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Appetite-suppressing effects of ammonia exposure in rainbow trout associated with regional and temporal activation of brain monoaminergic and CRF systems

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Summary

To assess whether the brain's monoaminergic and/or corticotropin-releasing factor (CRF) systems may be involved in mediating the appetite-suppressing effects of high environmental ammonia levels, we exposed rainbow trout to one of four NH_4Cl treatments (0, 500, 750 1000 μmol l^{-1}) for 24 or 96 h and monitored changes in food intake, brain serotonin (5-HT) and dopamine (DA) activity, CRF and urotensin I (UI) mRNA levels, and plasma cortisol levels. Food intake decreased in a dose-dependent manner after 24 h of ammonia exposure and partially recovered in all groups after 96 h. Ammonia also elicited dose-dependent increases in serotonergic activity in the hypothalamus (HYP), telencephalon (TEL) and posterior brain (PB). Whereas the increase in serotonergic activity was timed with the 24 h food intake inhibition, TEL and PB serotonergic activity increased after 96 h. In the PB, exogenous ammonia also elicited dose-dependent increases in dopaminergic activity after both 24 and 96 h of exposure. Transient increases in TEL CRF and UI mRNA levels, HYP UI mRNA levels, and plasma cortisol concentrations were evidence that the hypothalamic–pituitary–interrenal (HPI) stress axis was primarily stimulated in the first 24 h of ammonia exposure when food intake was depressed. Overall, the transient nature of the appetite suppression during chronic ammonia exposure, and the time-dependent changes in brain monoaminergic and CRF systems, implicate 5-HT, DA, CRF and UI as potential mediators of the appetite-suppressing effects of ammonia. Among these anorexigenic signals, our results specifically identify hypothalamic 5-HT as a potentially key neurobiological substrate for the regulation of food intake during exposure to high external ammonia concentrations.

Key words: feeding behavior, anorexia, HPI axis, stress, corticotropin-releasing factor, urotensin I, cortisol, rainbow trout, *Oncorhynchus mykiss*.

Introduction

Most fish, including rainbow trout *Oncorhynchus mykiss*, excrete the majority of their metabolic nitrogenous waste as ammonia through the gills (Wright, 1995; Kajimura et al., 2004). In these animals, nitrogen excretion is an essential process that counteracts the relative toxicity of ammonia accumulation and under low exogenous ammonia conditions it is readily achieved through passive diffusion (Wilkie, 2002). However, under natural and culture conditions, fish routinely encounter elevated levels of ammonia (Tomasso, 1994; Ip et al., 2001). While fish have evolved several strategies to cope with ammonia toxicity (Randall and Tsui, 2002), exposure to high ammonia levels causes a variety of negative physiological effects including a reduction in growth and food intake (e.g. Alderson, 1979; Lang et al., 1987; Beamish and Tandler, 1990; Atwood et al., 2000; Wicks and Randall, 2002a). Although the appetite-suppressing effects of high ammonia exposure are well documented, the mechanisms involved in mediating the effects of this environmental stressor are still poorly understood.

Among the many factors identified as potential regulators of food intake in fish (Lin et al., 2000), several indirect lines of evidence suggest that the monoamine-containing neuronal systems of the brain may be involved in mediating the appetite-suppressing effects of ammonia. In channel catfish *Ictalurus punctatus*, the growth-suppressing effects of chronic environmental ammonia are associated with exposure-dependent decreases in whole brain dopamine (DA) and serotonin (5-HT) concentrations and in an increase in 5-HT turnover (Atwood et al., 2000). Moreover, intracerebroventicular (icv) injections of 5-HT and DA inhibit feeding behaviour in goldfish *Carassius auratus* (De Pedro et al., 1998a,b) and intraperitoneal administration of the 5-HT-releasing agent, fenfluramine, induces a short-term inhibition of food intake in rainbow trout (Ruibal et al., 2002). Finally, in several species, including rainbow trout, there is an inverse relationship within dominance-based hierarchies between brain serotonergic activity and food intake where low-feeding fish have higher serotonergic activity (Winberg et al., 1993;

Alanara et al., 1998). Given this evidence, we investigated in this study the effects of high external ammonia exposure on food intake and on the regional brain turnover of 5-HT and DA in rainbow trout.

As indicated by the release of cortisol from the interrenal cells of the head kidney, most stressors result in an activation of the hypothalamic–pituitary–interrenal axis (HPI) in teleosts (reviewed by Wendelaar Bonga, 1997; Lovejoy and Balment, 1999; Bernier and Peter, 2001a). Similarly, the increase in plasma cortisol associated with ammonia exposure in a variety of different fish species suggests an activation of the HPI axis in response to this environmental stressor (Tomasso et al., 1981; Spotte and Anderson, 1989; Person-Le-Ruyet et al., 1998; Wicks and Randall, 2002b). Although the hypophysiotropic factors involved in stimulating the HPI axis in ammonia-exposed fish are not known, corticotropin-releasing-factor (CRF) and the related neuropeptide urotensin I (UI) are likely candidates (Lederis et al., 1994). In addition to their role as regulators of the HPI axis, CRF-related peptides have been identified as potent anorexigenic factors in fish (De Pedro et al., 1993; Bernier and Peter, 2001b). Therefore, we also examined the effects of high external ammonia exposure on the forebrain levels of CRF and UI mRNA in rainbow trout.

Thus, to get a better understanding of the central mechanisms controlling food consumption during ammonia toxicity in fish, one of the objectives of this study was to determine whether the appetite-suppressing effects of ammonia exposure are associated with changes in brain monoaminergic activity and/or CRF and UI mRNA levels. However, since the appetite-suppressing effects of chronic ammonia exposure can be transient (Beamish and Tandler, 1990; Wicks and Randall, 2002a) and there are known regional differences in the changes of both monoaminergic activity and CRF-related peptide gene expression in response to stressors (Winberg and Nilsson, 1993; Summers et al., 1998; Bernier and Peter, 2001b), a second objective of this study was to determine the temporal and regional changes in brain monoaminergic activity and/or CRF and UI mRNA levels associated with ammonia toxicity.

Materials and methods

Experimental animals

Rainbow trout *Oncorhynchus mykiss* Walbaum were transported from the Alma Aquaculture Research Station (Alma, ON, Canada) and weighed (122.6±3.4 g, N=84). The fish were maintained in indoor tanks (1000 l) at the University of Guelph (Guelph, ON, Canada) that were supplied with dechlorinated city well water. Eight weeks prior to the start of experimentation, trout were tagged with a passive integrated transponder (PIT; Biomark, Boise, ID, USA) and randomly assigned to 125 l tanks in groups of 10–12 fish. Each tank was provided with an air stone and the fish were exposed to a 12 h:12 h L:D photoperiod regime. Water temperature was maintained at 14.5°C throughout the experimental period and the water inflow for each tank kept at 6 l min^{-1}. In order to promote a conditioned feeding regiment, fish were hand-fed to satiety a commercially prepared trout chow (3PT Classic Sinking fish pellets, Martin Mills, Elmira, ON, Canada) at 10:00 h daily. Care was taken to ensure the food was distributed evenly throughout the tank to disrupt any dominance hierarchies.

Experimental design

Two separate, but similar experiments were performed. Experiment 1 was a flow-through 24 h ammonia exposure at three levels. Experiment 2 was a flow-through 96 h ammonia exposure at the same exposure levels. Experimental protocol and sampling procedure were identical for both and will be described together. In each experiment, groups of 10–12 fish were randomly assigned to one of eight tanks as described above. The eight tanks were paired to account for tank effects and to allow for four treatment conditions: control, 500, 750 and 1000 μmol l^{-1} ammonia. Throughout the experiments, fish were fed daily to satiety at 10:00 h and on the first day of the exposures the ammonia treatments began 2 h post-feeding. Using prepared stock solutions of ammonium chloride (NH_4Cl), ammonia levels in the tanks were increased instantaneously to their respective conditions and maintained using a peristaltic pump (Perista Pump SJ-1220, Rose Scientific, Mississauga, ON, Canada). Water samples were collected prior to feeding, immediately after the start of the experiment, and every 5 h thereafter to monitor pH and water ammonia levels. At the end of an experiment, fish were quickly netted from a tank, terminally anesthetized with an overdose of 2-phenoxyethanol (2.0 ml l^{-1}; Sigma, St Louis, MI, USA), weighed, and a blood sample collected by caudal puncture using a K_2-EDTA (0.5 mol l^{-1}, pH 8.0)-treated syringe. The brain was then dissected into three distinct regions, the telencephalon (TEL), hypothalamus (HYP), and posterior brain (PB; which includes the midbrain, optic tectum, cerebellum and brainstem), and immediately frozen in liquid nitrogen for subsequent measurement of both *CRF* and *UI* gene expression or serotonergic and dopaminergic activity. The sampling of each fish was completed in less than 2 min and an entire tank in less than 10 min. Blood samples were immediately centrifuged at 15 000 ***g*** for 5 min and the separated plasma frozen at –80°C for subsequent measurement of plasma ammonia and plasma cortisol concentrations.

Assessment of food intake

Using X-radiography (Jobling et al., 2001), food intake was assessed twice from each individual fish over the course of this study: 7 days prior to the start of the ammonia exposures (control food intake) and immediately after the ammonia exposures. In brief, the commercial trout food was grounded and re-pelleted with 450 μm steel beads (Draiswerke, Mahwah, NJ, USA) at a ratio of 5% by mass of dry powdered food. On days where control food intake was assessed, fish were fed to satiety at 10:00 h, anaesthetized 2 h later with tricaine methanesulfonate (0.1 g l^{-1}; MS-222; Syndel, Vancouver, BC, Canada), weighed, X-rayed using an Acu Ray

HFJ portable X-ray machine (1.05 mAs at 50 kV; Stern Medical Equipment, Brampton, ON, Canada), and returned to their original tanks for recovery. Post ammonia exposure, food intake was assessed as above immediately after terminal sampling of the fish. The beads located in the digestive tract of the fish were counted from the X-ray films and the amount eaten in grams was determined from a standard curve. Preliminary experiments showed that re-pelleting and diet labelling do not affect palatability and control food intake is not affected by the above procedures as long as the fish are given a 72 h recovery period between X-raying. Finally, during a 2-week acclimation period prior to the experiment, and between the control and post-exposure X-rays, the fish were fed the same re-pelleted diet without the label.

Ammonia and cortisol determinations

Water ammonia concentration was measured using the method described by Verdouw et al. (1978). Plasma samples were assayed for ammonia using the glutamate dehydrogenase enzymatic assay of Kun and Kearney (1971). Briefly, plasma samples were de-proteinized with 2 volumes of trichloroacetic acid (10% w/v) and centrifuged at 15 000 ***g*** for 10 min at 4°C. The supernatant was neutralized with potassium hydrogen carbonate ($KHCO_3$; 2 mol l^{-1}) and centrifuged at 7500 ***g*** for 30 s at 4°C. The enzymatic assay was adapted for 96-well microtitre plates. All samples were run in triplicate at 24°C with shaking in a SpectraMax 190 plate reader (Molecular Devices, Menlo Park, Ca, USA) and changes in absorbance were recorded until all reactions had gone to completion. Ammonia standards were prepared fresh in distilled water and the plasma ammonia concentrations determined from a linear regression of the standards using SOFTmax software 4.6 (Molecular Devices). Throughout, all ammonia concentrations reported refer to total ammonia-N.

Plasma cortisol concentration was measured using the enzyme immunoassay of Carey and McCormick (1998) with the following modifications. The primary antibody (rabbit anti-cortisol, cat. #F3-314, Esoterix Endocrinology, Calabasas Hills, CA, USA) and the cortisol–horseradish peroxidase conjugate (Clinical Endocrinology Laboratory, University of California Davis, Davis, CA, USA) were used in microtitre plates at a final dilution of 1:20 000 and 1:70 000, respectively. The plates were incubated at 26°C with shaking and read in a SpectraMAX 190 plate reader. All samples were run in triplicate and the cortisol concentrations determined from a 3-parameter sigmoidal standard curve (Sigma Plot 7.0; SPSS, Chicago, IL, USA). A serial dilution of rainbow trout plasma gave a displacement curve that was parallel to the standard curve and the lower detection of this assay was 0.6 ng ml^{-1}. Using a pooled plasma sample, the intra- and inter-assay variations were 5.7% (N=10) and 12.6% (N=5), respectively.

Monoamine determinations

For the three brain regions dissected, (TEL, HYP, and PB) dihydroxyphenylacetic acid (DOPAC), DA, 5-hydroxyindoleacetic acid (5HIAA) and 5HT were measured using high performance liquid chromatography with electrochemical detection as described by Renner and Luine (1984), with several modifications. Briefly, frozen tissue samples were weighed and placed in a 1.5 ml centrifuge tube. Sodium acetate buffer (pH 5) containing 1×10^{-7} dihydroxybenzylamine (internal standard) was added to each sample (10:1 v/w), the samples were sonicated on ice, and freeze–thawed. Samples were either processed directly for analysis or stored at –80°C until analysis. Prior to analysis, 10 µl of a 1 mg per 10 ml H_2O ascorbate oxidase solution (Sigma Co., St Louis, MO, USA) were added to each sample and the samples were centrifuged (17 000 ***g*** for 4 min). The supernatant was removed and 30 µl was injected into a Waters chromatography system (Waters Associates, Milford, MA, USA) and analyzed electrochemically using a LC-4B potentiostat and a glassy carbon electrode (Bioanalytical Systems, Inc., West Lafayette, IN, USA). The electrode potential was set at +0.65 V with respect to an Ag $AgCl^{-1}$ reference electrode. Separation was accomplished using a 4 µm C-18 radial compression cartridge (Waters Associates). The mobile phase consisted of 11 g citric acid, 8.6 g sodium acetate, 110 mg octylsulfonic acid (Sigma Co.), 250 mg EDTA and 100 ml methanol in 1 l of water.

The concentrations of the amines and amine metabolites were calculated with respect to the mean peak height values obtained from standard runs set in the internal standard mode using CSW32 software (Data Apex LTD, Prague, Czech Republic). The resulting values were corrected for volume and expressed as pg amine mg^{-1} tissue wet mass.

Total RNA extraction and first strand cDNA synthesis

Total RNA was extracted from the TEL and HYP brain regions using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) based on the acid guanidinium thiocyanate–phenol–chloroform extraction method. Total RNA concentrations were determined by ultraviolet spectrophotometry at 260 nm and diluted with RNAse-free water to a working dilution of 0.5 µg $µl^{-1}$. To eliminate potential genomic contamination, 1 µg of total RNA was treated with DNase I according to the manufacturer's protocol (DNase I amplification grade, Invitrogen). The DNase-treated total RNA samples were reverse transcribed to cDNA using SuperScript RNase H^- reverse transcriptase (Invitrogen).

Real-time reverse transcriptase-polymerase chain reaction

To quantify the mRNA expression of CRF and UI, the above cDNA products were amplified using the ABI Prism 7000 sequence detection system (Applied Biosystems; Foster City, USA). Each reaction contained 10 µl of SYBR green PCR master mix (part# 4309155; Applied Biosystems), 5 µl cDNA template, and 2.5 µl each of forward and reverse primers (0.4 µmol l^{-1}). Default cycling conditions were used: 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. This protocol was followed by a melting curve analysis to verify the specificity of the PCR products. To account for differences in amplification efficiency between the different

cDNAs, standard curves were constructed for each target gene using serial dilutions of cDNA samples known to have high expression levels of the target gene (Giulietti et al., 2001). Using the threshold cycle of each unknown, the relative dilution of a given sample was extrapolated using the linear regression of the target-specific standard curve. Finally, to correct for cDNA loading and RNA reverse transcriptase efficiency, each sample was normalized to the expression level of the housekeeping gene acidic ribosomal phosphoprotein A0 (ARP). All samples were assayed in triplicate and only one target was assayed per well.

Specific primers for either rainbow trout CRF (GenBank accession no. AY651777), UI (GenBank accession no. AY651778) and ARP (GenBank accession no. AY685220) were designed using the software program Primer Express (Applied Biosystems). To prevent co-amplification of genomic DNA, the sequence of each forward primer was chosen to flank an intron based on the position of known intron–exon junctions. To maximize amplification efficiency, the CRF (forward: 5′-ACA ACG ACT CAA CTG AAG ATC TCG-3′; reverse: 5′-AGG AAA TTG AGC TTC ATG TCA GG-3′), UI (forward: 5′-AGG AGA CAA AAT ACC GGG CA-3′; reverse: 5′-CTT CAT AGT GCT GGA CAG ACG G-3′) and ARP (forward; 5′-TGA AAA TCA TCC AAT TGC TGG A-3′; reverse 5′-CGC CGA CAA TGA AAC ATT TG-3′) primers were designed to amplify the shortest product possible (CRF, 54 bp; UI, 51 bp; ARP, 51 bp).

Statistical analysis

All data are presented as mean values plus one standard error of the mean (S.E.M.). Differences among treatments were assessed by one-way analysis of variance (ANOVA) followed by a pairwise Tukey multiple comparison test. Within a given treatment, the effects of treatment duration were assessed by unpaired Student's *t* test. Differences between the pre- and post-ammonia exposure food intake values were assessed by pairwise Student's *t* test. The software SigmaStat 3.0 was used for statistical analyses (SPSS, Chicago, IL, USA), while SigmaPlot 7.0 (SPSS) was used for correlation analyses. The significance level for all statistical tests was $P<0.05$.

Results

The measured water ammonia concentrations for both the 24 and 96 h exposures remained constant throughout the experimental period. In addition, the desired water ammonia levels of 500, 750 and 1000 μmol l^{-1} were achieved at both time periods with no significant difference between the 24 and 96 h exposures for all ammonia concentrations (Fig. 1A). The water pH remained constant at 7.9 in all tanks throughout both exposures. One mortality was noted in the highest NH_4Cl treatment (1000 μmol l^{-1}) for the 96 h exposure period. Also, early during the 96 h exposure, one fish displayed a partial loss of equilibrium but returned to an upright swimming position over the course of the experiment and was included in the data set. Relative to the control fish, after 24 h of exposure plasma ammonia increased significantly and in a dose-dependent fashion. Relative to the 24 h values, there was a significant recovery in plasma ammonia in the 750 and 1000 μmol l^{-1} treatments after 96 h exposure (Fig. 1B).

Prior to ammonia exposure, there was no difference in basal food intake between any of the 24 h (Fig. 2A) or 96 h (Fig. 2B) treatments. Food intake decreased both significantly and dose dependently after 24 h ammonia exposure, with complete appetite suppression in the highest dose (1000 μmol l^{-1}) (Fig. 2A). All doses did show some recovery after 96 h exposure, with the lowest dose (500 μmol l^{-1}) approaching control levels and the highest dose recovering by approximately 40% (Fig. 2B).

The NH_4Cl treatments were associated with changes in brain monoaminergic activity that were both brain region specific and dependent on exposure duration. In the HYP, serotonergic activity increased dose-dependently and peaked in the first 24 h (Fig. 3A). Although the 96 h activity level remained elevated relative to control, it was less pronounced than at 24 h, increasing significantly only in the highest dose (1000 μmol l^{-1}). By contrast, while there was no change in serotonergic activity in the TEL and PB after 24 h of ammonia exposure, there were significant increases in the 750 and

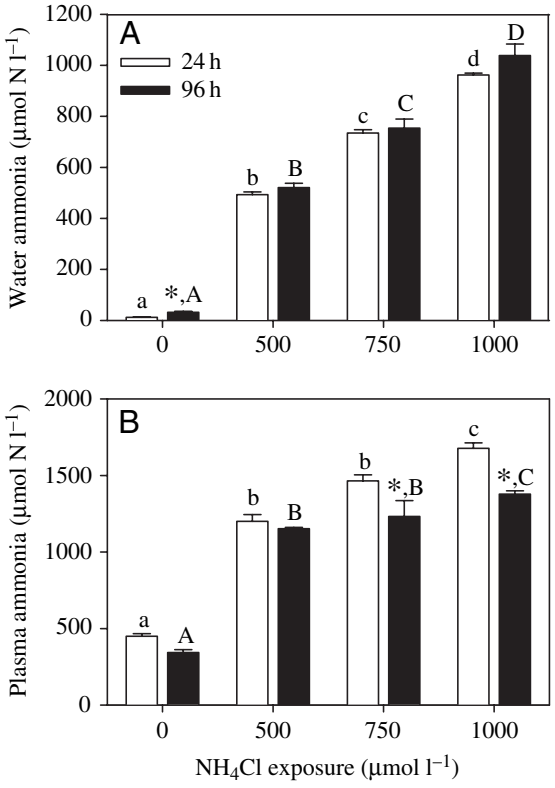

Fig. 1. Measured levels of total water (A) and plasma (B) [ammonia] in the 0 (N=10), 500 (N=10), 750 (N=10) and 1000 (N=10–12) μmol l^{-1} NH_4Cl treatments after 24 or 96 h exposure periods. Values are means + 1 S.E.M. Treatments that do not share a common letter for a given time are significantly different from each other. *Significant differences between exposure periods for a given treatment ($P<0.05$).

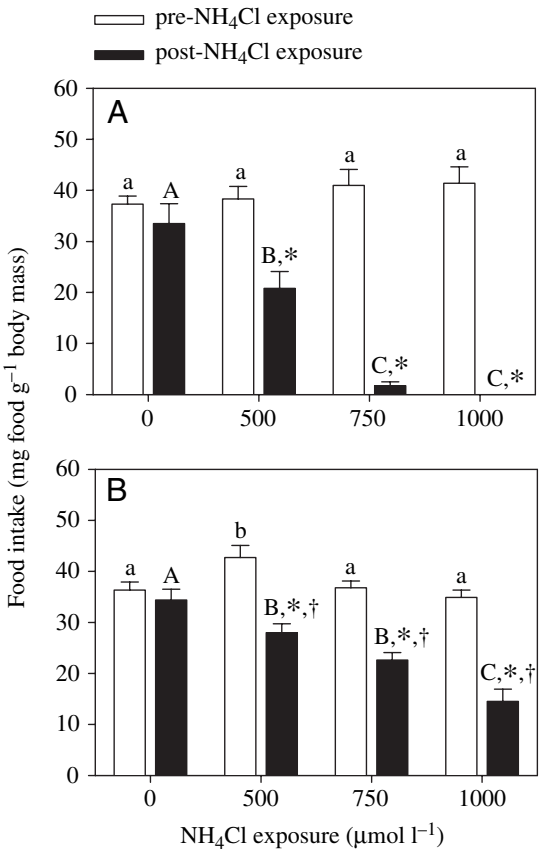

Fig. 2. Mean food intake (mg food g^{-1} body mass) of rainbow trout before and after exposure to 0 (N=10), 500 (N=10), 750 (N=10) or 1000 (N=10–12) μmol l^{-1} NH_4Cl for either 24 (A) or 96 (B) h periods. Values are means + 1 S.E.M. Treatments that do not share a common letter for a given feeding time and exposure period are significantly different from each other. *Significant differences between the pre- and post-NH_4Cl exposure values for a given treatment; †significant differences between the 24 and 96 h post-NH_4Cl exposure values for a given treatment (P<0.05).

1000 μmol l^{-1} doses after 96 h (Fig. 3B,C). Furthermore, in the TEL, there was a significant difference between sampling times in both the 500 and 750 μmol l^{-1} doses with the PB only displaying significant differences between exposure times in the 750 μmol l^{-1} dose. When plasma ammonia is regressed against 5-HT turnover after 24 h, although there is a significant linear relationship between these two variables in all three brain regions, the relationship is strongest in the HYP (r^2=0.39, P<0.0001, Fig. 3D) and more variable in the TEL (r^2=0.10, P=0.045) and PB (r^2=0.11, P=0.040). These relationships, however, disappear after 96 h of ammonia exposure in all three brain regions. There are also significant relationships between 5-HT turnover and water ammonia. Again the strongest relationship is in the HYP (r^2=0.40, P<0.0001), and more variable in the TEL (r^2=0.16, P=0.0114) and PB (r^2=0.12, P=0.0333). After 24 h of ammonia exposure significant linear relationships exist in the regression of plasma ammonia against 5-HT concentration in all brain regions; however, the relationship is strongest in the PB (r^2=0.31, P<0.0001), intermediate in the TEL (r^2=0.23, P<0.0001), and weakest in the HYP (r^2=0.11, P<0.0001). Overall, while both decreases in 5-HT and increases in 5-HIAA contributed to the significant increases in serotonergic activity in the HYP after 24 h of ammonia exposure, decreases in 5-HT concentrations appear to be the primary determinant of the changes in 5-HIAA:5-HT ratios in the HYP after 96 h of exposure and in the two other brain regions (Table 1).

Due to low DOPAC concentrations in the TEL and HYP, detection by high pressure liquid chromatography (HPLC) was not possible. DOPAC was, however, detectable in the PB, allowing dopaminergic activity to be measured in this region. Dopaminergic activity increased in the two highest ammonia dose samples after 24 h of exposure (Fig. 4A). Dopaminergic activity values remained elevated after 96 h and were not significantly different than after 24 h of ammonia exposure. A significant linear relationship exists in the regression of plasma ammonia against both dopaminergic activity (r^2=0.45, P<0.0001, Fig. 4B) and DA concentrations (r^2=0.31, P<0.0001) in the PB at 24 h. However, as previously observed with 5-HT turnover in the brain, the relationship between dopaminergic activity and plasma ammonia breaks down after 96 h of ammonia exposure. Overall, a decrease in DA concentration appears to be the primary determinant of the changes in DOPAC:DA ratios in the PB (Table 1).

In the HYP, ammonia exposure was associated with an increase in CRF mRNA levels after 96 h (Fig. 5A). This increase peaked at the highest ammonia dose (1000 μmol l^{-1}) and was significantly higher than the 24 h value. By contrast, only the lowest ammonia dose (500 μmol l^{-1}) was associated with an increase in TEL CRF mRNA levels after 24 h exposure (Fig. 5B). UI expression followed similar patterns in both the HYP and TEL, with the highest mRNA levels observed after 24 h in fish exposed to the lowest ammonia dose (500 μmol l^{-1}) and no apparent effect in the fish exposed to the highest ammonia dose (1000 μmol l^{-1}; Fig. 5C,D). After 96 h ammonia exposure, HYP and TEL UI mRNA levels in the ammonia-exposed fish did not differ from controls.

Changes in plasma cortisol levels were similar to the changes exhibited in plasma ammonia, with dose-dependent increases after 24 h exposure and a recovery towards control values after 96 h (Fig. 6A). After 24 h exposure, plasma cortisol values were respectively 3-, 9- and 14-fold higher in the low, medium, and high ammonia treatments than in the control fish. In contrast, plasma cortisol levels had returned to control values after 96 h in the 750 μmol l^{-1} treatment and decreased by more that 50% in the 1000 μmol l^{-1} treatment. There is a significant linear relationship between plasma ammonia and plasma cortisol after 24 h of ammonia exposure (r^2=0.41, P<0.001; Fig. 6B). Similarly, when 5-HT turnover is regressed against the 24 h plasma cortisol data, there are significant linear relationships between these variables in both the HYP and PB, however the relationship is stronger in the HYP (r^2=0.25, P=0.002, Fig. 6C) than in the PB (r^2=0.15, P=0.015).

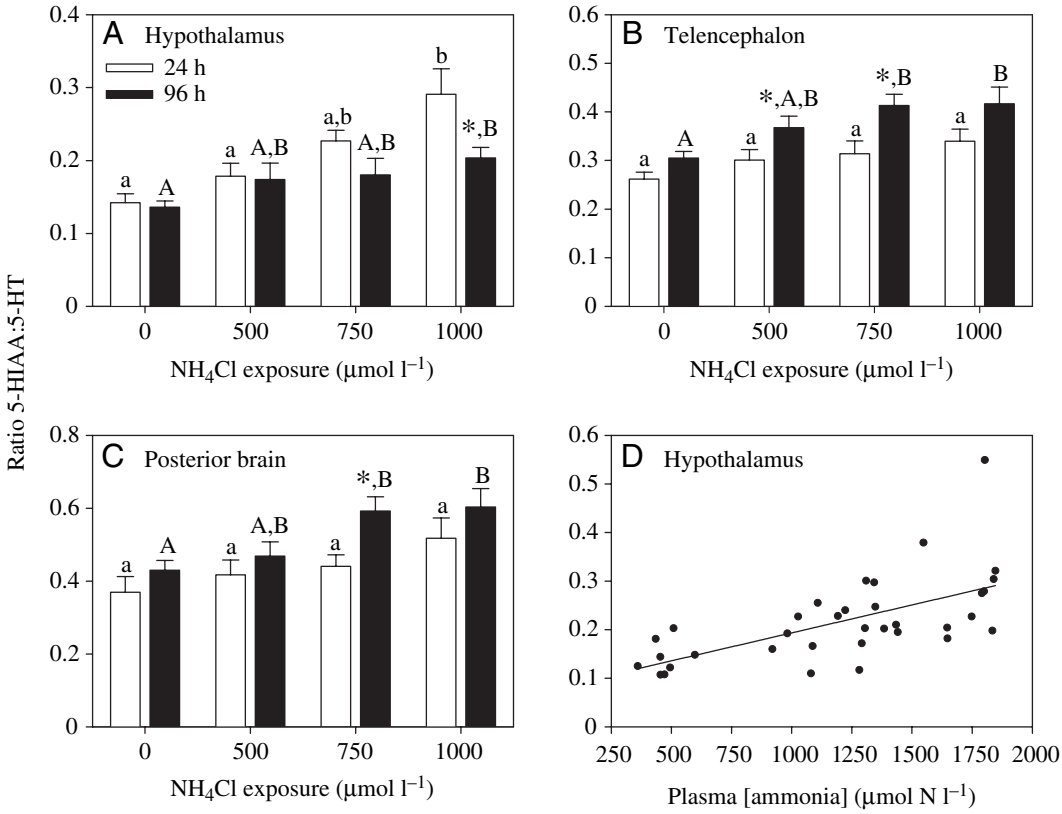

Fig. 3. Serotonergic activity in the hypothalamus (A), telencephalon (B), and posterior brain (C) regions of rainbow trout exposed to 0, 500, 750 or 1000 μmol l^{-1} NH_4Cl for either 24 or 96 h periods. Serotonergic activity is expressed as a ratio of the metabolite 5-hydroxyindole acetic acid (5-HIAA) to serotonin (5-HT). Values are means + 1 S.E.M. Treatments that do not share a common letter for a given time and brain region are significantly different from each other. *Significant differences between exposure periods for a given treatment (P<0.05; N=8–11). (D) Relationship between plasma [ammonia] and hypothalamic serotonergic activity after 24 h NH_4Cl exposure (r^2=0.39, P<0.001).

Discussion

Results from this study provide original evidence suggesting that the monoamines 5-HT and DA as well as the neuropeptides CRF and UI maybe involved in mediating the anorexia associated with ammonia toxicity in rainbow trout. While chronic ammonia exposure elicits changes in monoamine metabolism and increases in *CRF* and *UI* gene expression in various regions of the brain, we show that these responses are time-dependent and region-specific. Overall, when the regional changes in monoamine turnover and CRF-related peptide gene expression are compared over time to those in food intake and plasma cortisol, serotonergic activity in the HYP correlates best to the changes in food consumption and HPI axis activity that characterize the response to ammonia toxicity.

The appetite-suppressing effects of high ammonia exposure observed in this study are consistent with previous observations made in rainbow trout (Wicks and Randall, 2002a) and lake trout *Salvelinus namaycush* (Beamish and Tandler, 1990). While low levels of exogenous ammonia (≤225 μmol l^{-1}) have no effect on food intake (Wood, 2004), above a certain species-specific threshold, chronic increases in water ammonia concentrations elicit an initial dose-dependent reduction in food intake followed by a gradual recovery. The acute hyperammonemia-associated reduction in food intake will protect fish from the significant postprandial increase in plasma ammonia levels (Mommsen and Walsh, 1992; Wicks and Randall, 2002a). Therefore, the appetite-suppressing effects of ammonia may represent an immediate behavioural strategy aimed at minimizing ammonia toxicity. On the other hand, the gradual recovery in appetite associated with ongoing hyperammonemia probably results from biochemical adjustments aimed at converting ammonia into less toxic substances (Randall and Tsui, 2002).

The partial recovery in plasma ammonia levels after 96 h of chronic water ammonia exposure observed in this study and the similar results reported by Wicks and Randall (2002b), suggest that rainbow trout can detoxify ammonia. In fact, since all the fish in this study were sampled 2 h after being fed and all the ammonia-exposed fish ate significantly more after 96 h of ammonia exposure than after 24 h, the reductions in plasma ammonia levels after 96 h exposure are likely to be an underestimation. Similarly, the post-prandial increase in plasma ammonia levels likely contributed to the relatively high plasma ammonia concentrations of the control fish. In rainbow trout (Arillo et al., 1981; Vedel et al., 1998; Wicks and Randall, 2002b), as in several other species (Walsh and Milligan, 1995; Peng et al., 1998; Jow et al., 1999), available data suggest that an important mechanism of ammonia detoxification involves the conversion of excess ammonia to glutamate *via* glutamate dehydrogenase and then to neutral and storable glutamine *via* glutamine synthetase (GSase). In the brain of trout, there is a significant decrease in glutamate and increase in glutamine concentrations after 24 h of chronic ammonia exposure and these changes are maintained through 96 h (Vedel et al., 1988). The changes in brain glutamate and glutamine concentrations are associated with increases in brain and liver GSase and an increase in muscle glutamine levels, suggesting that the

Table 1. *Brain regional monoamines (5-HT, DA) and monoamine metabolites (5-HIAA, DOPAC) concentrations of rainbow trout exposed to a range of NH_4Cl concentrations for either 24 or 96 h*

Brain region and exposure duration	NH_4Cl treatments ($\mu mol\ l^{-1}$)	Monoamines and monoamine metabolites (pg mg^{-1} tissue)			
		5-HT	5-HIAA	DA	DOPAC
24 h					
HYP	0	1168.7±109.7[a]	159.1±8.9[a]		
	500	104.7±125.2[a]	186.2±15.8[a]		
	750	995.2±48.4[a]	225.1±17.4[a]		
	1000	857.4±105.3[a]	222.2±21.7[a]		
TEL	0	403.9±19.7[a]	104.3±5.3[a]		
	500	347.6±65.4[a]	94.6±10.0[a]		
	750	254.7±25.1[b]	79.5±8.8[a]		
	1000	270.4±22.4[b]	92.9±9.72[a]		
PB	0	129.7±16.4[a]	45.5±4.7[a]	70.1±5.9[a,b]	10.0±0.8[a]
	500	148.5±25.5[a]	60.0±8.8[a]	79.6±11.6[a]	13.7±1.9[a]
	750	104.6±14.7[a]	46.3±7.1[a]	44.7±4.8[b]	13.7±2.1[a]
	1000	97.4±7.9[a]	49.5±5.6[a]	44.3±4.4[b]	13.0±1.3[a]
96 h					
HYP	0	1428.6±92.1[a]	194.6±17.3[a]		
	500	1071.5±104.1[b]	177.4±21.4[a]		
	750	1119.1±101.0[a,b]	192.0±19.4[a]		
	1000	888.9±39.5[b]	177.4±9.8[a]		
TEL	0	324.4±24.7[a]	98.1±7.7[a,b]		
	500	237.0±23.9[a]	83.5±6.2[a]		
	750	284.4±30.3[a]	113.6±7.9[b]		
		263.9±42.5[a]	99.6±8.8[a,b]		
PB	0	137.8±16.7[a]	54.8±4.3[a]	76.7±5.0[a]	12.6±0.7[a]
	500	103.8±11.9[a]	46.8±4.6[a]	58.9±7.2[a,b]	14.1±2.0[a]
	750	94.3±9.3[a,b]	55.3±8.5[a]	50.0±5.0[b,c]	12.3±1.6[a]
	1000	82.3±7.6[b]	47.5±3.8[a]	38.7±3.1[c]	12.5±1.4[a]

Values are means ± 1 S.E.M. (N=8–12). Values for a given brain region and exposure duration that do not share a common letter are significantly different from each other (P<0.05).

HYP, hypothalamus; TEL, telencephalon; PB, posterior brain; 5-HT, serotonin; 5-HIAA, 5-hydroxyindole acetic acid; DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid.

ammonia detoxified by the brain and liver may be stored in the muscle (Wicks and Randall, 2002b).

Rainbow trout chronically exposed to high ammonia concentrations in this study displayed dose-dependent increases in brain 5-HIAA:5-HT ratios in the TEL, HYP, and PB. These results support the findings of Atwood et al. (2000) in channel catfish, where 9 weeks of exposure to environmental ammonia was associated with a dose-dependent increase in whole brain serotonergic activity. Besides environmental ammonia exposure, several other stressors have been shown to affect the 5-HT system in fish. For example, social subordination, chronic shallow water disturbances and predator exposure are all associated with an increase in brain serotonergic activity (Winberg and Nilsson, 1993). In mammals, the increased brain serotonergic activity associated with hyperammonemia appears to be caused by an increased availability in brain tryptophan (TRP). The high brain glutamine concentrations associated with hyperammonemia are known to stimulate blood–brain barrier transport of large neutral amino acids such as TRP, which in return can increase 5HT turnover (for reviews, see Bachmann, 2002; Felipo and Butterworth, 2002). Whether a similar glutamine-stimulated increase in brain TRP uptake or a different mechanism is responsible for the increase in the 5-HIAA:5-HT ratios observed in this study remains to be determined. However, after 96 h of ammonia exposure, rather than an increase in 5-HT turnover, the decreases in HYP and PB 5-HT concentrations without change in 5-HIAA levels suggest that 5-HT production is decreasing.

After 24 h of environmental ammonia, we observed exposure-dependent increases and decreases in hypothalamic serotonergic activity and food intake, respectively. In contrast, the exposure-dependent increases in serotonergic activity in the TEL and PB occurred after 96 h of chronic ammonia, at a time when food intake had partially recovered and hypothalamic serotonergic activity was almost back to control

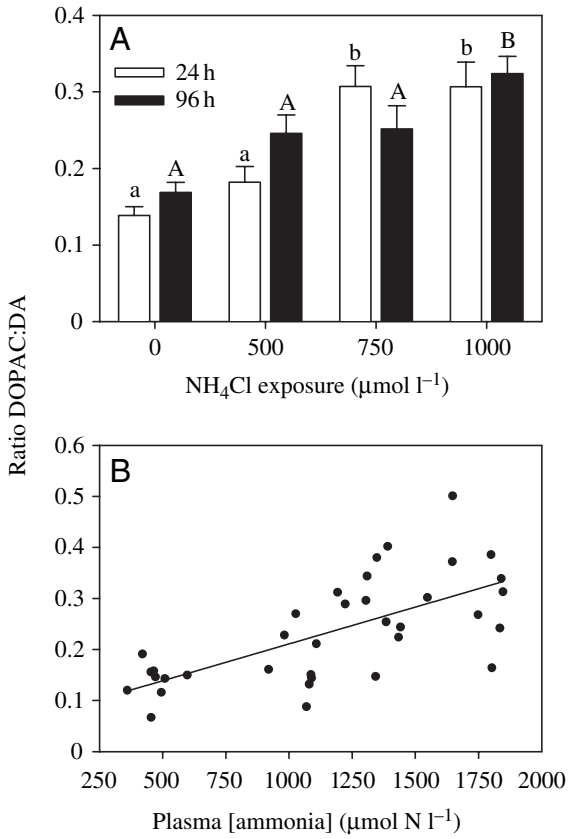

Fig. 4. Posterior brain dopaminergic activity (A) in rainbow trout exposed to 0, 500, 750 or 1000 μmol l^{-1} NH_4Cl for either 24 or 96 h periods. Dopaminergic activity is expressed as a ratio of the metabolite 3, 4-dihydroxyphenylacetic acid (DOPAC) to dopamine (DA). Values are means + 1 S.E.M. Treatments that do not share a common letter for a given time are significantly different from each other (P<0.05; N=8–11). (B) Relationship between plasma [ammonia] and posterior brain dopaminergic activity after 24 h NH_4Cl exposure (r^2=0.45, P<0.001).

levels. Although correlative, together this evidence does suggest that serotonergic activity in the HYP may play an important role in the regulation of food intake during ammonia toxicity in trout. In support of such a role for 5-HT, it is interesting to note that anorexia is also a characteristic feature of hyperammonemia in mammals (Bachmann, 2002) and that reducing dietary TRP supply counteracts the appetite-suppressing effects of hyperammonemia in humans (Hyman et al., 1986). Anatomically, although the largest density of serotonergic cell bodies are found in the raphe nuclei of the PB in fish (Parent, 1983), 5-HT immunoreactive cell bodies are also found in several hypothalamic nuclei in rainbow trout (Frankenhuis-van den Heuvel and Nieuwenhuys, 1984). These 5-HT positive cell bodies contribute fibers to the hypothalamic inferior lobe (Frankenhuis-van den Heuvel and Nieuwenhuys, 1984), a hypothalamic region previously identified as an important integration center for the regulation of appetite in fish (Peter, 1979; Wullimann and Mueller, 2004). In addition, central injection of 5-HT inhibits food intake in goldfish (De Pedro et al., 1998a), and intraperitoneal administration of the 5-HT-releasing agent, fenfluramine, simultaneously increases serotonergic activity in the HYP and temporarily stops food intake in rainbow trout (Ruibal et al., 2002).

Along with the changes in serotonergic activity, chronic ammonia exposure elicited an increase in PB dopaminergic activity. Unlike the regional changes in serotonergic activity that were either rapid (HYP) or delayed (TEL and PB), the dose-dependent increase in dopaminergic activity was significant after 24 h of ammonia exposure and maintained through 96 h. As previously observed by Atwood et al. (2000) in channel catfish, increasing ammonia exposure in this study resulted in significant decreases in whole brain DA and no changes in DOPAC concentrations, changes that are indicative of a decrease in DA production. As with 5-HT, the central DA system in fish appears to be sensitive to a variety of stressors (Overli et al., 2001; De Pedro et al., 2003). In general, results from these experiments and others suggest that the effects of stress on the central DA circuitry in fish is both stressor- and brain region-specific and may involve both changes in DA synthesis and metabolism. Although icv injections of DA agonists in goldfish have anorectic effects (De Pedro et al., 1998b), in mammals there is an extensive body of evidence showing that DA can both inhibit and stimulate food intake (for a review, see Meguid et al., 2000). In this study, after 24 h of ammonia exposure, the dose-dependent increase in dopaminergic activity in the PB concurred with the reduction in food intake. In contrast, the sustained increase in dopaminergic activity after 96 h exposure was associated with a partial recovery in food intake. Therefore, while the 24 h data suggest an involvement of DA in the reduction of food intake, the 96 h dopaminergic activity results suggest that DA is certainly not the only contributor to the observed changes in food consumption. Anatomically, several different dopaminergic nuclei have been located in the region identified in this study as the PB (which includes the midbrain, optic tectum, cerebellum and brainstem; Wullimann and Mueller, 2004). While none of these dopaminergic nuclei has specifically been linked to the regulation of appetite in fish, the posterior tuberculum in the midbrain is the origin of the ascending dopaminergic system in teleosts (Rink and Wullimann, 2001), a major activating/modulatory system in vertebrate brains known for its involvement in the regulation of feeding, drinking, and locomotion.

In addition to the potential involvement of central monoamines in the control of food intake during high ammonia exposure, our data implicate the CRF family of peptides in this regulatory process. Exposure to ammonia elicited an increase in CRF and UI mRNA levels in both the HYP and TEL. Since CRF-related peptides are potent anorexigenic signals in fish (De Pedro et al., 1993; Bernier and Peter, 2001b), our results suggest that CRF and UI may be involved in mediating at least a portion of the anorexigenic effects associated with ammonia toxicity. However, changes in gene expression do not necessarily predict peptide secretion, and besides its role in the regulation of food intake, the CRF system in fish is involved

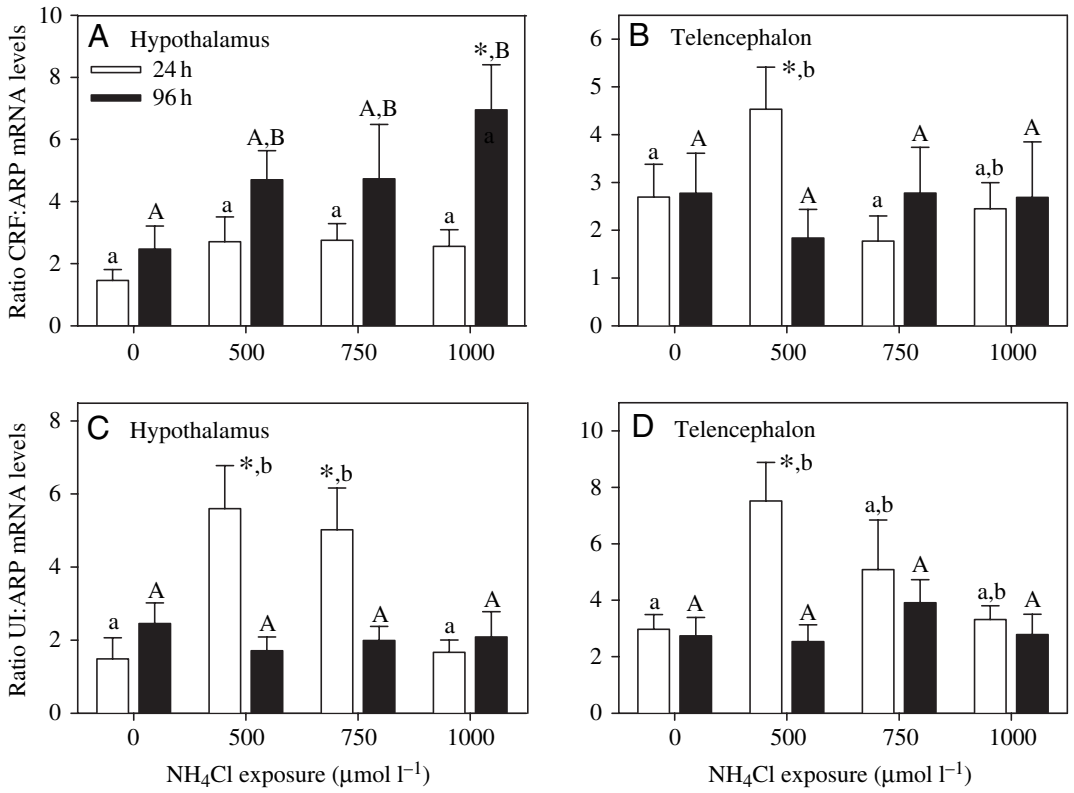

Fig. 5. The ratio of corticotropin-releasing factor (CRF; A,B) or urotensin I (UI; C,D) mRNA levels to the housekeeping gene acidic ribosomal phosphoprotein P0 (ARP) mRNA levels in the hypothalamus (A,C) and telencephalon (B,D) of rainbow trout exposed to 0, 500, 750 or 1000 μmol l^{-1} NH_4Cl for either 24 or 96 h periods. Values are means + 1 S.E.M. Treatments that do not share a common letter for a given time and brain region are significantly different from each other. *Significant differences between exposure periods for a given treatment (P<0.05; N=8–12).

in regulating several other aspects of the stress response (Lovejoy and Balment, 1999). Overall, the changes in *CRF* and *UI* gene expression with ammonia exposure were region-, dose- and time-dependent. Whereas the mRNA increases in TEL and HYP UI and in TEL CRF were only observed after 24 h of exposure and were inversely related to the ammonia concentration, the increases in hypothalamic CRF mRNA levels were observed after 96 h of exposure and were dose-dependent. Although the exact cause is not known, the mechanism responsible for the inverse relationship between the increase in mRNA levels and the dose of ammonia after 24 h of environmental ammonia may involve circulating glucocorticoids. In goldfish, cortisol exerts a negative feedback action on forebrain *CRF* and *UI* gene expression (Bernier et al., 1999, 2004). After 24 h of ammonia exposure in this study, plasma ammonia and cortisol levels were positively correlated. Therefore, the high circulating cortisol levels in the fish exposed to the highest ammonia concentration may have negated the stimulatory effects of hyperammonemia on TEL and HYP *UI* and on TEL *CRF* gene expression.

The stimulatory effects of environmental ammonia on plasma cortisol observed in this study are consistent with previous results in a variety of fish species (Swift, 1981; Spotte and Anderson, 1989; Person-Le-Ruyet et al., 1998; Wicks and Randall, 2002b). As in channel catfish (Tomasso et al., 1981), chronic exposure of trout to high ammonia concentrations was associated with an initial dose-dependent increase in plasma cortisol followed by a gradual return to basal values. The return of plasma cortisol concentrations to basal conditions in chronically exposed fish may be a reflection of ammonia detoxifying mechanisms. In fact, there is evidence to suggest that the ammonia exposure-elicited surge in plasma cortisol promotes the detoxification of ammonia. In the gulf toadfish *Opsanus beta*, dexamethasone injections increase hepatic GSase (Mommsen et al., 1992) and cortisol-synthesis inhibition prevents the acute hepatic GSase activation induced by confinement stress (Hopkins et al., 1995). Whether the ammonia exposure-elicited surge in plasma cortisol stimulates brain and liver GSase and contributes to the detoxification of ammonia to glutamine in rainbow trout needs to be ascertained. The stimulatory effects of cortisol implants on urea synthesis in rainbow trout do, however, suggest that cortisol can stimulate the enzymes of the uricolytic pathway in this species (McDonald and Wood, 2004). On the other hand, while there are still numerous questions as to the precise role of cortisol in the regulation of food intake in fish, since only chronic elevations in plasma cortisol appear to affect appetite, the ammonia-elicited surge in plasma cortisol was probably not a significant contributor to the control of food intake in this study (Bernier and Peter, 2001a; Bernier et al., 2004).

There is considerable evidence implicating CRF-related peptides and 5-HT in the control of the HPI axis in fish (e.g. Lederis et al., 1994; Winberg et al., 1997; Winberg and Lepage, 1998; Huising et al., 2004). In this study, relative to the changes in plasma cortisol, the timing of the increase in the mRNA levels of TEL CRF, TEL UI, HYP UI and HYP serotonergic activity suggest a potential involvement of both CRF-related peptides and 5-HT in the control of the HPI axis during environmental ammonia exposure. However, based solely on gene expression data that may be influenced by the

negative feedback effects of cortisol (see above), it is difficult to assess the extent of the CRF and UI contribution to HPI axis regulation during ammonia exposure. In contrast, the strong correlation between hypothalamic serotonergic activity and plasma cortisol levels after 24 h of exposure, suggest an important role of hypothalamic 5-HT in the regulation of the HPI axis in hyperammonemic fish. Interestingly, however, there is also evidence suggesting that the 5-HT system may be involved in suppressing the adrenocortical stress response in fish (Lepage et al., 2002).

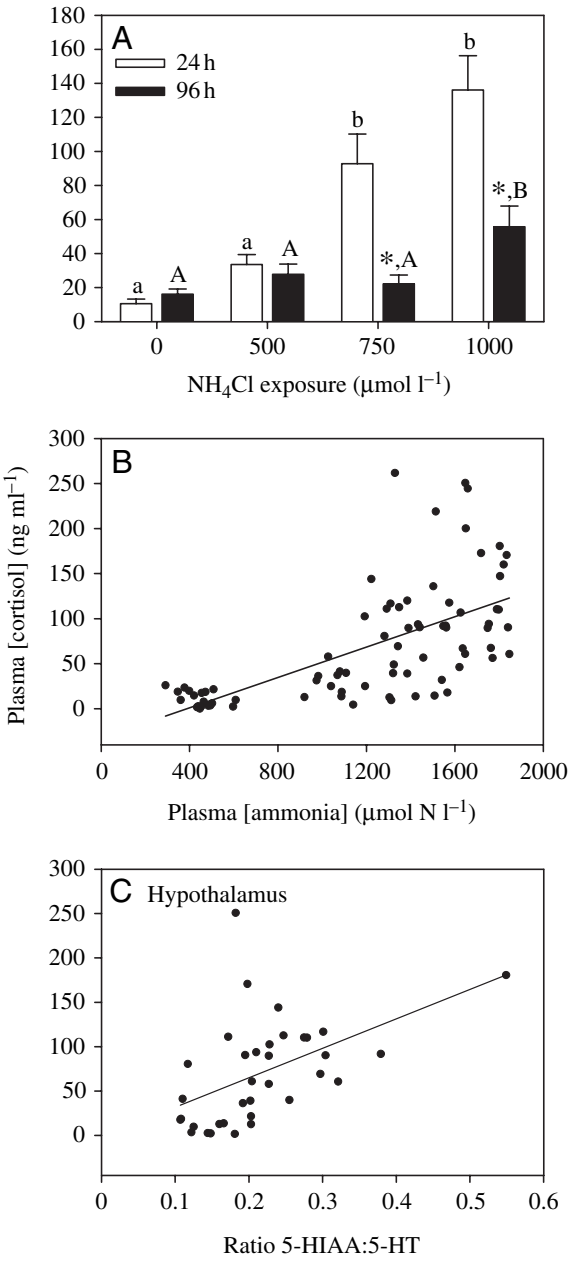

Fig. 6. (A) Plasma [cortisol] of rainbow trout exposed to 0, 500, 750 or 1000 μmol l^{-1} NH_4Cl for either 24 or 96 h periods. Values are means + 1 S.E.M. Treatments that do not share a common letter for a given time are significantly different from each other. *Significant differences between exposure periods for a given treatment (P<0.05; N=20–23). (B) Relationship between plasma [ammonia] and plasma [cortisol] after 24 h NH_4Cl exposure (r^2=0.41, P<0.001). (C) Relationship between hypothalamic serotonergic activity and plasma [cortisol] after 24 h NH_4Cl exposure (r^2=0.25, P=0.002). Serotonergic activity is expressed as a ratio of the metabolite 5-hydroxyindole acetic acid (5-HIAA) to serotonin (5-HT).

Pharmacological and anatomical studies have identified complex interactions between the dopaminergic, serotonergic and CRF systems in the brain of mammals (Meguid et al., 2000; Sullivan Hanley and Van de Kar, 2003; Herman et al., 2003). Although our understanding of the interplay between these various neuroendocrine systems in fish is cursory, there are a few examples of such interactions. In goldfish, for example, CRF may partially mediate the anorectic effects of 5-HT (De Pedro et al., 1998a) and the anorectic effects of CRF may involve the dopaminergic system (De Pedro et al., 1998b). Anatomical evidence also suggests potential interactions between the serotonergic and CRF systems in rainbow trout (Terlou et al., 1978; Frankenhuis-van den Heuvel and Nieuwenhuys, 1984). Hypothalamic regions known to contain CRF and UI cell bodies in fish, the preoptic area and the basal HYP (Lederis et al., 1994), are richly innervated by 5-HT fibers. Whether such interactions are important to the overall regulation of food intake and HPI axis during exposure to high ammonia concentrations remains to be determined.

In summary, exposure to high external ammonia concentrations over a 96 h period are characterized by a transient reduction in appetite and activation of the HPI axis in rainbow trout. Here we show that these responses to ammonia exposure are associated with regional and time-dependent increases in the activity of neurotransmitters and expression of neuropeptides with known anorexigenic and hypophysiotropic properties, namely 5-HT, DA, CRF and UI. These results implicate the brain's monoaminergic and CRF systems in the neuroendocrine circuitry involved in sensing and coordinating the response to hyperammonemia stress. More specifically, our findings show that among the many excitatory and inhibitory inputs that control food intake and HPI responsiveness, hypothalamic 5-HT may be a key neurobiological substrate for the regulation of these processes during exposure to high external ammonia concentrations.

List of abbreviations

5HIAA	5-hydroxyindoleacetic acid
5-HT	serotonin
ANOVA	analysis of variance
ARP	acidic ribosomal phosphoprotein
CRF	corticotropin-releasing factor
DA	dopamine
DOPAC	dihydroxyphenylacetic acid
GSase	glutamine synthetase
HPI	hypothalamic–pituitary–interrenal axis
HPLC	high pressure liquid chromatography
HYP	hypothalamus
icv	intracerebroventicular

PB	posterior brain
PIT	passive integrated transponder
RT-PCR	reverse transcriptase polymerase chain reaction
TEL	telencephalon
TRP	tryptophan
UI	urotensin I

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