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RESEARCH ARTICLE

CRF and urotensin I effects on aggression and anxiety-like behavior in rainbow trout

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SUMMARY

Corticotropin-releasing factor (CRF) is central in the stress response but also modulates several behaviors including anxiety-related behaviors and aggression. In this study, juvenile rainbow trout (*Oncorhynchus mykiss*) were tested for competitive ability, determined during dyadic fights for dominance, after intracerebroventricular (i.c.v.) administration of CRF, urotensin I (UI), the non-specific CRF antagonist α-helical RF_{9-41} (ahCRF) or the CRF receptor subtype 1-specific antagonist antalarmin, when paired with a mass-matched con-specific injected with saline. In addition, isolated fish received the same substances. Plasma cortisol and brain monoamines were monitored in all fish. Most fish receiving CRF showed a conspicuous behavior consisting of flaring the opercula, opening the mouth and violent shaking of the head from side to side. When this occurred, the fish immediately forfeited the fight. Similar behavior was observed in most fish receiving UI but no effect on outcome of dyadic fights was noted. This behavior seems similar to non-ambulatory motor activity seen in rats and could be anxiety related. Furthermore, fish receiving CRF at a dose of 1000 ng became subordinate, whereas all other treatments had no effects on the outcome of dyadic fights. In addition, isolated fish receiving ahCRF had lower brain stem concentrations of 5-hydroxyindoleacetic acid, serotonin, 3,4-dihydroxyphenylacetic acid and dopamine. In conclusion, CRF seems to attenuate competitive ability, and both CRF and UI seem to induce anxiety-like behavior.

Key words: aggression, anxiety, corticotropin releasing factor, cortisol, dopamine, dyadic fight, monoamine, urotensin I, UI, serotonin, stress.

INTRODUCTION

Aggression and stress appear to be linked by different but cooperative neuroendocrine circuitries (Summers and Winberg, 2006). Agonistic interactions are stressful, and previous studies have found that the brain monoaminergic systems, especially serotonin (5-hydroxytryptamine, 5-HT), play a key role in controlling and integrating aggressive behavior and stress responses during agonistic interactions (Summers and Winberg, 2006). Another factor that is central in the control of stress responses and that seems to interact with the serotonergic system is the neuropeptide corticotropin releasing factor (CRF). CRF belongs to the CRF family of neuropeptides, which includes urocortin I–III in mammals, sauvagine in anurans and urotensin I (UI) in teleosts (for reviews, see Fekete and Zorilla, 2007; Chang and Hsu, 2004). In addition to its hypophysiotrophic role (Fryer et al., 1983; Vale et al., 1981), CRF has also been reported to act as a neuromodulator within the central nervous system of various vertebrates (for a review, see Lowry and Moore, 2006), including teleost fish (e.g. Bernier, 2006; Carpenter et al., 2007; Clements et al., 2002; De Pedro et al., 1993). For instance, CRF has been reported to exert modulatory effects on neural activity (Lowry et al., 2000) and neurotransmitter function (Price and Lucki, 2001; Summers et al., 2003), and to play an important role in behavioral stress responses, being linked to fear and anxiety (Takahashi, 2001).

A stimulatory role of CRF on behavioral arousal and locomotor activity appears to have been conserved throughout the vertebrate subphylum (Lowry and Moore, 2006). This effect of CRF seems to be centrally mediated and not dependent on adrenal and interrenal cortisol secretion (Lowry and Moore, 2006). For instance, intracerebroventricular (i.c.v.) injections of CRF stimulate locomotor activity in chinook salmon (*Oncorhynchus tshawytscha* Walbaum) (Clements and Schreck, 2004; Clements et al., 2002; Clements et al., 2003) and rainbow trout (*Oncorhynchus mykiss* Walbaum) (Carpenter et al., 2007). In addition, CRF has been suggested to be involved in anxiety-related behaviors in mammals (Keck, 2006), and recently Carpenter et al. presented results suggesting that CRF may have a similar anxiogenic function in rainbow trout (Carpenter et al., 2007). In addition to its effects on motor activity CRF has also been reported to have an anorexic effect in various vertebrates (Lowry and Moore, 2006) including teleost fish (Bernier and Peter, 2001). Furthermore, in rats, central administration of exogenous CRF at lower doses induced aggression, whereas at higher doses aggression returned to control levels (Elkabir et al., 1990).

The CRF-related peptides also produce several similar effects to CRF. For instance, urocortin I and II have anxiogenic-like effects and attenuate feeding (Koob and Heinrichs, 1999; Pelleymounter et al., 2004), and urocortin III also attenuates feeding (Pelleymounter et al., 2004). The teleost UI, which is closely related to urocortin I (Fekete and Zorrilla, 2007), is involved in the control of cardiovascular activity and it increases dorsal aortic pressure after i.c.v. injection (Le Mevel et al., 2006). In addition, UI has been reported to attenuate feeding (Bernier and Peter, 2001).

In mammals, the effects of CRF and CRF-related peptides are mediated through two CRF receptor subtypes, CRF type I (CRF-R1) and CRF type II (CRF-R2) receptors (Bale and Vale, 2004). CRF-R1 and CRF-R2 have also been characterized in fish, and in fish an additional CRF receptor, CRF-R3, has also been described (Arai et al., 2001; Pohl et al., 2001).

The objective of this study was to investigate the involvement of the CRF system in competitive ability and stress responses. We report the effects of i.c.v. injections of CRF, UI, the nonselective CRF receptor antagonist α-helical CRF_{9-41} (ahCRF) and the CRF-R1 receptor antagonist antalarmin (Ant) (Heinrichs and Koob, 2004) on agonistic behavior and the outcome of dyadic fights for social dominance in juvenile rainbow trout. In addition, brain serotonergic and dopaminergic activities and plasma levels of cortisol were quantified to monitor interactions between CRF/UI and 5-HT, dopamine (DA) and hypothalamic–pituitary–interrenal (HPI) axis activity.

MATERIALS AND METHODS

Experimental animals

The experiments were performed on juvenile rainbow trout with a mass of 98.50±26.97 g (mean ± s.d., *N*=323). For at least 1 week before the experiments, the fish were kept indoors in a 1 m^3 holding tank at the Evolutionary Biology Centre, Uppsala University, Sweden, at a rearing density of approximately 0.02 $kg\,l^{-1}$, to minimize social interactions. The holding tank was continuously supplied with aerated (>90% O_2 saturation) Uppsala tap water (pH 7.6, HCO_3^- 5.2 $mmol\,l^{-1}$, Ca^{2+} 2.8 $mmol\,l^{-1}$, Mg^{2+} 0.4 $mmol\,l^{-1}$) at 8–11°C and the light:dark regime was continuously and automatically adjusted to latitude 51°N conditions. Fish were hand-fed with commercial trout pellets (EWOS ST40, Ewos AS, Bergen, Norway) at 1–2% of body mass per day.

Experimental protocol

Experiment 1

At the start of the experiment, fish, randomly selected from the holding tank, were lightly anesthetized with ethyl 4-aminobenzoate (0.25 $g\,l^{-1}$), mass-matched in pairs (deviations in mass within pairs were less than 5%) and tagged by a small cut in the caudal fin, either dorsally or ventrally. The fish were then transferred to social isolation in individual compartments of the experimental aquaria, keeping mass-matched pairs in neighboring compartments. The experimental aquaria (250 l) were divided into four equal-sized 62.5 l compartments (250×500×500 mm) using removable dark PVC walls. The aquaria were continuously supplied with aerated (oxygen saturation >90%) Uppsala tap water (0.8 $l\,min^{-1}$, 8–11°C), The light:dark regimen was 12 h:12 h (lights on at 08:00 h and lights off at 20:00 h) with the light provided by a 30 W Lumilux (Osram, Augsburg, Germany) daylight fluorescent tube placed 40 cm above the water surface of each aquarium. The fish were allowed to acclimate for at least 1 week during which they were hand-fed commercial trout pellets (EWOS ST40). At the end of the acclimation period all individuals consumed a number of pellets corresponding to 1% of their body mass.

Following the acclimation period, the behavioral experiment started by anesthetizing the fish with ethyl 4-aminobenzoate (as described above) and administering CRF, ahCRF or saline to the fish by an i.c.v. injection (described below), one fish in each pair receiving an injection of CRF or ahCRF and the other an injection of saline. The fish injected with saline in each pair were used as a control group. Following injection the fish were returned to their home compartments and were allowed to recover in isolation for 20 min. After this recovery period, the partitions separating the sized matched pairs of fish were removed and the fish were allowed to interact for 60 min. During this time they were filmed for later behavioral analysis. In all pairs, a clear dominant–subordinate relationship was established, with one fish being dominant and the other subordinate, as described by Øverli et al. (Øverli et al., 1999). At the end of the 60-min interaction period the social position of the fish (dominant or subordinate) was noted and the fish were sacrificed and samples of blood plasma and brain tissue were taken. Isolated fish were handled the same way, but were kept isolated for 60 min after the recovery period. The experiment was carried out during spring and autumn 2005.

Experiment 2

The protocol was identical to that described for experiment 1 except that the active substances were UI and the CRF antagonist Ant. The recovery period after i.c.v. injections was 30 min. The experiment was carried out during spring 2006.

The methodology of this study was approved by Uppsala Animal Research Ethical Committee.

Intracerebroventricular injection

Anesthetized (as described above) fish were immobilized in a holder and either an active substance (CRF, ahCRF, UI or Ant) or 0.9% saline was administrated directly into the third ventricle as described by Jönsson et al. (Jönsson et al., 2003; Jönsson et al., 2010). Briefly, the injection site, at an angle of approximately 30 deg, was in the midline at the posterior end of the two cranial dark areas corresponding to the two optic tecta and the base of the eyes. Injections were performed with a 30-gauge Microlance (VWR, Stockholm, Sweden) needle coupled to a 10-μl Hamilton (Bonaduz, Switzerland) syringe with a 20 PE cannula. The needle had a stop at 6 mm from the tip to reach the intended depth. The injection method was first validated, using dye injections and subsequent location of the dye in the fish brain. Later, the method was further validated by observing the behavior of fish after injection of saline. The method was found to have a success rate of ~90%. It was also noted that if the injection failed, the fish displayed typical behavioral disturbances and bleeding in the brain, and if this occurred during experiments the fish was killed and the results excluded from further analyses. The injection volume was 1 μl and the active substances ovine CRF (Sigma C3167, Stockholm, Sweden), ahCRF (Sigma C2917) and Ant (Sigma A8727) were dissolved in 0.9% saline. A stock solution of white sucker UI (Sigma U7253) was first dissolved in dimethyl sulfoxide (DMSO) and then further dissolved in 0.9% saline (1:8 and 1:16, respectively). In experiment 2, fish used as controls in experiments with UI received saline with the same DMSO concentration as the UI solution. All of these active substances were administrated in two different concentrations, 500 and 1000 $\mu g\,ml^{-1}$ for CRF (11 and 21 picomol, approximately 5 and 10 $ng\,g^{-1}$ body mass), 1000 and 2000 $\mu g\,ml^{-1}$ for ahCRF (26 and 52 picomol, approximately 10 and 20 $ng\,g^{-1}$ body mass), 62.5 and 125 $\mu g\,ml^{-1}$ for UI (1 and 3 picomol, approximately 0.6 and 1.3 $ng\,g^{-1}$ body mass), and 400 and 2000 $\mu g\,ml^{-1}$ for Ant (96 and 482 picomol, approximately 4 and 20 $ng\,g^{-1}$ body mass), respectively. Similar doses have previously been used for i.c.v. administration of CRF and ahCRF (Clements et al., 2002) and UI (Mimassi et al., 2000) in teleosts. The doses of Ant were based on doses used in studies on mammals (Habib et al., 2000). Ant has been used before (Lastein et al., 2008) but its specificity as a CRF-R1 antagonist in teleosts was not determined. Batches of CRF, ahCRF, UI and Ant were prepared once, divided into aliquots and kept frozen at –20°C until use.

Blood and tissue sampling

At the end of the 60-min interaction period the fish were netted and killed using ethyl 4-aminobenzoate (0.5 g l^{-1}). The fish was weighed and blood was collected through the caudal vasculature with a heparinized syringe. The blood was subsequently spun at 16,000 ***g*** for 10 min at 4°C and the plasma collected and stored at –80°C for later analysis. Thereafter, the spinal cord was cut and the brain stem removed. The brain stem was wrapped in aluminum foil, frozen in liquid nitrogen and stored at –80°C for later analysis.

Assays

Plasma was analyzed for cortisol using a commercial enzyme-linked immunosorbent assay (ELISA) kit (product no. 402710, Neogen Corporation, Lexington, KY, USA).

Tissue levels of 5-HT and the 5-HT metabolite, 5-hydroxyindoleacetic acid (5-HIAA), DA and the DA metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) were analyzed in brainstem samples using high-performance liquid chromatography with electrochemical detection (HPLC-EC) as described by Höglund et al. (Höglund et al., 2000). The ratios of 5-HIAA/5-HT concentration and DOPAC/DA concentration were calculated and used as an index of serotonergic and dopaminergic activity, respectively.

Behavioral observations

In our analysis of the dyadic agonistic interactions we recorded: (1) latency to first attack; (2) the individual performing the first attack; (3) the time until the dominant–subordinate relationship was established; (4) the number of aggressive acts performed before the dominant–subordinate relationship was settled (as well as number of aggressive acts per minute); (5) the number of aggressive acts performed by dominant individual after the dominant–subordinate relationship was settled (as well as number of aggressive acts per minute); and (6) the social rank obtained (the outcome of the fight for dominance).

Statistical analyses

Data presented are means ± s.e.m. if not stated otherwise. Physiological data (brainstem levels of 5-HT, 5-HIAA, DOPAC, DA and the 5-HIAA/5-HT and DOPAC/DA ratios and plasma concentrations of cortisol) of each experiment were first processed by a multivariate analysis of variance (MANOVA). If significant (P<0.05) effects were indicated by MANOVA, ANOVA and Tukey's *post hoc* analysis were also used. In some cases the data were transformed in order to fulfill the assumption of normal distribution.

Behavioral data (latency to first attack, time to establish the dominant–subordinate relationship, the number of aggressive acts performed before and following settlement of the dominant–subordinate relationship) of each experiment was analyzed by a two-way analysis of variance (two-way ANOVA) with social rank (dominant *vs* subordinate) and treatment as independent variables. A χ^2-test was used to analyze data on the effects of CRF, ahCRF, UI, Ant and saline on performing the first attack and the outcome of fights for social dominance. All statistics were processed with SYSTAT 8.0 software (Systat Systems Inc., Point Richmond, CA, USA).

RESULTS

In some cases food intake and growth during the acclimation period differed between pair members. If the deviation in mass between pair members was larger than 10% at the end of the experiment their data were excluded from further analysis. Mass and gender were included in the MANOVA and had no significant effects on any of the parameters analyzed.

Behavioral effects

In both experiments 1 and 2 agonistic interactions began with fish circling each other. After a variable amount of time, one of the fish attacked (an attack being a rapid approach followed by physical contact) the other fish. The time elapsed until this occurred was termed latency to first attack. Both fish continued with aggressive acts, usually taking turns attacking, for some time until one fish forfeited the fight for dominance. After this, the forfeiting fish was clearly subordinate and did not retaliate any attacks from the other pair member, the dominant fish. The dominant fish, however, continued attacking the subordinate fish even after the dominant–subordinate relationship was settled, although the frequency of aggressive acts declined. Furthermore, some of the fish given CRF or UI by i.c.v. injection displayed a stereotypic behavior consisting of flaring the opercula, opening the mouth and violent shaking of the head from side to side during dyadic interactions. When this head-shaking behavior was shown in fish receiving CRF (20 out of 27 individuals for both doses), they immediately lost the fight for social dominance and became subordinate. In these fish the head-shaking behavior occurred at 6±2 min after initiating dyadic interactions, i.e. 26±2 min after i.c.v. injection of CRF, and lasted a few seconds. Similarly, in fish treated with 62.5 ng UI (value after injection of 1 µl), 11 out of 16 fish showed the head-shaking behavior and of these, eight became subordinate and three dominant. However, in fish treated with 125 ng UI, 16 out of 18 fish showed the head-shaking behavior and of these, nine became subordinate and seven dominant. Therefore, in UI-treated fish, head-shaking behavior did not appear to have any significant effect on the outcome of dyadic fights for social dominance. The head-shaking behavior in UI-treated fish occurred at 21±5 and at 22±4 min after initiation of dyadic fights in fish receiving 62.5 and 125 ng UI, respectively, i.e. 51±5 and at 52±4 min after receiving UI. In contrast to CRF-treated fish, the UI-treated fish often showed the head-shaking behavior after the fight for social dominance was settled. In fish becoming subordinate, 14 out of 17 showed head shaking after dominance was established and in fish becoming dominant, 10 out of 10 showed head shaking after dominance was established.

Effects of CRF and ahCRF on dyadic fights for social dominance (experiment 1)

Treatment with CRF had dose-dependent effects on the outcome of dyadic fights for social dominance (Table 1). Fish receiving CRF at a dose of 1000 ng became subordinate significantly (P=0.008)

Table 1. The effect of CRF, UI, ahCRF and Ant on the outcome fights for social dominance in size-matched pairs of juvenile rainbow trout

Treatment	*N*	Dominant fish	Subordinate fish	*P*-value*
500 ng CRF	13	5	8	0.405
1000 ng CRF	14	2	12	0.008
1000 ng ahCRF	13	9	4	0.166
2000 ng ahCRF	16	6	10	0.317
62.5 ng UI	16	6	10	0.317
125 ng UI	18	8	10	0.363
400 ng Ant	6	2	4	0.414
2000 ng Ant	12	7	5	0.436

Ant, antalarmin; AhCRF, α-helical CRF_{9-41}; CRF, corticotropin releasing factor; UI, urotensin I.

*χ^2 analysis.

One fish in each pair received an intracerebroventricular injection of active substance (CRF, UI, ahCRF or Ant) whereas the other pair member received a similar injection of saline.

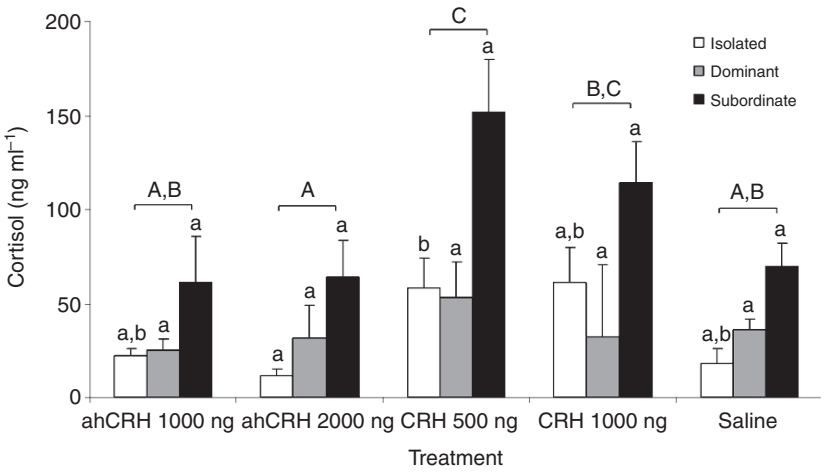

Fig. 1. Plasma cortisol concentrations in socially interacting and isolated juvenile rainbow trout given intracerebroventricular (i.c.v.) injections of CRF, ahCRF or saline (experiment 1). One fish in each interacting pair received an i.c.v. injection of active substance, CRF or ahCRF, whereas the other pair member received a similar injection of saline. Isolated trout received i.c.v. injections of CRF, ahCRF or saline. Values are means ± s.e.m. Different letters above the bars indicate significant differences (P<0.05, Tukey's *post hoc* analysis): capital letters indicate differences between overall treatments, whereas lowercase letters indicate differences within social ranks between the different treatments.

more often than their saline-injected opponents, whereas at a dose of 500 ng CRF had no significant effect on the outcome of fights for dominance (P=0.405). There was no significant effect of ahCRF on the outcome of dyadic fights for social dominance, either at a dose of 1000 ng or at 2000 ng.

There were no significant effects of treatment on attack latency, time until dominance or frequency of aggressive acts before and following establishment of the dominant–subordinate relationships. However, there was a significant interaction effect of treatment and social rank on time until settlement of the dominant–subordinate relationship ($F_{4,98}$=3.267, P=0.015) but no clear *post hoc* effects (data not shown).

Effects of UI and Ant on dyadic fights for social dominance (experiment 2)

Neither UI nor Ant had a significant effect on the outcome of dyadic fights for social dominance at any of the doses tested (Table 1).

There were no significant effects of treatment on attack latency, time until dominance or frequency of aggressive acts before and following establishment of the dominant–subordinate relationships. Neither were there any significant interaction effects of treatment and social rank (data not shown).

Physiological effects

Experiment 1

As expected, treatment had an overall effect on plasma cortisol levels, i.e. when including data from both interacting and isolated fish (MANOVA, $F_{4,130}$=781.646, P<0.001; Fig. 1). This effect was mainly due to an effect of treatment in isolated fish (MANOVA, $F_{4,42}$=3.185, P=0.023; Fig. 1), with isolated fish receiving CRF showing elevated plasma cortisol concentrations even though the *post hoc* test did not reveal any significant difference between CRF-treated, isolated fish and controls (Fig. 1). Furthermore, social interaction had a significant effect on plasma concentrations of cortisol (MANOVA, $F_{2,156}$=16.788, P<0.001; Table 2), subordinate individuals showing significantly higher plasma cortisol levels than both isolated and dominant fish (Tukey's *post hoc*, P<0.001). However, plasma cortisol concentrations of dominant and isolated fish did not differ (Table 2).

There were significant overall effects of injections on brain stem monoamines (Table 3). Specifically, injections had significant effects on brain stem DOPAC (MANOVA, $F_{4,130}$=3.530, P=0.009), DA (MANOVA, $F_{4,130}$=5.647, P<0.001) and 5-HT (MANOVA, $F_{4,130}$=10.342, P<0.001) concentrations, and 5-HIAA/5-HT ratios (MANOVA, $F_{4,130}$=6.307, P<0.001). However, there were no significant effects of injections on brain stem concentrations of 5-HIAA or DOPAC/DA ratios. The *post hoc* analyses showed that fish receiving ahCRF at a dose of 2000 ng had significantly lower concentrations of DA (Tukey's *post hoc*, P<0.013) and 5-HT in the brain stem (Tukey's *post hoc*, P<0.005) than fish receiving other treatments (Table 3). Also, brain stem concentrations of DOPAC were significantly lower in fish receiving 2000 ng ahCRF than in fish receiving 500 ng CRF (Tukey's *post hoc*, P=0.043) or 1000 ng ahCRF (Tukey's *post hoc*, P=0.023). Brain stem 5-HIAA/5-HT ratios were significantly higher in fish receiving 2000 ng ahCRF than in fish given 1000 ng ahCRF (Tukey's *post hoc*, P=0.012) or saline (Tukey's *post hoc*, P<0.001).

Furthermore, dyadic interactions also had effects on brain stem monoamines (Table 4) and significant effects were detected on DOPAC (MANOVA, $F_{2,130}$=3.251, P=0.042), DA (MANOVA, $F_{2,130}$=19.963, P<0.001) and 5-HT (MANOVA, $F_{2,130}$=42.665, P<0.001) concentrations, as well as on 5-HIAA/5-HT ratios (MANOVA, $F_{2,130}$=39.760, P<0.001). The *post hoc* analyses revealed that brain stem concentrations of DOPAC (Tukey's *post hoc*, P<0.05), DA (Tukey's *post hoc*, P<0.001) and 5-HT (Tukey's *post hoc*, P<0.001) were higher in socially interacting than in isolated fish, and that brain stem 5-HIAA/5-HT ratios were lower (Tukey's *post hoc*, P<0.001) in interacting than isolated fish (Table 4).

Table 2. Plasma cortisol concentrations (ng ml^{-1}) in socially interacting and isolated juvenile rainbow trout

	Rank	Cortisol	*N*
Experiment 1			
	Isolated	33±6^{a}	47
	Dominant	35±4^{a}	56
	Subordinate	89±9^{b}	56
Experiment 2			
	Isolated	39±4^{a,b}	60
	Dominant	33±3^{a}	52
	Subordinate	53±5^{b}	52

Values are means ± s.e.m.

One fish in each interacting pair received an intracerebroventricular (icv) injection of active substance (CRF, UI, ahCRF or Ant), whereas the other pair member received a similar injection of saline. Isolated trout received icv injections of CRF, UI, ahCRF, Ant or saline.

Different superscript letters indicate differences between social ranks (P<0.05, Tukey's *post hoc* analysis).

Table 3. Brainstem concentration of serotonin (5-HT), dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC) and 5-hydroxyindoleacetic acid (5-HIAA), and 5-HIAA/5-HT and DOPAC/DA ratios in socially interacting and isolated juvenile rainbow trout receiving intracerebroventricular injection of CRF, ahCRF or saline

Treatment	Rank	[DOPAC]*	[DA]* (*N*)	[DOPAC]/[DA] $\times10^{-3}$ (*N*)	[5-HIAA]* (*N*)	[5-HT]* (*N*)	[5-HIAA]/[5-HT] $\times10^{-3}$ (*N*)
1000 ng ahCRF							
	Isolated	39±34 (10)	298±100 (10)	61±27 (10)	57±10 (10)	113±13 (10)	507±68 (10)
	Dominant	26±4 (9)^{a}	447±68 (9)^{a}	69±18 (9)^{a}	54±8 (9)^{a}	491±57 (9)^{a}	121±22 (9)^{a}
	Subordinate	20±8 (4)	449±119 (4)	56±25 (4)	63±4 (4)	478±55 (4)	137±26 (4)
2000 ng ahCRF							
	Isolated	6±0 (10)	181±15 (10)	33±3 (10)	47±3 (10)	93±5 (10)	510±32 (10)
	Dominant	6±0 (6)^{b}	226±27 (6)^{b}	25±2 (6)^{a}	40±4 (6)^{b}	122±23 (6)^{b}	384±82 (6)^{b}
	Subordinate	8±1 (10)	219±23 (10)	38±5 (10)	43±4 (10)	113±16 (10)	435±60 (10)
500 ng CRF							
	Isolated	8±1 (9)	217±30 (9)	37±4 (9)	53±7 (9)	117±21 (9)	477±39 (9)
	Dominant	20±5 (5)^{a}	360±66 (5)^{a}	64±19 (5)^{a}	39±7 (5)^{a}	340±49 (5)^{a}	128±15 (5)^{a,b}
	Subordinate	27±3 (8)	482±55 (8)	60±8 (8)	42±8 (8)	401±39 (8)	120±12 (8)
1000 ng CRF							
	Isolated	6±1 (8)	196±24 (8)	34±3 (8)	48±5 (8)	91±16 (8)	627±127 (8)
	Dominant	4±3 (2)^{a,b}	454±183 (2)^{a}	10±10 (2)^{a}	62±28 (2)^{a}	261±60 (2)^{a}	233±54 (2)^{a,b}
	Subordinate	22±8 (12)	450±54 (12)	43±10 (12)	69±6 (12)	622±199 (12)	162±24 (12)
Saline							
	Isolated	8±1 (10)	223±23 (10)	37±6 (10)	49±5 (10)	120±20 (10)	460±54 (10)
	Dominant	27±9 (33)^{a,b}	392±41 (33)^{a}	68±22 (33)^{a}	77±20 (33)^{a}	358±43 (33)^{a}	268±36 (33)^{a}
	Subordinate	16±2 (22)	342±40 (22)	52±8 (22)	52±5 (22)	342±41 (22)	228±43 (22)

Ant, antalarmin; AhCRF, α-helical CRF_{9-41}; CRF, UI, urotensin I.
*Concentrations are in ng g^{-1}.
Values are means ± s.e.m.
One fish in each interacting pair received an intracerebroventricular (icv) injection of active substance, CRF or ahCRF, whereas the other pair member received a similar injection of saline. Isolated trout received icv injections of corticotropin releasing factor (CRF), α-helical CRF_{9-41} (ahCRF) or saline. Different superscript letters indicate differences between injections (P<0.05, Tukey's *post hoc* analysis).

There were also significant combined effects of injections and dyadic interactions on brain stem monoamines. Such effects were seen on the brain stem 5-HT concentration (MANOVA, $F_{8,130}$=3.575, P=0.001) and 5-HIAA/5-HT ratios (MANOVA, $F_{8,130}$=2.444, P=0.017). Injections did not have any significant effect on brain stem 5-HT concentrations in isolated fish, but socially interacting fish receiving ahCRF at a dose of 2000 ng showed lower brain stem 5-HT levels than socially interacting fish subjected to other treatments. Similarly, in isolated fish, injections had no effect on brain stem 5-HIAA/5-HT ratios, whereas socially interacting fish receiving ahCRF at a dose of 2000 ng showed higher brain stem 5-HIAA/5-HT ratios than socially interacting fish subjected to all other injections.

Experiment 2

Treatment had no significant effect on plasma cortisol levels (data not shown), but there was a significant effect of dyadic interactions (MANOVA, $F_{1,141}$=2005.775, P<0.001; Table 2). As expected, subordinate fish showed higher levels of plasma cortisol than dominant fish (Tukey's *post hoc*, P=0.006). There were, however, no significant differences in plasma cortisol concentrations between isolated fish and dominant or subordinate fish.

There was no significant combined effect of treatment and dyadic interactions.

Owing to technical problems we were not able to quantify monoamine and monoamine metabolite concentrations in brain stem samples from experiment 2.

DISCUSSION

The results of the present study show that juvenile rainbow trout treated with CRF lost staged fights for social dominance with a size matched opponent. This effect that could be related to a CRF-induced inhibition of aggressive behavior but also to an anxiogenic effect of CRF. By contrast, treatment with UI had no effect on the outcome of fights for social dominance, even though it appeared to induce anxiety-like behavior in the trout.

In rainbow trout as well as in other vertebrates, social subordination results in a general behavioral inhibition, including suppression of aggressive behavior, foraging and appetite (Blanchard

Table 4. Brainstem concentration of serotonin (5-HT), dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC) and 5-hydroxyindoleacetic acid (5-HIAA), and 5-HIAA/5-HT and DOPAC/DA ratios in socially interacting and isolated juvenile rainbow trout

Rank	[DOPAC]*	[DA]*	[DOPAC]/[DA] $\times10^{-3}$	[5-HIAA]*	[5-HT]*	[5-HIAA]/[5-HT] $\times10^{-3}$	*N*
Isolated	13±7^{a}	224±22^{a}	41±6^{a}	51±3^{a}	107±7^{a}	512±28^{a}	47
Dominant	22±5^{b}	383±28^{b}	59±13^{a}	61±11^{a}	349±30^{b}	230±25^{b}	56
Subordinate	17±2^{b}	371±24^{b}	46±4^{a}	49±3^{a}	379±48^{b}	218±25^{b}	56

*Concentrations are in ng g^{-1}.
Values are means ± s.e.m.
One fish in each interacting pair received an intracerebroventricular (icv) injection of active substance, corticotropin releasing factor (CRF) or α-helical CRF_{9-41} (ahCRF), whereas the other pair member received a similar injection of saline. Isolated trout received icv injections of CRF, ahCRF or saline. Different superscript letters indicate differences between social ranks (P<0.05, Tukey's *post hoc* analysis).

et al., 1993; Johnsson et al., 2005; Summers and Winberg, 2006). These behavioral effects are induced by the chronic social stress experienced by subordinate animals and are probably mediated by factors involved in the neuroendocrine stress response (Johnsson et al., 2005; Summers and Winberg, 2006). Subordinate animals are characterized by chronically elevated plasma levels of glucocorticoids along with elevated brain serotonergic activity (Blanchard et al., 1993; Johnsson et al., 2005; Summers and Winberg, 2006). Furthermore, social subordination induces anxiety in mammals (Arregi et al., 2006) and rats have been shown to have elevated expression of CRF mRNA in the paraventricular nucleus (Albeck et al., 1997). Social subordination results in a similar increase in the expression of CRF mRNA in the preoptic area (POA) of rainbow trout (Doyon et al., 2003), suggesting that CRF may be important in mediating the behavioral effects of social subordination. This suggestion is supported by the results of the present study showing that trout receiving 1000 ng CRF lost dyadic fights for social dominance. Exogenous CRF has been reported to reduce aggression in rodents (Gammie et al., 2004; Mele et al., 1987). However, there are also results suggesting that exogenous CRF administrated into the amygdala at low doses increase aggression in rats (Elkabir et al., 1990), and that the CRF-R1 antagonist, SSR12543A, reduces aggression in golden hamsters (*Mesocricetus auratus* Waterhouse) (Farrokhi et al., 2004). In teleost fish, previous results of the effects of treatment with CRF on aggression were unclear, with a reduced number of attacks but also decreased latency to attack (Carpenter et al., 2009).

The observation that trout receiving CRF lost fights for social dominance might also be related to anxiogenic effects of CRF. Both CRF and UI, at low as well as high doses, induced head-shaking behavior. A similar behavioral effect of i.c.v. CRF in rainbow trout was reported by Carpenter et al. in a study using the same doses of CRF and a very similar experimental design (Carpenter et al., 2007). In rats, i.c.v. injections of CRF induce both ambulatory and non-ambulatory motor activity (Lowry and Moore, 2006). The non-ambulatory motor activity, which is especially apparent when animals are tested in their home environment, consisted of head movements, non-ambulatory limb movements and shifts in body position (Butler et al., 1990). The CRF-induced elevation of non-ambulatory motor activity has been suggested to represent an increase in anxiety state and risk assessment behaviors (Blanchard and Blanchard, 1989). Interestingly, the CRF-induced head-shaking behavior in rainbow trout observed in the present study and by Carpenter et al. appears similar to the effects of CRF on non-ambulatory motor activity in rats (Carpenter et al., 2007). Therefore, the head-shaking behavior of rainbow trout could be analogous to the anxiety-related elevation of non-ambulatory motor activity in rats, and it may reflect an anxiogenic effect of CRF in fish also.

Interestingly, UI also induced the head-shaking behavior even though UI had no effect on the outcome of fights for social dominance. There were also some differences in performance of the head shake between CRF- and UI-treated fish. In trout receiving CRF, at both doses, all the fish showing the head shake behavior immediately lost the dyadic fight and became subordinate, and after that they never showed a second bout of head shaking. In contrast, in UI-treated fish the head-shaking behavior was observed both in fish losing and in fish winning fights for social dominance. Thus, it seems as if UI has a stronger head-shake-inducing effect than CRF, and that the suppression of competitive ability (aggression) and induction of head-shaking behavior are mediated by different mechanisms. Other investigations have indicated that the CRF1 receptor subtype has anxiogenic effects. For instance, knockdown of CRF-R1 had an anxiolytic-like effect in rats (Heinrichs et al., 1997), whereas CRF-R2 knockout mice showed anxiety-like behavior (Bale et al., 2000). However, some studies indicate that CRF-R2 is involved as well. For instance, treatment with urocortin II, a CRF-R2 agonist, in mice induced anxiogenic behavior in an elevated plus maze (Pelleymounter et al., 2004). In addition, urocortin III, another CRF-R2 agonist, seems to induce anxiety-related behavior in rats (Zhao et al., 2007). These results indicate that CRF-R2 is also involved in mediating anxiogenic effects. In the present study, anxiety-like behavior was induced by both CRF and UI. This makes it tempting to suggest that CRF-R1 mechanisms mediated CRF-induced effects on aggression, whereas the head-shaking behavior was induced by activation of CRF-R2. In the present study CRF was used at higher doses than UI. Consequently, CRF may have interacted with both CRF-R1 and CRF-R2 receptors, whereas UI, which was administrated at lower doses, interacted preferentially with CRF-R2 receptors. These results are also in agreement with the divergent affinities of the CRF-R1 and CRF-R2 receptors for CRF and urocortin 1 in mammals (Lovejoy and Balment, 1999). However, a similar divergence in affinity for CRF and UI might not be present in teleost CRF receptors (Arai et al., 2001; Pohl et al., 2001). Moreover, the interpretation of the results is further complicated by the use of non-specific peptides in the present study. Ovine CRF has been used previously and was found to effect behavior (Carpenter et al., 2007), whereas white sucker UI has, to our knowledge, not been used previously in rainbow trout. White sucker UI shares 37 out of 41 amino acids with rainbow trout UI and could in fact be more potent than ovine CRF, only sharing 27 out of 41 amino acids with rainbow trout UI. Thus, this potency difference could in part compensate for the different doses of CRF and UI applied.

A neuromodulatory role has been suggested for CRF. Thus, instead of having a direct effect on specific motor patterns CRF may adjust behavioral responses induced by environmental cues (Lowry and Moore, 2006). Such a modulatory role of CRF could be mediated at least in part *via* effects on brainstem neuromodulatory systems, such as the dopaminergic and serotonergic systems. It is well known that CRF has a modulatory effect on the serotonergic system in mammals (Price et al., 2002; Price and Lucki, 2001). For instance, CRF administrated into the raphe nuclei has been shown to modulated 5-HT release in rats (Price and Lucki, 2001). CRF also modulates 5-HT release during swimming stress in rats (Price et al., 2002). An interaction between CRF and the brain 5-HT system has been reported also in teleosts. Clements et al. showed that fluoxetine (a selective 5-HT reuptake inhibitor) potentiated the CRF-induced stimulation of locomotor activity, whereas the 5-HT_{1A} receptor antagonist, NAN190, had the opposite effect, reducing CRF-induced effects on locomotion in Chinook salmon (Clements et al., 2003). The suggestion that the effects of CRF on locomotor activity are mediated through interactions with serotonergic and dopaminergic systems was further supported by a study by Carpenter et al. (Carpenter et al., 2007). They found that an i.c.v. dose of 2000 ng CRF stimulated locomotion and at the same time increased serotonergic and dopaminergic activity in amygdalo-striatal pallium, POA and raphe nuclei of rainbow trout. There was also a positive correlations between the dose of CRF and DA, 5-HT and 5-HIAA levels, and positive correlations between the concentration of these monoamines and locomotion.

In the present study we could not detect any effects of CRF on monoamine or monoamine metabolite concentrations. There was, however, an effect of ahCRF. Fish receiving the high dose of the non-selective CRF receptor antagonist, ahCRF, showed lower brain

stem concentrations of 5-HT and DA as well as the metabolites 5-HIAA and DOPAC. Similar results, with CRF antagonists reducing 5-HT, have been reported in nucleus accumbens of rats (Lukkes et al., 2008). Furthermore, it seems that there was an interaction effect of social interaction and treatment, hence the effects of ahCRF on 5-HT and 5-HIAA/5-HT ratios seemed to be related to effects in socially interacting fish. During dyadic fights for social dominance both winners and losers show elevated DA and 5-HT activity (Øverli et al., 1999). Also, various stressors other than social interaction result in an activation of these brain monoaminergic systems (reviewed by Johnsson et al., 2005). Thus, in the present study stimulatory effects of exogenous CRF on brain stem 5-HT and DA activities may have been masked by the stress induced by i.c.v. injections and subsequent dyadic interaction. Also, in the present study the concentrations of monoamines and monoamine metabolites were only assayed in the brain stem, and exogenous CRF may have had effects on 5-HT and DA in telencephalic areas as reported by Carpenter et al. (Carpenter et al., 2007).

As expected, social interaction had effects on brain stem 5-HT and DA. Socially interacting trout showed higher 5-HT and DA concentrations as well as higher DOPAC and 5-HIAA levels than isolated fish. Similar effects of short-term interaction have been reported previously (Winberg and Nilsson, 1993; Øverli et al., 1999) and suggest a stress-induced activation of DA and 5-HT in fish engaged in dyadic fights for social dominance. The social stress experienced by interacting fish is also reflected in elevated plasma cortisol concentrations in subordinates. Furthermore, as expected (Fryer et al., 1985; Vale et al., 1981) fish receiving CRF showed elevated levels of plasma cortisol. However, this effect was relatively modest and most obvious in isolated fish. The behavioral effects of CRF are more likely to have been mediated centrally, and possibly through interactions between CRF and brain monoaminergic systems. Fish treated with UI did not show any increase in plasma cortisol concentrations as compared with controls, even though earlier studies have shown that UI can act as an ACTH secretagogue (Fryer et al., 1985; Lederis et al., 1985). This lack of effect of UI on plasma cortisol concentrations is probably the result of the low dose of UI used in the present study. Furthermore, modest effects of UI on plasma cortisol levels may have been masked by stress induced by the i.c.v. injection procedure (Carpenter et al., 2007; Clements et al., 2002).

In conclusion, the results of the present study show that trout receiving i.c.v. CRF lose dyadic fights for social dominance. This is probably because of an inhibitory effect of CRF on aggressive behavior. By contrast, UI did not have any effects on the outcome of fights for social dominance even though both CRF and UI induced a pronounced head-shaking behavior that is likely to reflect an anxiogenic effect of these peptides. Furthermore, our results suggest that effects on aggressive behavior and anxiety may be mediated by different receptor subtypes.

LIST OF ABBREVIATIONS

5-HIAA	5-hydroxyindoleacetic acid
5-HT	serotonin (5-hydroxytryptamine)
AhCRF	α-helical CRF_{9-41}
Ant	antalarmin
CRF	corticotropin releasing factor
CRF-R1	CRF subtype type I
CRF-R2	CRF subtype type II
DA	dopamine
DOPAC	3,4-dihydroxyphenylacetic acid
UI	urotensin I

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