsen et al., 2003; Essex-Fraser et al., 2005). Cerebral glutamine levels increase in fish in response to exposure to elevated levels of ammonia in the environment (Levi et al., 1974; Arillo et al., 1981; Iwata, 1988; Vedel et al., 1998; Peng et al., 1998; Wang and Walsh, 2000; Veauvy et al., 2005) or feeding (Wicks and Randall, 2002). Brain GSase activity in three batrachoidid fish species (*O. beta*, *O. tau*, *Porichthys notatus*) was positively correlated with ability to tolerate ammonia (Wang and Walsh, 2000). Moreover, pretreatment with methionine sulfoximine (MSO), an inhibitor of GSase, reduced survival in toadfish subjected to sub-lethal levels of external ammonia, suggesting that GSase is critical to the ammonia stress response (Veauvy et al., 2005). In other studies, induction of brain GSase in fish exposed to sub-lethal ammonia concentrations was relatively small or absent (Peng et al., 1998; Wang and Walsh, 2000; Wicks and Randall, 2002).

Our understanding of the role of GSase in the ammonia stress response in fish should be enhanced by new molecular

information. In mammals, GSase is encoded by one gene that is expressed at relatively high levels in brain tissue (Mearow et al., 1989). Gene sequences for GSase have been reported in several fish species, including *O. beta* (Walsh et al., 1999; Walsh et al., 2003), *Oreochromis niloticus* (Mommsen et al., 2003), *Danio rerio* (see Murray et al., 2003) and elasmobranchs (Laud and Campbell, 1994). In rainbow trout, four GSase isoforms have been identified (*Onmy-GS01–GS04*) (Murray et al., 2003), and mRNA levels for each isoform have been measured in early stages of development (Essex-Fraser et al., 2005). An initial induction of *Onmy-GS01* and *-GS03* prior to hatching in trout embryos was correlated with rising levels of ammonia. In adult trout, only *Onmy-GS01* and *-GS02* were expressed at appreciable levels in liver and expression was generally very low in skeletal muscle tissue (Essex-Fraser et al., 2005). Little information is available on mRNA levels in the brain, where GSase is thought to play a very important role.

In the present study, two hypotheses were tested. First, we hypothesized that the full complement of GSase isoforms (*Onmy-GS01–GS04*) are expressed in cerebral tissues of the rainbow trout, where GSase activity is 200–500 times higher than other tissues (Essex-Fraser et al., 2005). Second, we hypothesized that GSase activity and mRNA levels are upregulated in the brain of the ammonia-intolerant rainbow trout during ammonia stress. In previous studies, GSase activity measurements on whole brain homogenates in fish may have masked more subtle changes in specific regions, therefore we subdivided the brain into its three primary areas (fore-, mid- and hindbrain). Trout were exposed to control water or 670 μmol l^{-1} NH_4Cl for 9 h or 48 h. Ammonia concentrations were measured in plasma, brain (fore-, mid- and hindbrain) and liver samples. GSase activity and mRNA levels for *Onmy-GS01–GS04* were determined in brain (fore-, mid- and hindbrain) and liver tissue.

Materials and methods

Animals

Rainbow trout *Oncorhynchus mykiss* Walbaum (mean mass 88±3 g) were purchased from Rainbow Springs Trout Farm (Thamesford, ON, Canada). Fish were randomly assigned to eight 130 l tanks (8–9 fish each) supplied with continuous flow (4 l min^{-1}) local well water (pH 8.2; 14°C; 411 mg l^{-1} hardness as $CaCO_3$; Ca^{2+}, 5.24 mequiv l^{-1}; Cl^-, 1.47 mequiv l^{-1}; Mg^{2+}, 2.98 mequiv l^{-1}; K^+, 0.06 mequiv l^{-1}; Na^+, 1.05 mequiv l^{-1}). The fish were fed standard trout pellets daily (2% of body mass per day) and held in a 12 h:12 h light:dark photoperiod. Fish were left to acclimate in the tanks for approximately 4 weeks before the experiment began and were not fed during the 48 h experimental period.

Experimental protocol

For each exposure period, fish were divided into four groups. Two tanks of fish were randomly assigned to the control group (no NH_4Cl added, pH 8.2) while the remaining two tanks were assigned to the 670 μmol l^{-1} NH_4Cl exposure group (pH 8.2; NH_3 concentration ~31 μmol l^{-1}; ammonia N, 12 mg l^{-1}). This level of ammonia was 75% of the 96 h-LC_{50} value (0.89 mmol l^{-1}, pH 8.2) (Thurston et al., 1981). This sublethal level of ammonia was used because in preliminary experiments, where trout were exposed to lower ammonia levels under the same conditions, there was no significant change in brain GSase activity. At the start of the experiment, the water in each of the ammonia exposure tanks was spiked with 5.24 g of NH_4Cl dissolved in 2 l of tank water in order to immediately bring the tank concentration up to 670 μmol l^{-1} NH_4Cl. To maintain this concentration in a flow-through system for the duration of the 48 h exposure period, a 1.2 mol l^{-1} NH_4Cl solution was added to each ammonia exposure tank at a rate of 2.5 ml min^{-1} using a peristaltic pump (Minipuls 3, Villiers Le Bel, France). The freshwater flow rate was ~4 l min^{-1}. Water samples (5 ml) were collected 5 min after the initiation of the experiment (time 0 h) and every 6 h thereafter. Water samples were stored at –20°C for later analysis (within 1 week).

At the end of the 9 h or 48 h exposure period, all fish were terminally anaesthetized in 2 ml l^{-1} 2-phenoxyethanol. Blood samples (1 ml) were collected by caudal venipuncture in 0.5 mol l^{-1} Na_2EDTA-coated syringes (to prevent coagulation), placed on ice, then centrifuged at 11 000 ***g*** for 5 min. Plasma was decanted and stored at –80°C for later analysis (within 1 week). Whole brains were removed and dissected into three regions: (i) the telencephalon (forebrain), (ii) the preoptic area, optic tectum, hypothalamus and midbrain (midbrain) and (iii) the cerebellum and hindbrain (hindbrain), according to the figure published elsewhere (Doyon et al., 2003). Although it would have been ideal to measure GSase activity in each of the nine distinct brain tissues (Doyon et al., 2003), tissues were pooled in the midbrain and hindbrain in order to obtain sufficient mass to detect GSase activity. The olfactory bulbs, optic nerves, pituitary gland and spinal cord were not included in these brain sections. Liver tissue was also collected for comparison with brain values. Livers were removed, and tissue samples were immediately frozen in liquid nitrogen and stored at –80°C for later analysis (within 2 weeks).

Analyses

Ammonia

Ammonia concentration in water samples was determined using a colorimetric assay (Verdouw et al., 1978). Brain regions and liver samples were ground to a fine powder in liquid nitrogen using a mortar and pestle and deproteinized by adding 0.5 w/v 8% perchloric acid (PCA). Plasma (500 μl) was deproteinized in 250 μl 8% PCA. Samples were centrifuged at 16 000 ***g*** (4°C) for 5 min. The resulting supernatant was neutralized using 0.5 v/v saturated potassium bicarbonate and centrifuged at 16 000 ***g*** (4°C) for 5 min. Ammonia levels in the resulting supernatant were analyzed according to the enzymatic method described by Kun and Kearney (Kun and Kearney, 1974). All spectophotometric measurements were performed on a SpectraMax 190 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

Glutamine synthetase activity

Brain regions and liver samples were prepared for enzyme analysis as previously described (Steele et al., 2001), with the exception that all samples were homogenized in approximately 70 volumes of ice-cold homogenization buffer. After centrifugation, endogenous substrates were removed from the homogenate by passage through Sephadex columns as described

(Felskie et al., 1998). Glutamine synthetase activity was determined as the production of γ-glutamyl hydroxamate from 0 to 3 min at 26°C as previously described (Shankar and Anderson, 1985), using a Ultrospec 3100 pro UV/Visible spectrophotometer (Biochrom Ltd, Cambridge, UK).

RNA extraction and cDNA synthesis

Total RNA was isolated from brain regions and liver using TRIzol Reagent (Invitrogen, Carlsbad, CA, US). Samples (<100 mg) were homogenized in 1 ml TRIzol by repeatedly drawing the mixture into a sterile syringe (3 ml) fitted with a 20 G needle. After following the manufacturer's instructions, RNA pellets were reconstituted in 30 μl water and stored at –80°C. To eliminate possible genomic DNA contamination, total RNA (3 μg) was treated with Deoxyribonuclease (DNase) I, amplification grade (Invitrogen, Carlsbad, CA, USA). The DNase-treated total RNA samples were reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and primer, Poly-T. Non-reverse transcribed controls were synthesized using the same reaction but substituting water for the SuperScript enzyme.

Real-time PCR

mRNA expression of *Onmy-GS01–GS04* was quantified from the above cDNA products using the ABI Prism 7000 sequence detection system (Applied Biosystems, Forster City, CA, USA). The coding sequences of these isoforms are very similar while the 3′ UTRs are more diverged. The nucleotide sequences of the 3′ UTR for *Onmy-GS01* and *-GS03* were 81% homologous, whereas *Onmy-GS02* and *-GS04* were 79% homologous. The low level of variation in the coding sequence of the isoforms made probe and primer design for mRNA analysis more difficult. The approach we selected was real-time PCR using a gene-specific probe and set of primers that matched unique 3′ UTR sequences in each isoform. In preliminary trials, one PCR reaction from each primer set was purified using a QIAquick PCR purification kit (Qiagen Inc., Hilden, Germany) and sequenced to ensure that each primer set was only amplifying the target sequence.

Primers and dual-labeled probes (Table 1) were designed for each gene using PrimerExpress software (v. 2.0, Applied Biosystems). All probes were dual-labeled with 6-FAM fluorescent reporter at the 3′ end and TAMRA quencher at the 5′ end. Each PCR reaction contained 5 μl template, 12.5 μl Taqman Universal PCR Master Mix (no AmpErase UNG, Applied Biosystems, Foster City, CA, USA), and 2.5 μl each of forward and reverse primers (9 μmol l^{-1}) and probe (2.5 μmol l^{-1}). The following conditions were used: 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. To correct for variability in amplification efficiency between different cDNAs, a standard curve was performed (Giulietti et al., 2001) for each glutamine synthetase gene using serial dilutions of cDNA samples from whole trout brain. The relative dilution of a given sample was extrapolated by linear regression using the threshold cycle of each unknown. To account for differences in cDNA loading and RNA reverse transcriptase efficiency, each sample was normalized to the expression level of the control gene β-actin. The expression of β-actin was not significantly different between any of the

Table 1. *Primer and probe sequences for detection of glutamine synthetase (Onmy-GS01–GS04) and β-actin gene expression in rainbow trout Oncorhynchus mykiss using real time PCR*

Gene*	Forward primer 5′–3′	Reverse primer 5′–3′	Probe 5′–3′	Length of amplicon (bp)
Onmy-GS01	CTGCAGTCTGTGTTCAGGGTAGA	CATCTGTCTGGAATTGTTAAGTCCATA	TACCTTTTGATCACTGCCAACATTGCCC	101
Onmy-GS02	GGCAGTGTCTTTAAATGGCAACA	ACGCTACAATTGGCAAGACTGA	CTGTCTCCAGATTTGACACATTCCTGGATCAT	136
Onmy-GS03	GTGTATCAATTTGCTACTCATGTTTAACAT	AAAATGGGTTCTTGATACAACTTCTACTAA	AAGGATCCAAGGTGCATCTGTGTTTTTATACATG	191
Onmy-GS04	TTAATGAAAGATGGTGGCTGACA	CTGCAGGAAACGCGAGATC	CATTGTCTTCCCCTTTTGAGTCTTCTAGTGGG	105
β-actin	GACCCAGATCATGTTTGAGACCTT	CGTAGCCCTCGTAGATGGGTACT	ACTCCGGTGACGGCGTGACCC	152

*GenBank accession numbers: *Onmy-GS01*, AF390021; *Onmy-GS02*, AF390022; *Onmy-GS03*, AF390023; *Onmy-GS04*, AF390024; β-actin, AJ438158.

tissues nor between control and ammonia exposed individuals (data not shown).

Samples were assayed in triplicate with only one target gene assayed per well. Non-reverse transcribed RNA and water-only controls were run to ensure that no genomic DNA was being amplified and that reagents were not contaminated.

Statistical analysis

Statistical analyses of GSase activity and ammonia concentrations were performed using Sigma Stat (Version 3.0, SPSS Inc., Chicago, IL, USA). For GSase activity and ammonia concentrations, differences between control and ammonia treated groups as well as differences within groups were analyzed using one-way analysis of variance (ANOVA). General Linear Model (GLM) analysis was used to compare mRNA levels of individual GSase genes (*Onmy-GS01–GS04*) between control and ammonia-exposed groups, as well as to compare control levels of individual genes between tissues and between genes within a tissue type. A Tukey *post-hoc* test was applied if statistical differences were detected with the above analysis. Results were declared to be significant if $P<0.05$.

Results

Ammonia

The average ammonia concentration in the water of the control tanks was 10±2 μmol l^{-1} and 671±7 μmol l^{-1} in the experimental tanks. In control fish, brain and liver ammonia concentrations were significantly higher than plasma levels in both the 9 h and 48 h experiments (Fig. 1). In addition, liver ammonia content in control fish was modestly higher (~1.4-fold) compared to brain levels in most regions at both the 9 h and 48 h fish (Fig. 1).

In NH_4Cl-exposed fish, ammonia concentration was significantly elevated in forebrain (fourfold), midbrain (fourfold), hindbrain (fourfold), liver (twofold) and plasma (fourfold) following 9 h and 48 h of exposure relative to control fish (Fig. 1). In addition, the liver ammonia concentration following 9 h and 48 h of external ammonia treatment were significantly lower compared to the brain regions.

GSase activity

In control fish, GSase activity in brain tissue was, on average, 270-fold higher relative to liver values (Fig. 2). The mid- and hindbrain had significantly higher GSase activities relative to the forebrain in both control groups of fish (9 h and 48 h).

In NH_4Cl-exposed fish, there was a significant increase in GSase activity after 9 h in mid- and hindbrain (1.3- to 1.5-fold), as well as in liver (1.5-fold) (Fig. 2). After 48 h of ammonia exposure, GSase activity was elevated to an even greater extent in all three brain regions (1.4- to 1.8-fold) and in liver (1.7-fold) relative to control fish.

GSase gene expression

All four GSase isoforms, *Onmy-GS01–GS04*, were expressed in brain tissue of control fish (Fig. 3A,B). Each isoform was also detected in the liver, but the level of expression of *Onmy-GS04* was very low relative to the brain (Fig. 3A,B). In control fish (9 h experiment), *Onmy-GS03* mRNA levels were significantly higher (Fig. 3A) relative to *Onmy-GS02* in all three brain regions and *Onmy-GS04* in the forebrain and hindbrain, but these differences were not apparent in the 48 h experiment (Fig. 3B).

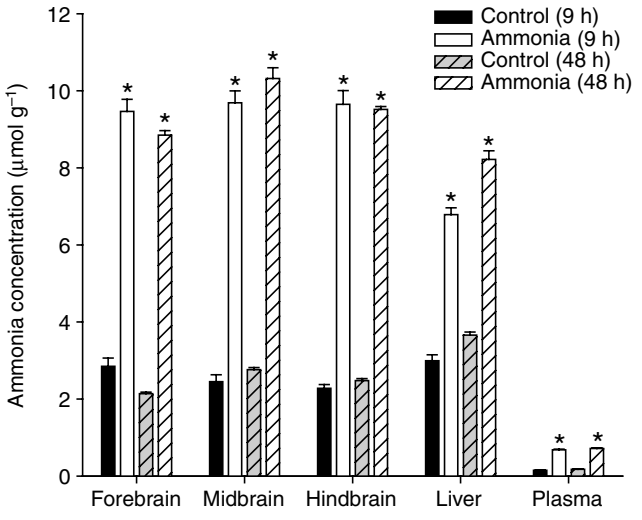

Fig. 1. Ammonia concentration in rainbow trout *Oncorhynchus mykiss* brain regions (fore-, mid- and hindbrain), liver and plasma in fish exposed for 9 or 48 h to control water (9 h control, black bars; 48 h control, shaded hatched bars) or 670 μmol l^{-1} NH_4Cl (9 h ammonia, open bars; 48 h ammonia, open hatched bars). An asterisk indicates significant differences between control and NH_4Cl-exposed fish at a given sampling time. For clarity, statistical comparisons between ammonia concentrations in control fish were omitted from the figure and are presented in the Results. Values are means ± s.e.m. (N=8–12).

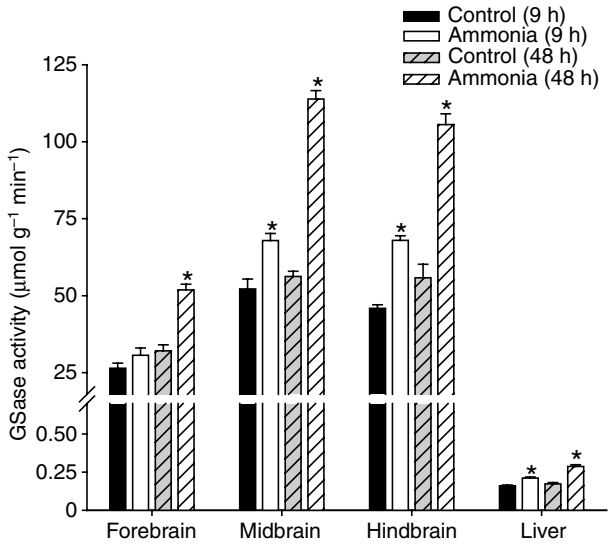

Fig. 2. Glutamine synthetase (GSase) activity measured by the transferase assay in rainbow trout *Oncorhynchus mykiss* brain regions (fore-, mid- and hindbrain) and liver in fish exposed for 9 or 48 h to control water (9 h control, black bars; 48 h control, shaded hatched bars) and 670 μmol l^{-1} NH_4Cl (9 h ammonia, open bar; 48 h ammonia, open hatched bars). An asterisk indicates significant differences between control and NH_4Cl-exposed fish at a given sampling time. For clarity, statistical comparisons between GSase activities in control fish were omitted from the figure and are presented in the Results. Values are means ± s.e.m. (N=8-16).

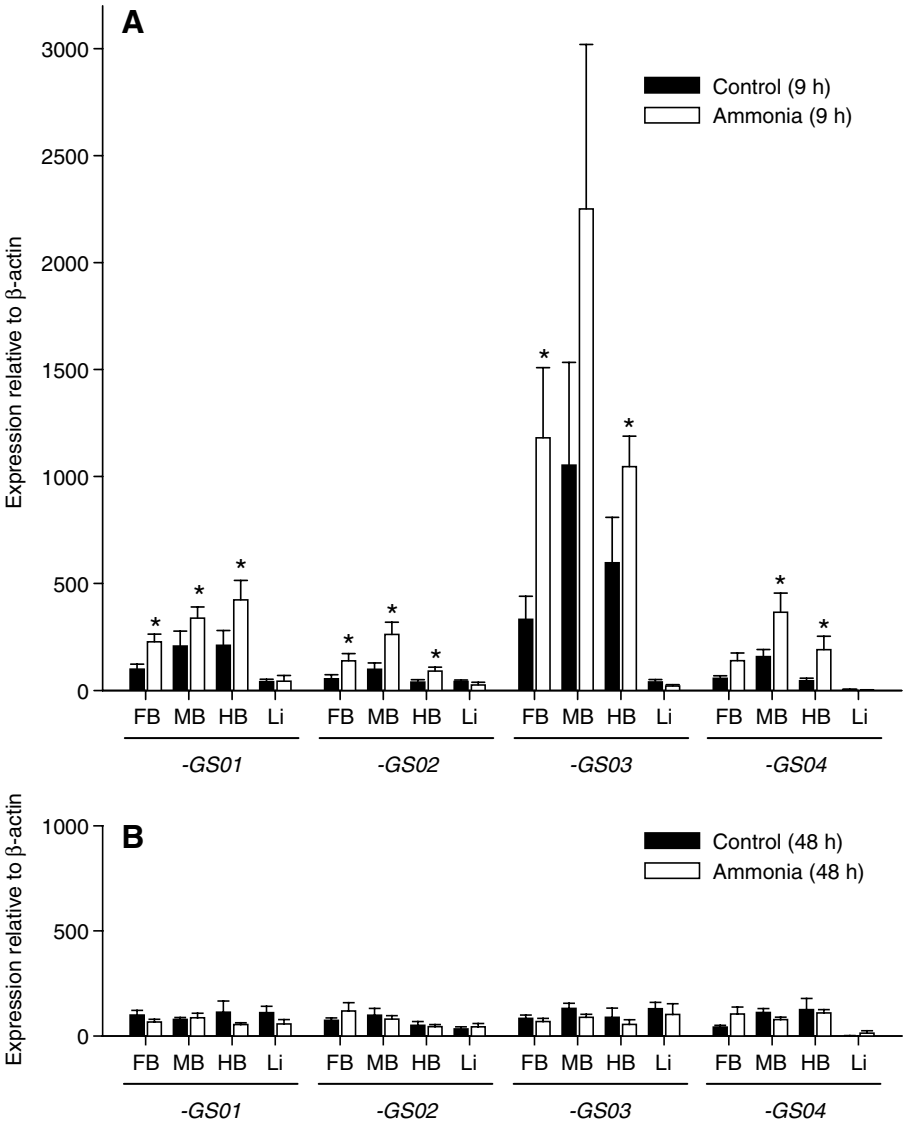

Fig. 3. Changes in mRNA levels of glutamine synthetase (GSase) genes (*Onmy-GS01*, *-GS02*, *-GS03*, *-GS04*) in rainbow trout *Oncorhynchus mykiss* brain regions relative to the expression of a control gene, β-actin. FB, forebrain; MB, midbrain; HB, hindbrain; Li, liver. Fish were exposed for 9 h (A) or 48 h (B) to either control water or 670 μmol l^{-1} NH_4Cl. An asterisk indicates significant differences between control and NH_4Cl-exposed fish. Values are means ± s.e.m. (*N*=6).

Brain GSase mRNA expression was induced relative to control fish following 9 h of exposure to external ammonia (Fig. 3A), but by 48 h mRNA levels had returned to control values (Fig. 3B). There were no significant changes in liver GSase expression at 9 h (Fig. 3A) or at 48 h (Fig. 3B). *Onmy-GS01* and *-GS02* mRNA levels were significantly increased (~twofold) in ammonia-exposed fish in all three brain regions (Fig. 3A). *Onmy-GS03* mRNA levels were fourfold higher in the forebrain and twofold higher in the hindbrain of ammonia-exposed fish relative to control fish (Fig. 3A). *Onmy-GS04* mRNA levels were also increased by >twofold in the mid- and hindbrain of the experimental (9 h ammonia exposure) compared to control fish (Fig. 3A).

Discussion

The results of this study support our first hypothesis that all four GSase isoforms are expressed in trout brain. Two distinct evolutionary lineages of GSase have been identified in rainbow trout, *Onmy-GS01/03* and *Onmy-GS02/04*, which likely represent at least two rounds of gene duplications (Murray et al., 2003). The GSase gene pairs in each lineage are thought to be the result of the ancient salmonid tetraploidization event that occurred between 25 and 100 mya (Allendorf and Thorgaard, 1984). We have recently reported that of the four GSase isoforms, only *Onmy-GS01* and *-GS03* are initially induced during early developmental stages of rainbow trout (Essex-Fraser et al., 2005). After the yolk is absorbed (80 days post-fertilization), the full expression of all four GSase isoforms is evident in whole trout homogenates. We show here that all four GSase isoforms are co-expressed in three trout brain regions, with much lower levels detected in the liver using real time PCR. These results agree with our preliminary findings using northern analysis (Murray et al., 2003). It was surprising that *Onmy-GS03* mRNA levels in the brain were higher than other GSase isoforms in both control and ammonia-exposed fish in the 9 h experiment, but not the 48 h experiment. Further work is necessary to determine if the four GSase isoforms have specific functions at the enzyme level.

We hypothesized that GSase is induced in the brain of rainbow trout during ammonia stress. With exposure to sub-lethal NH_4Cl in the external water, ammonia concentrations and GSase activities and mRNA levels were significantly enhanced

in each brain region. The time course of these changes indicates that tissue and plasma ammonia concentrations reached their peak (~9–10 μmol g^{-1}) in 9 h or less. High levels of body ammonia directly or indirectly stimulated the induction of brain GSase mRNA levels, indicated by the twofold higher *Onmy-GS01–GS04* levels in almost all brain regions after 9 h of ammonia exposure. In addition, there was a significant elevation of brain GSase activity in two of the three brain regions by 9 h. A further step increase in brain GS activity was observed after 48 h of high external ammonia, but by this time mRNA levels had returned to control levels. Hence, the response to elevated external ammonia in trout involves a relatively rapid induction of four GSase isoforms and upregulation of the functional protein in all regions of the brain. It may not be surprising that harsher ammonia exposures in more ammonia-tolerant species resulted in relatively modest increases (11–26%) or no change in brain GSase activities; however, baseline GSase activities in mudskippers, *Periophthalmodon schlosseri* and *Boleophthalmus boddaerti* (Peng et al., 1998), and marine toadfishes, *O. beta* and *O. tau* (Wang and Walsh, 2000), were several fold higher than rainbow trout (Fig. 2).

GSase in mammals is expressed in astrocytes, but in goldfish and amphibians it is thought to be localized in the ependymoglial cells that line the ventricles and spinal canal (Norenberg, 1983). Although the ventricular system is fairly extensive in the teleost brain (Meek and Nieuwenhuys, 1998), the higher GSase expression in the midbrain and hindbrain is possibly due to a higher population of ependymoglial cells or to the expression of GSase in other neural cells. In future studies, *in situ* hybridization would be invaluable in localizing GSase isoforms to specific cells in the trout brain.

External ammonia exposure resulted in an elevation of hepatic ammonia content and GSase activities but not mRNA levels. Liver ammonia content was twofold higher after 9 h of external ammonia treatment and remained high for the 48 h exposure. Although the absolute level of GSase activity in liver tissue is very low relative to brain, activities were significantly elevated at both 9 and 48 h of ammonia treatment. These small changes in liver GSase activity were not correlated with changes in the level of mRNA at 9 or 48 h and therefore may be due to post transcriptional regulation (Labow et al., 1999) or possibly changes in transcription occurred very quickly and had returned to control levels by 9 h. Anderson et al. (Anderson et al., 2002) found a 5- to 20-fold elevation of hepatic GSase mRNA, protein and activity in the sleeper (*Bostrichthys sinensis*) exposed to 15 mmol l^{-1} NH_4Cl for 48 h. Higher concentrations of external ammonia (25 mmol l^{-1} NH_4Cl) induced hepatic GSase by ~threefold in the air-breathing catfish (*Clarias batrachus*) (Saha et al., 2002), but liver GSase of other ammonia-tolerant species does not respond to relatively high levels of external NH_4Cl (Peng et al., 1998; Wang and Walsh, 2000; Ip et al., 2004). The increase in liver GSase activity in response to ammonia exposure in the present study is in agreement with findings in trout under similar conditions (Wicks and Randall, 2002).

Plasma and tissue ammonia content reached a steady state by 9 h of ammonia exposure because there was no further change after 48 h of exposure. The total ammonia content in a fish exposed continuously to elevated external ammonia will depend on four parameters: (1) the rate of influx across the gills, (2) the rate of ammonia production by the liver, (3) the rate of ammonia efflux (against the gradient) and (4) the rate of conversion of ammonia to another compound (e.g. glutamine). Brain ammonia content remained elevated at 48 h despite a significantly higher GSase activity at this time period. As more and more ammonia enters the brain, glutamine accumulates over glutamate levels (Vedel et al., 1998; Veauvy et al., 2005), presumably because of the relatively high activity of brain GSase compared to GDH activity (see Introduction). The extra- to intracellular ammonia gradient in the brain would be maintained by the incorporation of ammonia into glutamine and ammonia would continue to move into brain cells. The key role of GSase, therefore may not be to lower brain ammonia levels but rather to prevent a rise in glutamate, a major excitatory neurotransmitter. Indeed, in studies on *O. beta* exposed to sublethal water ammonia, brain GSase activity was reduced by 80% by pretreatment with the inhibitor MSO, brain glutamine:glutamate levels were significantly reduced relative to the ammonia treatment minus MSO, and fish died 40 h into the experiment. These experiments underline the importance of brain GSase activity in the overall response to elevated external ammonia in *O. beta* and comparative studies in rainbow trout are warranted.

Our results provide an interesting contrast to studies on the mammalian brain response to an ammonia load. It should be noted that arterial ammonia levels in mammals are typically well below 100 μmol l^{-1} (Felipo and Butterworth, 2002) and hyperventilation and convulsions were observed in rats with experimentally manipulated brain ammonia concentrations of 3–4 μmol g^{-1} (Kensenko et al., 1994; Kensenko et al., 1995), levels comparable to control brain ammonia concentrations in rainbow trout (Fig. 1). Thus, mammalian brain tissue is far more sensitive to elevated ammonia. In mammals, cerebral GSase activity is an order of magnitude lower relative to trout (Kosenko et al., 1995) and is inhibited in acute ammonia exposure (Felipo and Butterworth, 2002; Kosenko et al., 2003). High plasma ammonia activates NMDA glutamate receptors in the brain and the subsequent release of nitric oxide is thought to induce a reversible covalent modification of GSase, which in turn reduces enzyme activity (Miñana et al., 1997; Monfort et al., 2002; Kosenko et al., 2003). Blocking NMDA receptors with antagonists such as MK-801 has been shown to prevent death, as well as to increase cerebral GSase activity in hyperammonemic rats (e.g. Hermenegildo et al., 1996; Monfort et al., 2002). MK-801 had a protective effect in plainfin midshipman *Porichtys notatus* (Walsh et al., 2007) and loach *Misgurnus anguillicaudatus in vivo* (Randall and Tsui, 2002) given an ammonia load. It is not clear how NMDA receptors are involved in the brain's response to elevated ammonia in fish; however, both *in vitro* and *in vivo* experiments are required to understand the mechanisms more thoroughly.

In conclusion, all four genes coding for trout GSase are expressed in fore-, mid- and hindbrain of rainbow trout. Exposure to sublethal ammonia results in a relatively rapid, but transient increase in the transcription of the four GSase genes in the brain and a corresponding increase in brain GSase activity. The fact that brain GSase is induced in response to hyperammonemia in the ammonia intolerant rainbow trout indicates that GSase plays a key role in the cerebral response to ammonia stress.

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