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Differential Increase in Forebrain and Caudal Neurosecretory System Corticotropin-Releasing Factor and Urotensin I Gene Expression Associated with Seawater Transfer in Rainbow Trout

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Transfer to seawater (SW) in rainbow trout elicits an increase in plasma cortisol and a bout of anorexia. Although the corticotropin-releasing factor (CRF) system has known hypophysiotropic and anorexigenic properties, it is not known whether CRF-related peptides originating from either the forebrain or the caudal neurosecretory system (CNSS) play a role during SW acclimation. Therefore, we examined the effects of SW transfer on food intake, plasma osmolality, hypothalamic-pituitary-interrenal axis activity, and the expression of CRF and urotensin I (UI) in the forebrain and the CNSS. While SW transfer chronically suppressed food intake over a 2-wk period, it transiently increased plasma osmolality, ACTH, and cortisol. Similarly, 24 h after SW transfer, hypothalamic and preoptic area CRF mRNA levels were significantly increased but recovered to pretransfer levels within 7 d. Conversely, SW transfer elicited a delayed increase in hypothalamic UI mRNA levels and had no effect on preoptic area UI expression. In the CNSS, SW exposure was associated with parallel increases in CRF and UI mRNA levels from 24 h post transfer through 7 d. Finally, *in situ* hybridization demonstrated an extensive and overlapping pattern of CNSS CRF and UI expression. These results differentially implicate specific neuronal populations of the CRF system in the acute and chronic responses to a hyperosmotic stress and suggest that forebrain and CNSS CRF-related peptides have different roles in the coordinated response to fluid balance disturbances. (*Endocrinology* 146: 3851–3860, 2005)

ACCLIMATION TO SEAWATER conditions in euryhaline teleosts involves several physiological, morphological, and behavioral changes that are controlled and coordinated by endocrine and neuroendocrine systems (1, 2). In salmonids, abrupt transfer from fresh water (FW) to seawater (SW) is characteristically accompanied by a temporary decrease in food intake. This transient period of anorexia after direct transfer to SW takes place in both juveniles and adults and has been observed in a variety of species, including rainbow trout, *Oncorhynchus mykiss* (3, 4). Although available evidence suggests that SW transfer-induced anorexia is not a direct result of osmoionoregulatory failure (5, 6), interference with drinking (7), suppressed digestive enzymatic activity (8), or changes in social interactions (6), the causative agents are not known.

Among the endocrine signals involved in osmoregulation in fish, cortisol has been identified as an important SW-adapting hormone, and several studies have reported increased circulating levels and metabolic clearance of this glucocorticoid after SW exposure (1, 9, 10). Because cortisol is the primary product of the hypothalamic-pituitary-interrenal (HPI) axis in fish (11), SW transfer likely involves a stimulation of this neuroendocrine pathway. However, although the related hypothalamic neuropeptides corticotropin-releasing factor (CRF) and urotensin I (UI) are key hypophysiotropic agents (12), few studies have examined their role in the regulation of the HPI axis during SW transfer (13). Similarly, whereas CRF-related peptides have also been identified as potent anorexigenic factors in fish (14, 15), whether they contribute to the regulation of food intake after SW transfer has not been investigated.

In addition to the forebrain, fish possess a second major source of CRF-related peptides that releases its content to the circulation, the caudal neurosecretory system (CNSS) (16). The CNSS, a unique fish neuroendocrine organ, consists of an arrangement of neurosecretory cells, known as Dahlgren cells, with caudally projected axons terminating on capillaries located in the caudal vertebrae (17). Traditionally known as a location of high UI expression, the CNSS has now been identified as an equally important source of CRF in flounder (*Platichthys flesus*) (18). To date, whereas no precise physiological role has been attributed to the CNSS, an involvement in osmoregulation has been suggested. Several studies have shown that changes in salinity can alter the immunoreactive pattern of UI in the CNSS and the ultrastructural appearance of the Dahlgren cells (19–22). Moreover, UI can directly elicit the release of cortisol from the interrenals (23, 24) and has various effects on the active transport of ions across a number of epithelial surfaces (25, 26). Therefore, in response to SW transfer, available evidence suggests that the CNSS may be a source of circulating CRF-related peptides that could, at

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Abbreviations: CNSS, Caudal neurosecretory system; CRF, corticotropin-releasing factor; CRF-R, CRF receptor type; EIA, enzyme immunoassay; FW, fresh water; HPI, hypothalamic-pituitary-interrenal; Na^+/K^+-ATPase, Na^+/K^+-adenosine triphosphatase; PFA, paraformaldehyde; SW, seawater; UI, urotensin I.

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least, contribute to the control of interrenal cell activity and ionic regulatory processes.

Thus, to assess the potential contribution of the CRF system in rainbow trout to the physiological and behavioral responses associated with an hyperosmotic stress, we investigated the effects of SW transfer on food intake, the activity of the HPI axis, the hypoosmoregulatory ability of the animals, and the preoptic area, hypothalamus, and CNSS CRF and UI mRNA levels. Furthermore, as a means of determining the importance of the CNSS as an additional source of CRF expression in rainbow trout, we characterized the CNSS distribution of CRF and UI mRNA by *in situ* hybridization.

Materials and Methods

Animals

Rainbow trout of either sex weighing 86–200 g (121 ± 3 g; n = 66) for experiment 1, 98–292 g (184 ± 6 g; n = 87) for experiment 2, and 97–197 g (135 ± 6; n = 12) for experiment 3 were obtained from Rainbow Springs Trout Farm (Thamsford, Canada) and transported to the Hagen Aqualab at the University of Guelph. Fish were acclimated for at least 4 wk in 650-liter fiberglass tanks continuously supplied with well-aerated local well water, maintained at 14 C, and exposed to a 12-h light, 12-h dark photoperiod regime. Fish were fed daily with commercial trout pellets (3PT Classic Sinking; Martin Mills, Elmira, Canada). All procedures used were approved by the local Animal Care Committee and conform to the principles of the Canadian Council for Animal Care.

Experimental design

Experiment 1: SW transfer and forebrain CRF-related peptide gene expression. Fish anesthetized in buffered tricaine methanesulfonate (0.1 g/liter; MS-222; Syndel, Vancouver, Canada) were weighed and injected with a 125-kHz implantable passive integrated transponder tag (11.5 × 2.1 mm; Biomark, Inc., Boise, ID) into the epaxial musculature anterior to the dorsal fin. Fish were then placed in flow-through, 125-liter tanks in groups of 8–10 and allowed to acclimate for 2 wk. Using x-radiography, individual food intake was assessed twice, once before SW/FW exposure and at the time of terminal sampling. For the exposures, fish were removed from their tank and either returned to their respective FW tank or relocated to flow-through, 125-liter tanks containing artificial SW (33 ppt; pH 8.2; 11 C). Except for the control group, which was left undisturbed, all treatments were handled in an identical fashion. In total, there were seven different treatments consisting of a control group and three different groups exposed to either FW or SW for a period of 24 h, 168 h (7 d), or 336 h (14 d). At the end of the FW or SW exposure periods, fish were terminally anesthetized with 2-phenoxyethanol (2 ml/liter; Sigma, St. Louis, MO), and a blood sample was immediately drawn via caudal puncture and spun down at 14,000 × *g* for 5 min. Plasma was aliquoted and frozen at −20 C for analysis of osmolality and cortisol. Additionally, to quantify CRF and UI mRNA levels, the brain was removed and regionally dissected to isolate the preoptic area and the hypothalamus as per Doyon *et al.* (27).

Experiment 2: SW transfer and CNSS CRF-related peptide gene expression. Fish were weighed, tagged, acclimated to their respective tanks, assessed for their food intake, and handled throughout in identical fashion as in experiment 1. In total, there were nine treatments consisting of a control group and four different groups exposed to either FW or SW for a period of 6, 24, 72, or 168 h. At the end of the exposures, fish were terminally anesthetized, and a blood sample was drawn for analysis of plasma osmolality, ACTH, and cortisol levels. A gill sample of eight to 10 filaments from the first arch was excised, immersed in 100 μl ice-cold SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3), and stored at −80 C for analysis of Na^+/K^+-adenosine triphosphatase (Na^+/K^+-ATPase) activity. The CNSS was excised to quantify CRF and UI mRNA levels. The region of the caudal spinal cord removed consisted of the five most caudal vertebrae and the urostyle. The CNSS was placed in a solution of 4% paraformaldehyde (PFA) in PBS (pH 7.4) and gently rocked for 24 h at 4 C.

Experiment 3: FW transfer from 14 to 11 C and CRF-related peptide gene expression. To account for the difference in temperature between the FW and the SW tanks, two groups of nine fish were placed in 125-liter FW tanks at 14 C and allowed to acclimate for 2 wk. Fish were weighed, tagged, assessed for control food intake, and handled throughout in identical fashion as in experiment 1. Fish were then removed and either returned to their respective tank or transferred to a new 125-liter FW tank maintained at 11 C. Twenty-four hours after transfer, fish were terminally anesthetized, and food intake was assessed. A blood sample was drawn for later analysis of plasma cortisol; and the preoptic area, the hypothalamus, and the CNSS were dissected to quantify CRF and UI mRNA levels.

Quantification of food intake

During the acclimation period, and between the first (control) and second (post exposure) assessment of food intake, fish were fed to satiation daily at 1100 h using repelleted 3PT fish food. On days where individual food intake was assessed, fish were fed a labeled food that could be detected by x-radiography. Briefly, the diet was ground to a fine powder and repelleted with 450-μm hardened cast carbon steel spheres (Draiswerke, Mahwah, NJ) at a ratio of 5% by mass of dry powdered food. Approximately 90 min after feeding, fish were anesthetized in a buffered MS-222 (0.5 g/liter) solution and x-rayed using an ACU-RAY HFJ portable x-ray unit (50 kV peak; 1.05 mA sec at 90 cm; Sterne, Brampton, Canada). After development of the radiographs, the individual steel spheres present in the gastrointestinal tract were tallied, and the amount of food eaten was determined by a calibration curve. Initial experiments indicated that the steel sphere did not alter palatability of the diet, and the x-ray procedure could be repeated at 72-h intervals without affecting food intake.

Plasma analysis

Plasma osmolality was measured in duplicate on a Vapro vapor pressure osmometer (Wescor, Inc., Logan, UT). Plasma ACTH was determined in duplicate using a ^{125}I-ACTH RIA kit (MP Biomedicals, Inc., Irvine, CA). Plasma cortisol was determined in triplicate using an enzyme immunoassay (EIA) as per the protocol of Carey and McCormick (28), with the following modifications: microtitre plates were coated with rabbit anticortisol antibody (catalog no. F3-314; Esoterix Endocrinology, Calabasas Hills, CA) at a dilution of 1:20,000, and the cortisol-horseradish peroxidase conjugate (Clinical Endocrinology Laboratory, University of California Davis, Davis, CA) was added at a dilution of 1:60,000. The concentration of ACTH and cortisol in the plasma samples was calculated using three-parameter sigmoid curve regression equations (SigmaPlot 7.0; SPSS, Inc., Chicago, IL) as obtained from the standard curves.

Na^+/K^+-ATPase activity

Gill Na^+/K^+-ATPase activity was determined using the microassay method of McCormick (29). Both Na^+/K^+ ATPase and bicinchoninic acid protein assays (no. 23225; Pierce Chemical Co., Rockford, IL) were run on a SpectraMAX 190 microplate reader using SOFTmax software 4.6 (Molecular Devices, Menlo Park, CA).

Total RNA extraction

Total RNA from the preoptic area and hypothalamus was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA) based on the acid guanidinium thiocyanate-phenol-chloroform extraction method. A modified protocol described by Masuda *et al.* (30) was used to extract total RNA from the CNSS. Briefly, the urophysis and attached portion of the spinal cord were removed from the PFA-fixed vertebral column and dehydrated in a series of ethanol washes and incubated overnight at 4 C. The ethanol was replaced with 1 ml digestion buffer (200 mM Tris-HCl, 200 mM NaCl, 1.5 mM $MgCl_2$, 2% sodium dodecyl sulfate, pH 7.5) containing 500 μg proteinase K (Invitrogen) and the mixture incubated at 37 C until complete tissue lysis (60–90 min). Tissue lysate was incubated in 0.5 ml phenol/chloroform/isoamyl alcohol (25:24:1) at room temperature, centrifuged, and the upper phase precipitated overnight at −20 C in isopropranol. Total RNA concentrations from all

tissues were determined by UV spectrophotometry at 260 nm, and samples were stored at −80 C until used.

Quantification of mRNA by real-time RT-PCR

One microgram of total RNA was treated with DNase I according to manufacturer's protocol (DNase I amplification grade, Invitrogen) and reverse transcribed to cDNA using SuperScript II RNase H^- reverse transcriptase (Invitrogen). The cDNA product was amplified using the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). Each reaction contained 10 μl SYBR green PCR master mix (Applied Biosystems), 5 μl cDNA template, and 2.5 μl each of forward and reverse primers (0.4 μM). Default cycling conditions were used: 10 min at 95 C followed by 40 cycles of 15 sec 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 using serial dilutions of cDNA samples known to have high expression levels of the target gene (31). 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. To correct for differences in RNA loading and reverse transcriptase inefficiencies, each sample was normalized to the expression level of the housekeeping gene β-actin. All samples were assayed in triplicate, and only one target was assayed per well. Finally, nonreverse transcribed RNA and water controls were run to ensure that no genomic DNA was being amplified and the reagents were not contaminated. Primers for rainbow trout UI (GenBank accession no. AJ005264), CRF (GenBank accession no. AF296672), and β-actin (GenBank accession no. AF296672) were designed using Primer Express (Applied Biosystems); and to prevent potential coamplification of genomic DNA, the sequence of either the forward or reverse primer was based on the position of a known exon-exon junction (UI, GenBank accession no. AY651778; CRF, GenBank accession no. AY651777) or a deduced junction based on sequence identity (β-actin). 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 β-actin (forward: 5′-GCC CCC CTC AAC CCC-3′; reverse: 5′-GAA GGT CTC AAA CAT GAT CTG GGT C-3′) primers were also designed to amplify the shortest product possible (CRF, 54 bp; UI, 51 bp; β-actin, 60 bp).

In situ hybridization

In situ hybridization was performed to localize the expression of CRF and UI in the CNSS of rainbow trout. The CNSS from three untreated control fish was fixed in a solution of 4% PFA in PBS (pH 7.4) overnight and sequentially transferred over 3 d to 30% sucrose in PBS, 1:1 30% sucrose in PBS:Cryomatrix (Thermo Shandon, Inc., Pittsburgh, PA), and Cryomatrix at 4 C. The tissues were embedded and cut in a cryostat to produce 7-μm-thick sections. Riboprobe templates were generated by subcloning 455- and 409-bp fragments that span the coding and 3′ untranslated regions of the rainbow trout CRF and UI cDNAs, respectively, into the pGEM-T Easy vector (Promega Corp., Madison, WI). The plasmid DNA templates were linearized with either *Sal*I or *Nco*I (Invitrogen) and the digested products reverse transcribed in the presence of digoxygenin-11-UTP using either T7 or SP6 RNA polymerases (Roche Diagnostics Canada, Laval, Canada) to synthesize sense and antisense probes for each cDNA. Adjacent cryosections were hybridized overnight with the sense or antisense probes as described by Sciavolino *et al.* (32), with the following modifications: sections were postfixed in 4% PFA and 0.2% glutaraldehyde after permeabilization and incubated in prehybridization solution for 2 h, and the alkaline-phosphatase staining reaction was developed for a period of 6 h. Slides were visualized using a Leica DM LA microscope (Leica Microsystems, Wetzlar, Germany), and images were captured using Openlab imaging software (version 3.5.1; Improvision, Coventry, UK).

Statistical analysis

All data are presented as mean ± SE. Differences within a treatment were assessed by one-way ANOVA followed by a Dunnett's multiple-comparison test to compare the time-zero control data point with values at subsequent times. Differences between the FW and SW treatments were assessed by a Student's *t* test. The significance level for all statistical tests was $P < 0.05$.

Results

Experiment 1: SW transfer and forebrain CRF-related peptide gene expression

Exposure of FW-acclimated rainbow trout to full-strength SW elicited a severe and chronic reduction in food intake (Fig. 1A). In contrast, transferring FW-acclimated trout to another FW tank had no effect on next-day food consumption (Fig. 1A). The anorexigenic effects of the SW treatment were first observed 24 h post transfer and were maintained through 336 h. Relative to the FW controls, SW exposure also resulted in a significant osmotic disturbance and stress response. Plasma osmolality and cortisol increased significantly in the first 24 h post SW transfer and returned to control values after 168 h (Fig. 2, A and B). Whereas FW transfer had no short- or long-term effect on the forebrain

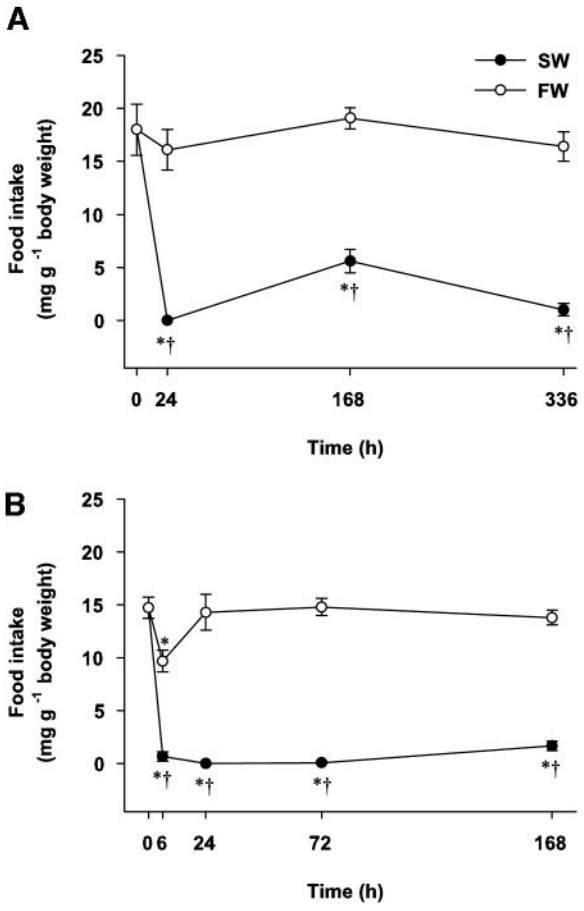

FIG. 1. Effects of FW or SW transfer on food intake in FW-acclimated rainbow trout. Food intake was monitored for either (A) 336 h (experiment 1) or (B) 168 h (experiment 2) post transfer. Whereas transfer to FW elicits a small transient reduction in food intake, SW exposure chronically suppresses appetite up to 14 d post transfer. *, Significant difference from the time-zero control value as determined by one-way ANOVA and by Dunnett's test; †, difference at a given time between FW and SW treatments as determined by Student's *t* test (n = 8–10; $P < 0.05$).

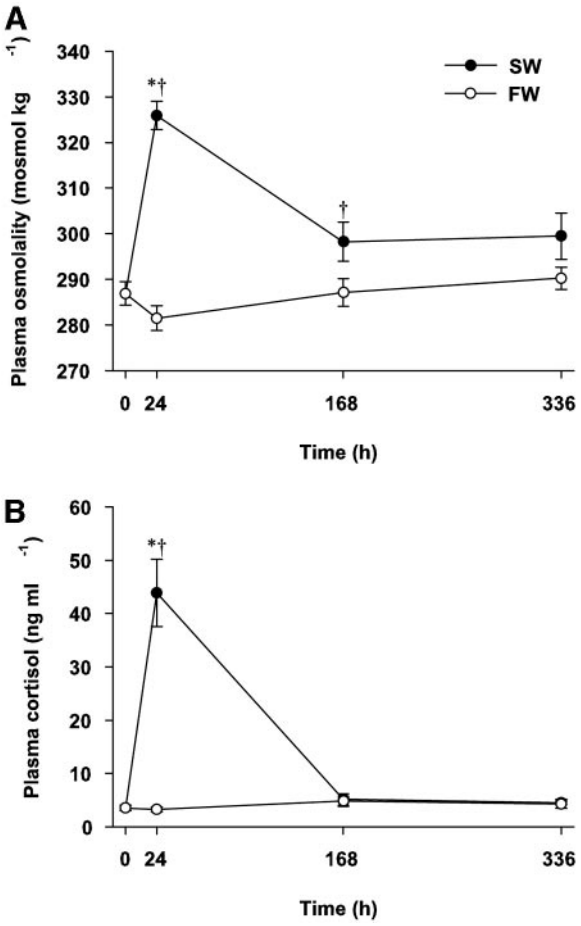

FIG. 2. Effects of FW or SW transfer in experiment 1 on plasma (A) osmolality and (B) cortisol in FW-acclimated rainbow trout. Plasma osmolality and cortisol increase sharply within the first 24 h after SW transfer; and whereas cortisol values are back to control levels after 168 h, plasma osmolality recovers after 336 h. *, Significant difference from the time-zero control value as determined by one-way ANOVA and by Dunnett's test; †, difference at a given time between FW and SW treatments as determined by Student's *t* test (n = 8–10; $P < 0.05$).

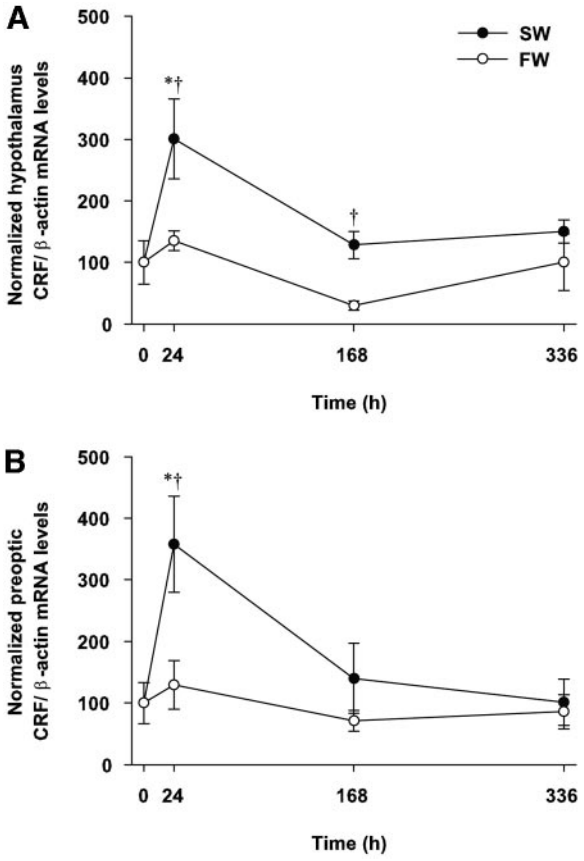

FIG. 3. Effects of FW or SW transfer in experiment 1 on (A) hypothalamic and (B) preoptic area CRF mRNA levels in FW-acclimated rainbow trout. SW exposure elicits transient increases in CRF expression that are fully recovered at 336 h post transfer in the hypothalamus and 168 h post transfer in the preoptic area. *, Significant difference from the time-zero control value as determined by one-way ANOVA and by Dunnett's test; †, difference at a given time between FW and SW treatments as determined by Student's *t* test (n = 8–10; $P < 0.05$).

mRNA levels of CRF and UI, SW exposure affected the expression of both genes. Twenty-four hours after transfer to SW, there were 3- and 3.5-fold increases in CRF mRNA levels in the hypothalamus and preoptic region, respectively (Fig. 3, A and B). The effects of SW transfer on forebrain CRF gene expression appeared to be relatively short-term, however, because the mRNA levels were back to control levels at 168 h post transfer. In contrast, SW transfer elicited a delayed 6.2-fold increase in UI mRNA levels in the hypothalamus after 336 h of exposure (Fig. 4A) but had no effect on this transcript in the preoptic region (Fig. 4B).

Experiment 2: SW transfer and CNSS CRF-related peptide gene expression

Whereas FW transfer only elicited a small and transient reduction in food intake 6 h post transfer, SW transfer severely reduced food consumption throughout the 168-h exposure period (Fig. 1B). Fish transferred into FW did not experience any change in either plasma osmolality or gill Na^+/K^+-ATPase activity (Fig. 5). In the SW-exposed fish, relative to the time-zero control group, plasma osmolality steadily rose during the first 72 h post transfer and nearly recovered to control levels at 168 h post transfer (Fig. 5A). Conversely, there was no significant change in gill Na^+/K^+-ATPase activity in the SW-exposed fish until 168 h post transfer, at which point the activity increased by 2.4-fold (Fig. 5B). SW exposure also resulted in changes in plasma ACTH and cortisol (Fig. 6). Plasma ACTH significantly increased, 1.8-fold, 72 h after SW transfer and fully recovered to control values at 168 h post transfer (Fig. 6A). For the plasma ACTH RIA, all samples were analyzed in one assay that had a lower detection limit of 10 pg/ml and an intraassay variation of 5.1% (n = 8), and a serial dilution of rainbow trout plasma gave a displacement curve parallel to the ACTH standard curve (data not shown). Plasma cortisol increased 3.9-, 5.9-, and 8.7-fold at 6, 24, and 72 h after SW transfer, respectively, and fully recovered to control values at 168 h post transfer (Fig. 6B). The plasma cortisol EIA had a lower detection limit of 0.60 ng/ml, intra- and interassay variations of 5.7% (n = 10) and 12.6% (n = 5), respectively; and a serial dilution of rainbow trout plasma gave a displacement curve that was parallel to the cortisol standard curve (data not shown).

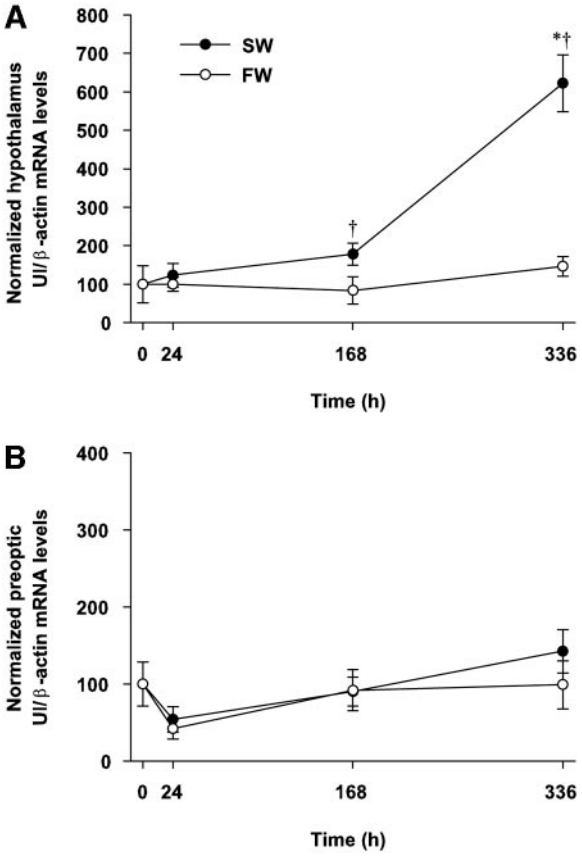

FIG. 4. Effects of FW or SW transfer in experiment 1 on (A) hypothalamic and (B) preoptic area UI mRNA levels in FW-acclimated rainbow trout. Whereas SW exposure has no effect on preoptic area UI expression, it elicits a delayed and robust increase in UI mRNA levels in the hypothalamus. *, Significant difference from the time-zero control value as determined by one-way ANOVA and by Dunnett's test; †, difference at a given time between FW and SW treatments as determined by Student's *t* test (n = 8–10; $P < 0.05$).

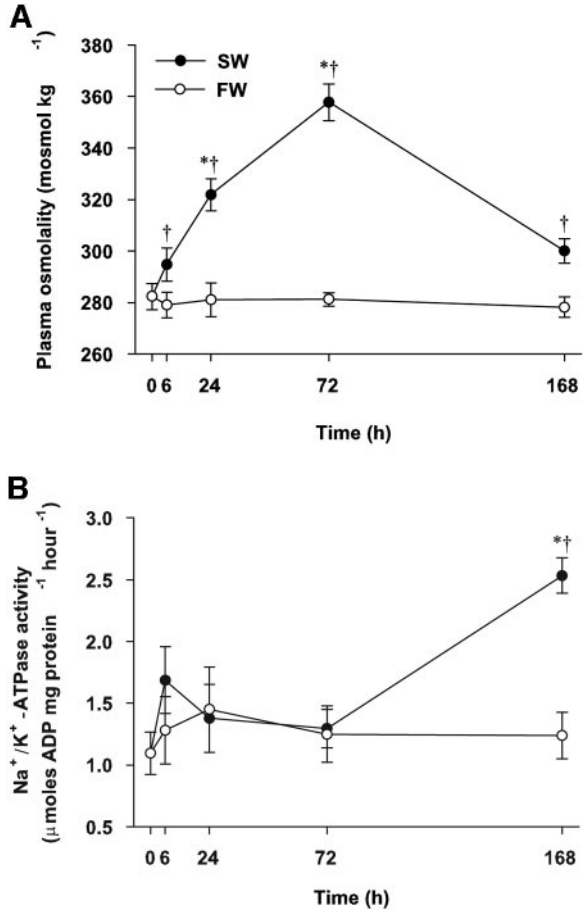

FIG. 5. Effects of FW or SW transfer in experiment 2 on (A) plasma osmolality and (B) gill Na^+/K^+-ATPase activity in FW-acclimated rainbow trout. SW transfer elicits a transient increase in plasma osmolality, and the return toward control conditions is associated with a mark increase in gill Na^+/K^+-ATPase activity. *, Significant difference from the time-zero control value as determined by one-way ANOVA and by Dunnett's test; †, difference at a given time between FW and SW treatments as determined by Student's *t* test (n = 8–10; $P < 0.05$).

Finally, whereas FW transfer had no short- or long-term effect on the mRNA levels of CRF and UI in the CNSS, exposure to SW conditions elicited marked increases in the levels of both transcripts at 24, 72, and 168 h post transfer (Fig. 7).

Experiment 3: FW transfer from 14 to 11 C and CRF-related peptide gene expression

Transferring trout from 14 C to 11 C FW had no effect on next-day food intake, plasma cortisol, or on the preoptic region, hypothalamus, and CNSS CRF and UI mRNA levels (data not shown).

In situ hybridization

In situ hybridization of the caudalmost portion of the spinal cord with digoxigenin-labeled CRF and UI riboprobes revealed an extensive pattern and high levels of gene expression for both transcripts (Fig. 8, A and B). Overall, CRF and UI expression was restricted to the cell body of the Dahlgren cells, the neurosecretory neurons of the CNSS. The expression pattern of CRF and UI formed a band around the central canal and was absent from the external surface of the spinal cord, from the nerve axons, and from the urophysis. Adjacent sections, hybridized with the CRF and UI antisense probes, gave strong signals for each mRNA that often colocalized to the same Dahlgren cells (Fig. 8, C and D). The sense probes showed no hybridization signal (Fig. 8, E and F).

Discussion

We have shown that SW transfer in rainbow trout is associated with a significant, but differential, up-regulation of CRF-related peptide gene expression in the preoptic area, the hypothalamus, and the CNSS. In general, although the appetite-suppressing effects of SW transfer were chronic over the duration of our studies, the period of HPI axis activation after the osmotic stress was transient and paralleled the osmoregulatory response. This clear separation between HPI axis activity and feeding during SW adaptation was also reflected in the differential up-regulation of the different CRF and UI neuronal populations investigated.

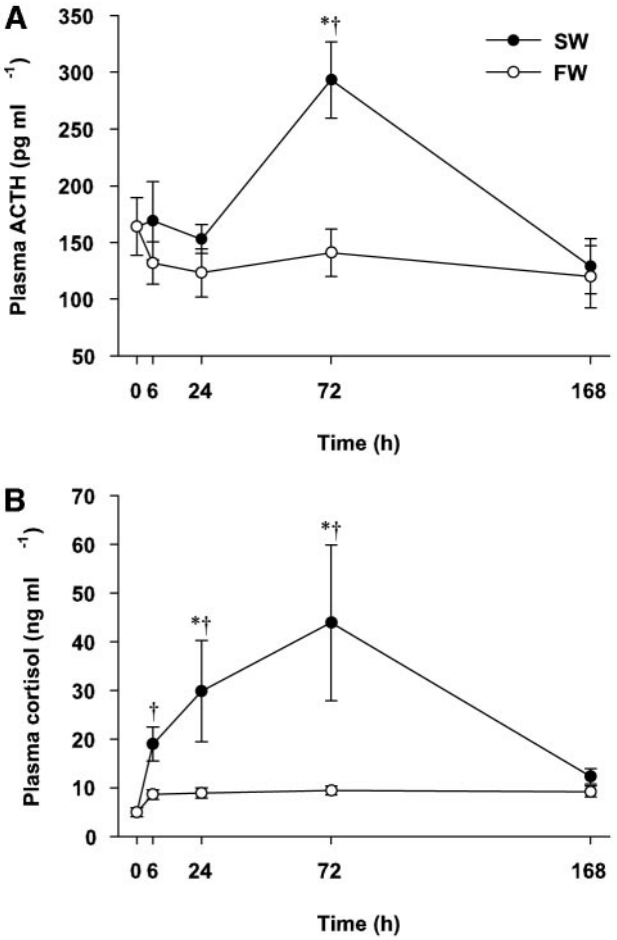

FIG. 6. Effects of FW or SW transfer in experiment 2 on plasma (A) ACTH and (B) cortisol in FW-acclimated rainbow trout. Whereas transfer to FW has no effect on the circulating levels of ACTH and cortisol, SW exposure elicits increases in the plasma concentration of both stress hormones that are fully recovered at 168 h post transfer. *, Significant difference from the time-zero control value as determined by one-way ANOVA and by Dunnett's test; †, difference at a given time between FW and SW treatments as determined by Student's *t* test (n = 8–10; $P < 0.05$).

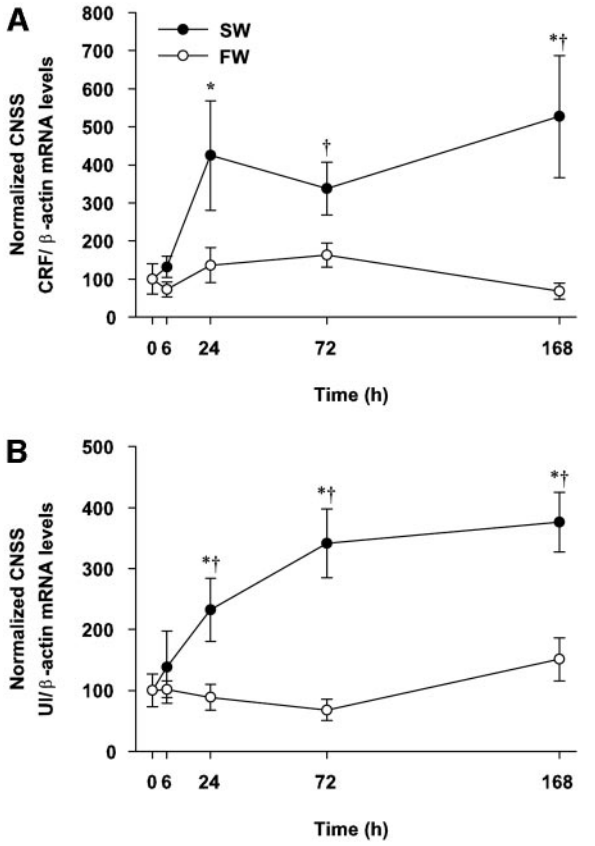

FIG. 7. Effects of FW or SW transfer on CNSS CRF (A) and UI (B) mRNA levels in FW-acclimated rainbow trout. In experiment 2, SW transfer elicited rapid and sustained increases in CNSS CRF and UI mRNA levels through 168 h post transfer. *, Significant difference from the time-zero control value as determined by one-way ANOVA and by Dunnett's test; †, difference at a given time between FW and SW treatments as determined by Student's *t* test (n = 8–10; $P < 0.05$).

Although SW transfer elicited transient increases in preoptic area and hypothalamus CRF mRNA levels, it had no effect on preoptic area UI expression, and it was associated with a delayed increase in hypothalamus UI expression and chronic elevations in CNSS CRF and UI mRNA levels. These results suggest considerable variation in the transcriptional regulation of different CRF and UI neuronal populations in response to an osmotic stressor and provide a novel outlook on the potential roles of the CRF system during both the acute and chronic phases of the stress response in fish.

SW transfer and forebrain CRF-related peptide gene expression

We present original evidence demonstrating changes in forebrain CRF-related peptide gene expression in response to SW transfer in fish. Within 24 h of the hyperosmotic stress, 3- and 3.5-fold increases in CRF expression were observed in the preoptic area and hypothalamus, respectively. Similarly, previous studies in rainbow trout have observed an increase in preoptic area CRF expression in response to subordination (27) and handling stress (33) and identified the magnocellular portion of the preoptic nucleus (NPO) in trout and other salmonids as an important site of CRF gene expression (34) and CRF immunoreactive perikarya (34, 35). The timing of the increases in forebrain CRF mRNA levels and return to control conditions corresponds with the transient increase and recovery in plasma osmolality. These observations suggest that components of the neural circuitry involved in regulating preoptic area and hypothalamus CRF gene expression may be osmotically responsive. Similarly, as previously shown in rainbow trout (36) and brown trout (*Salmo trutta*) (37) over a more detailed time-course than in this study, the gradual return to resting plasma osmolality and cortisol levels and the opposing increase in gill Na^+/K^+-ATPase activity between post transfer d 5 and 10 indicate that the fish are compensating for the immediate osmotic stress of SW transfer. Overall, although *in situ* hybridization is needed to identify the specific forebrain nuclei involved, our data implicate CRF-expressing neuronal populations from the preoptic region and hypothalamus in the acute phase of the response to a hyperosmotic stress in rainbow trout.

Unlike CRF, forebrain UI mRNA levels in trout were not

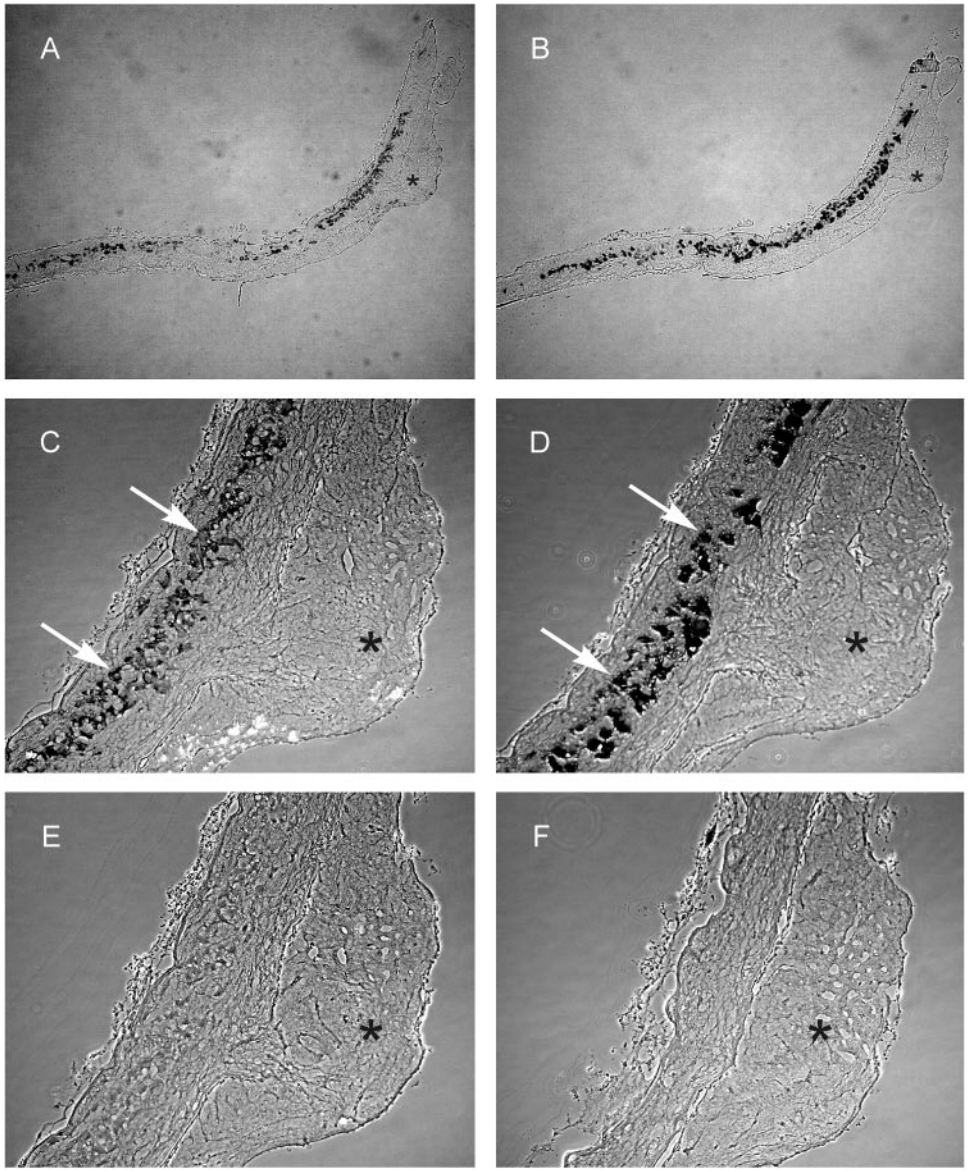

FIG. 8. *In situ* hybridization of CRF and UI in the CNSS of rainbow trout. The (A) CRF and (B) UI gene expression pattern with digoxigenin-labeled antisense riboprobes forms a band around the central canal that extends throughout the caudalmost portion of the spinal cord and is absent from the external surface, the nerve axons, and the urophysis. *Arrows*, (C) CRF and (D) UI gene expression localized to the same group of Dahlgren cells in adjacent sections. *In situ* hybridization of (E) CRF and (F) UI with digoxigenin-labeled sense riboprobes (negative controls). *, Urophysis.

immediately affected by SW emersion, and increased expression was detected in the hypothalamus only after prolonged SW exposure once plasma osmolality had returned to control values. Although detection of UI transcripts within the forebrain's preoptic region and hypothalamus is consistent with previous observations in other fish species (18, 38), our results provide original evidence that hypothalamic UI is implicated in the response to a hyperosmotic challenge. However, the delayed response to the hyperosmotic stimulus indicates that hypothalamic UI may not be regulated by the same osmotically responsive neuronal circuits as forebrain CRF.

SW transfer and CNSS CRF-related peptide gene expression

This study is the first characterization of CRF and UI expression in the CNSS of trout and investigation into the impact of an osmoregulatory stress on the expression of CRF-related peptides in this neurosecretory organ. The *in situ* hybridization results indicate a broad, overlapping expression pattern of CRF and UI, extending along the spinal cord beyond the region of the urophysis. These results, in association with our real-time gene expression data, suggest that the CNSS is a major site of CRF and UI production outside of the forebrain's preoptic area and corroborate the recent findings of Lu *et al.* (18) in the European flounder. This high capacity for CRF-related peptide expression in the CNSS may be the source of the high circulating CRF levels observed after confinement stress in tilapia (39). In response to SW transfer, we observed a chronic increase in the CNSS expression of CRF and UI. Previous studies also report changes in the immunoreactive pattern of UI and in the ultrastructural appearance of the CNSS in response to salinity alterations (19, 20, 22). In general, transfer to a hyperosmotic environment results in an increase in Dahlgren cell UI immunoreactivity, a decrease in urophysis UI signal, and a reduction in the abundance of urophysis neurosecretory granules. Although

measurements of circulating CRF and UI levels in response to SW transfer are needed, our findings, together with the above immunohistochemical observations, suggest that the CNSS is a source of circulating CRF-related peptides. Additionally, it appears that the altered levels of expression for both CRF and UI may be stress specific. In the European flounder, whereas CRF expression levels within the CNSS increased 3 h post handling stress, there was no change in UI gene expression (18). In contrast, SW transfer in this study elicited an increase in both transcripts, indicating perhaps that both CRF and UI function in an osmoregulatory capacity. In association with the rise in plasma osmolality, we see a parallel increase in CRF and UI gene expression in the CNSS. However, this increased expression is maintained beyond the initial osmotic stress response, indicating that factors other than plasma osmolality contribute to the regulation of CRF and UI expression in the CNSS. The apparent colocalization of UI and CRF in the CNSS of rainbow trout and the parallel changes in gene expression in response to SW transfer suggest a common Dahlgren cell activation by osmotic stress. Whether these cells or another population of Dahlgren cells have the capacity for differential secretion of UI and CRF, as previously observed in the European flounder (18), remains to be ascertained.

SW transfer, CRF-related peptides, and the regulation of food intake

Rainbow trout in this study experienced a drastic reduction in food intake upon SW emersion. These results are consistent with similar studies involving Atlantic salmon (*Salmo salar*), Arctic char (*Salvelinus alpinus*), and rainbow trout (3–6, 40). Although the changes in trout forebrain CRF expression are transient relative to the chronic appetite suppression, given the potent anorexigenic properties of CRF in fish (14, 15), our results do suggest a potential role for either preoptic or hypothalamic CRF nuclei in the regulation of food intake during SW transfer. In mammals, whereas the anorexigenic properties of CRF-related peptides are often associated with the CRF and urocortin cell bodies of the paraventricular nucleus (41, 42), gene expression changes in CRF neurons of the lateral hypothalamic area correlate best with the anorexic effects of dehydration stress in rats (43). Although UI also has potent anorexigenic properties in fish (15), our results do not implicate this neuropeptide in the acute anorexigenic response to SW transfer. Instead, if involved, hypothalamic UI may sustain the anorexia beyond the period of time required for the development of hypoosmoregulatory capacity. Clearly, given the complex interplay of hormones and hypothalamic neuropeptidergic substrates involved in mediating dehydration-induced anorexia in rats (44), CRF-related peptides are unlikely to be the only neuropeptides involved in regulating food intake during SW adaptation in fish.

Through direct actions on the gastrointestinal system, CRF-related peptides may also contribute to SW transfer-induced anorexia. In rodents, where urocortin is expressed in the enteric nervous system (45), peripheral injections of either urocortin or CRF significantly delay gastric emptying (46, 47), and this response is mediated through the CRF receptor type (CRF-R)2 found in the gastrointestinal tract (48). Although neither CRF-R1 nor CRF-R2 have been identified in the gastrointestinal tract of fish (49, 50), adaptation of goldfish (*Carassius auratus*) to dilute SW is accompanied by a marked increase in ^{125}I-labeled UI binding sites in the anterior intestine, suggesting a role for CRF-related peptides in regulating intestinal transport during osmotic stress (51). Furthermore, drainage of CRF-related peptides from the urophysis into the caudal vein makes it likely that the gastrointestinal system is exposed to physiological concentrations of CRF-related peptides in response to specific stressors. The rapid and chronic reduction in food intake upon SW exposure in trout was associated with chronic increases in CNSS CRF and UI expression. Assuming that the changes in CRF-related peptide expression translate into increases in the circulating levels of CRF and UI, the appetite-suppressing effects of SW transfer may be mediated through CRF- and/or UI-regulated changes in gastrointestinal function.

SW transfer, CRF-related peptides, and the regulation of cortisol secretion

Given the potent ACTH-secretory properties of CRF and the fact that ACTH is the principle cortisol secretagogue in fish (11, 12), the significant increases and recovery in preoptic and hypothalamus CRF mRNA levels, plasma ACTH, and cortisol in the first 7 d post SW transfer implicate forebrain CRF in the regulation of the HPI axis after an abrupt hyperosmotic stress. In contrast, although UI is also a potent hypophysiotropic factor in fish (12), our results do not support a role for either preoptic or hypothalamus UI in the regulation of the HPI axis after SW transfer. In the first 24 h post SW transfer, because plasma ACTH remains unchanged despite a rapid rise in plasma cortisol, our results also suggest that extra-HPI axis factors are involved in stimulating cortisol secretion during the acute phase of SW transfer. Although not observed in every species (39), CRF-related peptides can directly stimulate cortisol secretion from the interrenal tissue in some fish, including rainbow trout (23, 24). Given the rapid increase in CNSS CRF and UI expression after SW transfer, there is a possibility that CRF-related peptides of CNSS origin directly contribute to the regulation of cortisol secretion during a hyperosmotic challenge. Similarly, angiotensin II can directly stimulate interrenal secretion of cortisol in rainbow trout (23), and SW transfer is associated with an activation of the renin-angiotensin system in this species (52).

Perspectives

Although discussed separately, there is physiological and anatomical evidence suggesting that the CRF and UI neurons of the forebrain and the CNSS are not independent from one another. For example, relative to sham-operated fish, excision of the CNSS, or urophysectomy, results in a significant elevation in hypothalamic immunoreactive UI in goldfish (53) and white sucker (*Catostomus commersoni*) (54). Moreover, the CNSS receives extensive and diverse descending projections from different levels of the brain stem, and some of these brain stem nuclei are known to send ascending fibers to the hypothalamus (17, 55). Therefore, there is a possibility

that brain stem nuclei are involved in coordinating the cranial and caudal responses of the CRF system in fish and that the response from one end of the central nervous system to SW transfer is not independent from the other.

We have examined the effects of an environmental stressor on the expression of CRF-related peptides within the brain and the CNSS of a fish. The differential response of the CRF-related peptide-expressing neurons of the preoptic area, the hypothalamus, and the CNSS in response to SW transfer in rainbow trout provide a novel outlook on the potential roles of the CRF system. In general, our results suggest that forebrain CRF and UI may both be involved in fluid homeostasis. Similarly, the chronic increases in Dahlgren cells CRF and UI expression after SW transfer are consistent with the previously proposed involvement of the CNSS in osmoregulatory functions (17). Finally, although several factors are involved in controlling glucocorticoid secretion (23) and food intake (56) in fish, and future studies are needed to provide more direct evidence, our results implicate forebrain and CNSS CRF-related peptides in the regulation of cortisol secretion after SW transfer and as potential mediators of the anorexigenic response.

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